A double-edged sword：Complement component 3 in *Toxoplasma gondii* infection

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Abstract

We artificially infected Sprague Dawley (SD) rats and Kunming (KM) mice with type II *Toxoplasma gondii* (*T. gondii*) strain Prugniaud (Pru) to generate toxoplasmosis, which is a fatal disease mediated by *T. gondii* invasion of the central nervous system (CNS) by unknown mechanisms. We aimed to explore the mechanism of differential susceptibility of mice and rats to *T. gondii* infection. Therefore, we established a strategy of isobaric tags for relative and absolute quantitation (iTRAQ) to identify differentially expressed proteins (DEPs) in the rats' and the mice's brains compared to the healthy groups. Complement component 3 (C3) was upregulated and the tight junction (TJ) pathway showed a disorder in KM mice, which was susceptible to *T. gondii* infection. In the CNS, we presumed that *T. gondii*-stimulated C3 disrupts the TJ of the blood-brain barrier (BBB). This effect allows more *T. gondii* passing to the brain through the intercellular space.

Statement of significance of the study

Rat and mouse models for *T. gondii* infection are proper pathophysiological models to study the toxoplasma encephalopathy, and the consequent severe mental disorders, because these two animals have different susceptibility to *T. gondii* infection. To explore the reason for this difference, we analyzed the cerebral proteome in rats and mice during chronic *T. gondii* infection. Interestingly, our results underlined alterations of complement and coagulation cascades, and tight junction pathways in the mouse brain. Specifically, we evaluated the relationship between C3 and TJ during *T. gondii* infection, which supports the paracellular entry mechanism.

Keywords: Blood-brain barrier; Central nervous system; Complement component 3; *Toxoplasma gondii;* Paracellular entry mechanism

1. Introduction

Toxoplasma gondii (*T. gondii*), a critical zoonotic parasite, infects almost all warm-blooded animals, including human-beings, worldwide[1]. The infection rates of this parasite are estimated at 30% in humans, but it geographically differs from region to region[2, 3]. Because the most severe complications occur due to infection of the central nervous system (CNS)[4], it is important to elucidate how *T. gondii* transits across the blood-brain barrier (BBB) and enters into the CNS. Previous studies have reported two primary mechanisms explaining how *T. gondii* goes through the BBB, including Trojan-horse like mechanism and a transcellular crossing mechanism[5, 6].

T. gondii invades the host cells and persists as intraneuronal cysts, resulting in damage to the CNS in varying degrees, because the parasites are suppressed by the immune system but not eliminated[7]. Rats and mice are widely used as experimental animals for *T. gondii* infection due to their different susceptibility[8, 9]. Specifically, adult rats have an innate resistance to *T. gondii*[10]. Tissue infections induced by cysts appeared in a subclinical form in the immunocompetent rats, though they were infected with the RH strain (type I genotype)[9]. On the other hand, mice died when infected with type I genotype strains, irrespective of the infective dose[10]. Compared to type I stains, types II and III strains are less virulent to mice, and the infective doses of those strains are significantly higher for rats than for mice, irrespective of the administration routes[11]. Moreover, the *T. gondii* cysts are abundantly found in the brains of mice but infrequently found in the brains of rats[9, 12]. In agreement the rat brain has lower risk of *T. gondii* infection, with no visible histological damage[12, 13]. Compared to rats, the learning capacity of mice is much more conspicuously retarded by a *T. gondii*-infection[14].

However, at present, no research has been performed on this kind of difference, and the exact mechanism is still poorly understood.

Our goal was to explore the mechanisms of the different susceptibility of mice and rats to *T. gondii*. Comparing to control groups, we established an iTRAQ-based strategy to identify differentially expressed proteins (DEPs) of infected Sprague Dawley (SD) rats and Kun Ming (KM) mice brains. Afterwards, we used KEGG pathways to analyze the DEPs patterns. Interestingly, in mice brain, we identified two specific pathways: the complement and coagulation cascades pathway and the tight junction (TJ) pathway, related to the host immune system and the brain intrusion of *T. gondii*, respectively. The two pathways contained 9 and 10 significant DEPs, among them the complement component 3 (C3), whose expression was increased 11 times. We hypothesize that C3 mediates the differences in this parasite-host interaction mechanism through disrupting TJ of the BBB to allow *T. gondii* invasion of the brain. Repression of the pharmacology or genetics may reduce the damage in the host, suggesting a therapeutic opportunity.

2. Materials and Methods

2.1 Animals and Parasites

The SD rats and KM mice were purchased from the Guangdong Medical Laboratory Animal Center, and were bred under specific pathogen-free conditions at South China Agricultural University. Parasites were harvested from the brains of KM mice that had been chronically infected with the Pru strain (preserved in our laboratory), a type II strain of *T. gondii*. The brain tissue of these animals was

dispersed in normal saline. The final concentration of the infectious agent was adjusted to a dose of 10 cysts/0.2 ml for mice and a dose of 50 cysts/0.5 ml for rats [11, 13], which were administered orally by gavage.

2.2 Sample collection for quantitative proteomic analysis

In total, 18 SPF KM mice (9 males, 9 females, 56 days old), and 9 SPF rats (4 males, 5 females, 60 days old) were infected with type II *T. gondii* Pru strain, and were randomly divided into 3 groups. Each group contained 6 mice and 3 rats. After 30 days of infection, we collected brain tissue from the infected KM mice and rats. Simultaneously, we collected brain tissue from 18 uninfected KM mice and 9 uninfected rats at similar age and sex to serve as uninfected control group. Each of the brain tissue samples was divided into 3 equal parts; two parts were processed for proteomics analysis (one as the biological replicate, the other as the technical replicate), while the last part was used for qRT-PCR validation. All animal care procedures were conducted in conformity with NIH guidelines (NIH Pub. No. 85-23, revised 1996) and were approved by the Animal Ethics Committee of South China Agricultural University (SCAUAEC-2015-054).

Using a mortar and pestle, 100 mg brain tissue from each animal was ground into a fine powder and preserved in liquid nitrogen. Then, we used the Radio immunoprecipitation assay (RIPA) Lysis Buffer (50mM Tris-HCl, 150mM Nacl, 1% SDS, 1% SDC pH 8.0, 0.1% Triton X-100) to extract total proteins from each sample. The samples were sonicated (20 Watts, 10 times) and centrifuged (4ºC, 12,000 rpm, 20 min).

2.3 Trypsin digestion and iTRAQ labeling

We used the BCA method (BCA Protein Assay Kit, Biotech) to determine the protein concentrations[15], and utilized the filter-aided sample preparation (FASP) method to digest proteins[16]. Briefly, we reduced the alkylated, and subjected it to the tryptic hydrolysis of each protein sample (100 μg). iTRAQ labeling was performed according to manufacturer's protocol (Applied Biosystems, Sciex). The samples of infected micewere labeled with iTRAQ tag 114 and 115, while the samples of control mice were labeled with tag 113 and 116. The samples of infected rats were labeled with iTRAQ tag 119 and 121, while the samples of control rats were labeled with tag 117 and 118. All labeled samples were pooled together and vacuum-dried.

2.4 High-pH Reversed-Phase Chromatography

We carried out a high pH Reverse Phase Fractionation (hpRP) chromatography using a Dionex UltiMate 3000 high-performance Liquid Chromatography (LC) system, whose autosampler and ultraviolet (UV) detection was equipped with collection option of the micro fraction. We diluted the iTRAQ labeled samples in buffer A (20 mM NH_4HCO_2, pH 10) before High-Performance Liquid Chromatography (HPLC) on a Gemini-NX C18 columns (3 μm, 2 × 150 mm, 110 A, Phenomenex) with buffer A (mobile phase A), and buffer B (80% ACN/20% 20 mM NH_4HCO_2, mobile phase B). At a flow rate of 0.2 mL/min, the peptides were eluted with a gradient of 0%–5% mobile phase B for 10 min, 5%–10% mobile phase B for 10 min, 15%–37% mobile phase B for 60 min, 37%–95% mobile phase B for 5 min, and 95% mobile phase B for 5 min. The UV absorbance was set at 214/280

nm to collect fractions every 1 min for 12 fractions per each sample. The fractions were dried for further analysis.

2.5 LC-MS/MS Analysis

The samples were separated by a linear gradient, which was formed by mobile phase A (0.1% FA, 5% ACN) and mobile phase B (0.1% FA, 80% ACN). In 40 min, the mobile phase B was changed from 5% to 35% at a flow rate of 300 nL/min. We used a Triple TOF 5,600 system (AB SCIEX) with an information dependent mode to perform the Mass Spectrometer (MS) analysis. Using 100 ms accumulation time per spectrum, in high-resolution mode (70,000), we dynamically acquired MS spectra across the mass range of 350-1,800 m/z. Up to 20 precursors per cycle was selected for fragmentation from each MS spectrum, where each precursor has a minimum accumulation time of 120 s and a dynamic exclusion of 20 s. The high sensitivity mode (resolution: 17,500) of tandem mass spectra were used, which has rolling collision energy and an iTRAQ reagent collision energy adjustment.

2.6 Protein identification and data analysis

Via Proteome Discoverer 1.4 (Thermo Fisher Scientific), the raw data were converted to peak lists, which are MASCOT generic format (.mgf) files. For in-depth proteome analysis and protein quantitation analysis, we searched the Uniprot-mouse database and the Uniprot-rat database (www.uniport.org) with the ProteinPilot^{TM} Software 5.0 (Applied Biosystems Sciex). This software is based on Paragon Algorithm to convert the peptide analysis data into differential protein analysis

data (Unused > 1.3, Peptides（95%）≥ 2). To ensure up- and downregulation authenticity, a *P-value* < 0.05 and a fold change ≥ 2 (FC ≥ 2) and a FC ≤ 0.5 were used in the analysis. To understand the relationships between the mice and rats infected with *T. gondii*, we used Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) and DAVID (https://david.ncifcrf.gov) to compare the DEPs and their biological functions. KEGG database was used for biological pathway analysis of these DEPs (http://www.genome.jp/kegg/), where every protein's name, sequence and functional information are provided by UniProt Databases (http://www.uniprot.org/).

2.7 Quantitative real-time PCR analysis

We used quantitative real-time PCR (qRT-PCR) analysis to verify the expression of DEPs from the iTRAQ analysis. Using the manufacturer's protocols of RNAiso Plus (TaKaRa, Dalian, China), total RNA was extracted from the infected and control KM mice's and rats' brains. Then, we resuspended the final total RNA into RNase-free water and measured its concentration and purity using the ultra-microspectrophotometer (Thermo Scientific Nanodrop 2000, Waltham, MA, USA). cDNA was synthesized using the SYBR PrimeScript™ RT Master Mix (Perfect Real-Time) Kit (TaKaRa, Dalian, China). Gene-specific primers for qRT-PCR were designed with Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). According to the manufacturer's instructions (TaKaRa Dalian, China), qRT-PCR was performed on a Rotor-Gene Q (Qiagen) real-time system with SYBR Green master mix (SYBR Premix Ex Tag TMII; TaKaRa Bio; http://www.TaKaRa-bio.com). The thermal profile of qRT-PCR was 5 min at 95ºC followed by 40 cycles of 30 s at 95ºC and 1 min at 60ºC. Each sample was run in triplicates. For normalization of gene expression, we used *β*-actin as a

reference gene, and we established a blank control, which was set as 1. Relative gene expression was calculated by using the formula $2^{-\triangle\triangle Ct}$.

3. Results

3.1 Detection of differentially expressed proteins in mice and rats

To find out the effects of *T. gondii* infection in the host brain at the protein level, we infected KM mice and SD rats with *T. gondii* Pru strain. We established an iTRAQ-based strategy to analyze the brains of mice and rats. On the basis of the analysis with ProteinPilot^{TM} 5.0 search engine in the mice and rats respectively, we searched out 173,032 and 166,557 identified spectra, 80,329 and 76,902 distinct peptides, 17,081 and 16,974 proteins before grouping, and 5,033 and 4822 proteins with a minimum unused score of > 1.3 (Unused > 1.3), which indicates > 95% confidence in correct sequence identification. In total, 4,862 and 4,659 proteins were acquired in mice and rats, respectively, with at least two peptides identified (Peptides ≥ 2). About 86.81% and 74.7% of these proteins had a coefficient of variation (CV) of ≤ 50% among replicates in the mice and rats, respectively. All data related to this study has been publicly available on iProX (www.iprox.org) with id IPX0001302000 /PXD011192. The following analysis was based on proteins with CV ≤ 50%. In total, we determined 461 DEPs ($P < 0.05$, FC ≥ 2, and FC ≤ 0.5) in infected mice, among them 215 were upregulated (46.63%), and 246 downregulated (53.37%). Whereas, in infected rats 292 DEPs were detected ($P <$ 0.05, FC ≥ 2, and FC ≤ 0.5), among them 95 upregulated (32.53%), and 197 downregulated (67.47%) (shown in supplementary information). Considering the names of mice's proteins, which differ from rat's, we used the "BLAST" software to find homogeneous DEPs (HDEPs), which included 381

proteins in KM mice and 269 in rats (Figure 1). These results suggested that the DEPs of our focus should be further narrowed so that the cerebral proteomic differences between rats and mice infected with *T. gondii* could be displayed more directly. In addition, further studies should be needed to verify the reliability of the DEPs.

3.2 Validation of differentially expressed proteins by quantitative real-time PCR

To determine the reliability of the iTRAQ results in mouse and rat, we selected 5 proteins with different expression level for qRT-PCR analysis. We found total agreement between the results of qRT-PCR and iTRAQ results (Figure 2). Data were statistically analyzed and were presented as a mean ± standard deviation. The differences in protein expression patterns could be possibly argued by the higher sensitivity of iTRAQ comparing to qRT-PCR suggesting that the iTRAQ results are more reliable.

3.3 Complement component 3 upregulation in mice infected with *T. gondii*

Because of the massive number of data, we had to reduce the amount of information if we wanted to make a differential analysis. To find the key proteins that differentiate the phenotypes of the two hosts, we firstly, compared the HDEPs from rats and mice (Figure 3a). Sixty-seven HDEPs were detected to be common to both two hosts, while 202 and 314 HDEPs were specific to SD rats and KM mice, respectively. To explore the mechanism of differential susceptibility, we therefore focused on the particular HDEPs. Subsequently, we further investigated biological functions of these HDEPs and mapped them into 71 pathways of rats, 37 pathways of KM mice and 28 homo-pathways using

DAVID database. Performing Venn analysis of all these pathways, we further narrowed the range of data to find specific protein pathways in rats and mice (Figure 3b). We focused only on specific pathways of typical HDEPs. We identified 10 mouse-specific pathways and 44 rat-specific pathways, of which the top 10 enriched pathways are shown in Figure 4. The top 10 enriched pathways of rats were Rap1 signaling pathway, Endocytosis, Neurotrophin signaling pathway, Cholinergic synapse, Alcoholism, Ras signaling pathway, Chemokine signaling pathway, GnRH signaling pathway, Melanogenesis, and Alzheimer's disease. Whereas, the top 10 enriched pathways of mice were Protein processing in endoplasmic reticulum, TJ, Complement and coagulation cascades, Parkinson's disease, Platelet activation, Glutathione metabolism, Phosphatidylinositol signaling system, Bacterial invasion of epithelial cells, Amoebiasis, and *Staphylococcus aureus* infection. Upon infection, the parasites persist as intraneuronal cysts in the CNS for the lifetime of the host[7]. Interestingly, the *T. gondii* cysts are more frequently found in the brain of mice than in the brain of rats[9, 12]. The control of cysts depends on the immune system of hosts, and the ongoing inflammation is required to control *T. gondii* in chronic cerebral infection[7]. The complement system contributes to the defense of infection, as an important aspect of host immune system[17]. Additionally, the brain invasion of *T. gondii* relates to TJ[18]. Consequently, we considered that the primary cause of the phenotypic difference between mouse and rat may be associated to the immune system and the brain invasion of *T. gondii*. So we focused on the complement and coagulation cascades pathway and TJ, which had 9 and 10 DEPs, respectively (Table 1 and 2). The most significant HDEP among them was C3 therefore, these results suggested that the C3 protein may be a crucial protein that differentiate the phenotype of the two hosts.

4. Discussion

In this study, we infected rats and mice with type II *T. gondii* strain (Pru). We then used an iTRAQ-based strategy to identify 381 HDEPs in rats' brain and 269 in KM mice's brains (Figure 1). By using the KEGG pathway analyses, we specifically found the complement-related pathway and TJ pathway in the infected mice's brains, which contained 19 HDEPs. Among them, expression of C3 was increased 11 times as compared with the control group (Table 1 and 2).

Strain type, host genetic background, and route of infection contribute obviously to the differential susceptibility to *T. gondii* infection[19]. Because of the differences in the virulence and geographical occurrence, this parasite contains three major types: I, II, and III[19, 20] . Type II strains are the most prevalent in the United States and Europe[21]. Mice are good models for *T. gondii* due to their high sensitivity to many different strains[8]. Type I *T. gondii* strains can lethally infect mice, while type II and III strains cause chronic-progressive or latent infection [2, 22]. Comparatively, rats are more resistant to toxoplasmosis and mimic *T. gondii* disease in human[8]. Type I, type II and III strains cause chronic subclinical infection in immunocompetent rats[9, 10, 23-25], but the pathology and physiology of *T. gondii* infection in rats are still poorly understood[11]. Taken together, type II strains are appropriate experimental materials to study the different outcome of *T. gondii* infection in mice and rats.

Scientists attribute the increasing understanding of *T. gondii* protein expression to the advances of

proteomic technologies. Using 2-dimensional electrophoresis (2-DE) and matrix-assisted laser-desorption/ionization time of flight mass spectrometry (MALDI- TOF-MS), Cohen *et al.* constructed the first proteome map of *T. gondii* tachyzoite in 2002[26]. Recently, Zhou *et al*. used the same strategy to observe the proteomic changes in the hippocampus tissue of rat brain with chronic *T. gondii* infection[27]. Furthermore, Zhou *et al*. explored the proteomic profiles of brain tissues of KM mice at 7, 14, 21 d after infection with Pru strain cysts[28]. Some researchers used two-dimensional difference gel electrophoresis (2D-DIGE) and MALDI-TOF-MS to identify DEPs of 4 different genotypes of *T. gondii* tachyzoites [29]. In recent years, iTRAQ was reported to be the most accurate labeling method for quantification of the relative abundance of proteins with good accuracy and repeatability[30]. Because of its high-throughput, it has been widely used in proteomics to study the relative and absolute quantification of up to 8 samples at the same time[30]. Sahu *et al.* firstly utilized iTRAQ labeling for a global quantitative proteomic study of the proteome profile of human brain that was co-infected with *T. gondii* and HIV[31]. Later, the liver proteome of the mouse following acute *T. gondii* infection and the proteome of *T. gondii* oocysts during sporulation were reported[32]. Interestingly, Wang *et al.* compared DEPs from different stages of *T. gondii* Pru strain, including tachyzoite, bradyzoites-containing cyst, and sporulated oocyst[33]. Moreover, we have previously reported the DEPs of *Mongolian gerbil* brains infected with *T. gondii*[34]. Notably, the new research technology of iTRAQ has provided improvements in three aspects: high throughput, sensitivity and accuracy[35]. With the advancement of the technology, iTRAQ is still widely used in bio-materials such as microbes, animals, plants and biomedicine[36]. Most importantly, iTRAQ-based strategy has been used for the identification of biomarkers in a plethora of diseases[37]. A recent study, validated

271 the role of CD44 in hosts chronically infected with *T. gondii* based on iTRAQ technology, and
272 reported that CD44 mediates the production of IFN-γ and Ca2+, and the parasite prefers to invade
273 cells with high levels of CD44[38]. In brief, this is the first study to establish an iTRAQ-based strategy
274 to compare DEPs from infected rats' and KM mice's brains, which aimed to explore the mechanism
275 of different susceptibility of mice and rats to *T. gondii*.
276
277 *T. gondii* can infect a variety of tissues including the brain, and because of preference for the neurons,
278 it results in *Toxoplasma* encephalitis (TE)[7]. TE, characterized by both focal and diffuse neurological
279 lesions, concurs with behavior disorders and mental symptoms, such as schizophrenia, Alzheimer's
280 disease (AD) and Parkinson's disease (PD)[39]. In agreement, *T. gondii*-infected rodents exhibit a
281 number of modifications in their behavior. For example, *T. gondii* causes a shift in the emotional
282 response of rats and increases the timidity in mice[24, 40]. Most notably, they display an altered
283 response to feline predator odor, from aversion to attraction[23, 41]. At present, the proposed hypotheses
284 of TE pathogenesis include host dopamine pathways, direct damages to neurons, and parasite-
285 mediated acetylation[7, 42], but the mechanisms explaining how *T. gondii* exactly affects the CNS are
286 not precisely demonstrated. During *T. gondii* chronic infection, the cysts persist in the CNS for the
287 life time of the host[43]. The changes observed in the hosts with *T. gondii* on the CNS are related to
288 the tissue cyst tropism, size and number[44]. Compared to rats, the behavioral disorders of mice are
289 much more conspicuously affected by a *T. gondii*-infection[14]. Because of the interest in behavioral
290 effects of hosts infected with *T. gondii*, several authors have claimed that the parasite shows a tissue
291 tropism for the amygdalar region in the brains of chronically infected rodents [23, 24, 45]. However,

amygdala tropism is not clear for tissue cysts in rats [11]. In addition, some previous studies in mice reported that tissue cysts are distributed unevenly in all regions of the brain with no specific tropism to amygdaloid regions[13]. But other studies indicated that tissue cyst distribution is not random and tropism could vary with the duration of infection[44, 46]. Dubey *et al*. (2016) indicated that these differences of tissue cyst tropism are related to techniques, host, parasite strain, route of infection, duration of infection, number of rodents, method of infection and development stage of the parasite[11]. Consequently, in order to avoid tropism differences between mice and rats to influence the proteomic results, we used the same parasite strain (Pru) freshly collected in one mouse, infected animals through the same way (oral), and included the whole brain of each experimental animal after 30 days of infection. Dubey *et al*. (2016) added that the tissue cyst size is dependent on the duration of infection, the type of host cell parasitized, development strain of the parasite and the cytological method used for measurement[11]. When both hosts are given the same inoculum, tissue cysts in brains of rats are of the same size as in mice[25]. However, the *T. gondii* cysts are abundantly found in the brain of mice but less frequently found in the brain of rats[9, 12]. According to a research on reactivation of latent infection, *T. gondii* tachyzoites are present in the brains of chronically infected mice but the frequency of the phenomenon is unknown[47]. Instead, in experimentally infected rats, tissue cyst rupture was frequent, but there was no evidence for reactivation and the presence of tachyzoites[11]. Moreover, the rats have lower brain infestation and no visible histological damages[12, 13]. These phenomena of *T. gondii* infection brought us to consider what differences exist between rat and mouse brain.

The infiltrating peripheral immune cells in the CNS provide a continuous immune response to prevent TE and avoid reactivation of TE[48]. Since the 1980s, some researchers have shown that the complement pathway is either a critical response factor for an initial immune response to *T. gondii* or a target for parasite evasion[49]. These observations support that complement pathways are particularly changed in the KM mice brain with *T. gondii* infection, unlike in rats, and it may explain at least partially why mice and rats have different susceptibility to *T. gondii* infection. Previous reports showed that C1q, C3, and C4b are upregulated in some brain disorders associated with TE, including schizophrenia, AD, aging and multiple sclerosis[34, 50, 51]. In the current study, we identified several pathways, like the AD in the rat and PD in the mice (Figure 4), which is in agreement with the previous studies. Although Xiao *et al.* (2016) have reported alterations in the level of C1q in mice[52], this is the first study that directly identified C1q, C3, and C4b from the brain of infected KM mice.

Complement is an innate immune system pathway recognizing and eliminating cellular debris and pathogens. Among them, C1q is a part of the C1-complex and is an immune protein that bridges adaptive and innate immunity. The basic functions of C1q are to clear antigen-antibody immune complexes in systemic circulation [53]. Furthermore, C1q initiate the proteolytic reactions to generate molecules, which participate in phagocytic responses and inflammation, and induce the activation of the classical complement pathway [17]. In the classic immune responses, C1q leads to activation of C3 and cleaves C3 into C3a and C3b, which is a central protein in the complement and coagulation cascade[54]. C3a can promote inflammatory responses and gliosis, which is an anaphylatoxin. C1q

and C3b fragments can bind to the surface proteins of microorganisms to promote phagocytosis[55]. Additionally, C1q-C3 can modulate the expression of the interleukin 1 beta (IL1β) and tumor necrosis factor alpha (TNFα), and the hyperplasia of microglia and astrocytes[56, 57], after SE or in epilepsy[58]. Considering studies on the classical complement pathway in *T. gondii* infection using quantitative PCR, Xiao *et al.* (2016) firstly claimed that C1q activation is a part of the host immune response to *T. gondii* chronic infection, and primarily recognizes degenerate cysts as targets for the parasite elimination[52]. In our study, we used iTRAQ strategy to confirm the variation of C1q in the KM mice with *T. gondii* infection. As a factor for SE or epilepsy, the *T. gondii* increases C1q and C3 in the host brain, which may remarkably contribute to the neurogenic inflammation.

For non-inflammatory mechanisms, on the one hand, microglia express complement receptors, and C1q, while C3 locate synapses, which collectively mediate phagocytic microglia to prune synapses in developing brains[59]. However, this process is significantly down-regulated in the mature brain[59]. C4 also is a crucial part of the complement cascade, which is expressed by neurons and is secreted by dendrites, axons and synapses[51]. During postnatal development, C4 promotes synapse elimination in mice[59]. Accordingly, C4 activity in the event of schizophrenia will reduce the number of synapses[51, 60]. Emerging research implicates activation of the synaptic pruning pathway in synapses loss in the AD brain[61]. Accordingly, we hypothesized that by increasing C1q, C4b, and C3 expression, chronic *T. gondii* infection mediates damage of nerve cells, such as neurodegeneration, possible synapse loss, and neuronal death. On the other hand, C1q and iC3b proteins can mediate phagocytic responses of microglia, which consequently, means that C1q and iC3b attract microglia

to the orchestrate elimination of foreign bodies, called as “eat-me” signals [17, 62]. To illustrate, C1q binds to cellular debris or apoptotic cells to promote microglial clearance and prevent further inflammation[56, 63]. Therefore, some researchers reported that the activation of complement protects nerve cells in epilepsy and SE[64]. For *T. gondii* infection, this mechanism may protect the nerve cells as well. In conclusion, the complement is a two-edged weapon, which can be beneficial and detrimental for the host when *T. gondii* infects the brain.

The basal lamina, pericytes, endothelial cells and astrocytic end-feet comprise the BBB, which is a selective barrier. The tight junctions in the BBB are adhesive structuresthat seal the gap between endothelial cells to selectively prevent the entry into the brain of pathogenic microbes and antigens from the blood[65]. Pathogens must cross the BBB to enter the CNS, which is a challenge[66]. A plausible explanation for the development of TE is that some *T. gondii* tachyzoites enter the CNS and form into cysts to avoid the host immune responses and anti-toxoplasma drugs. Nevertheless, how does *T. gondii* cross TJs to enter the brain? Two mechanisms have been proposed. The first one is the transcellular crossing mechanism, in which tachyzoites from the bloodstream can adhere to, invade, replicate and pass from the endothelial cells of CNS to the CNS parenchyma. This mechanism was discovered by utilizing multiphoton *in vivo* imaging and transgenic reporter systems (Figure 5B) [6]. Although the parasite infection of the CNS precedes immune cells infiltration[67], some studies reported that mouse brain is more quickly infected by dendritic cells and macrophages previously infected with *T. gondii* tachyzoites than by *T. gondii* tachyzoites only[68]. This findings are in agreement with a second mechanism called the Trojan-horse like mechanism, which implicates

infected immune cells as a carriers of the intracellular parasites through the BBB (Figure 5A)[5, 68]. In addition, we thought of a third mechanism in which *T. gondii* tachyzoites may directly cross the BBB through the brain TJ (Figure 5C). Previous studies have described that the *T. gondii* tachyzoites can show "gliding motility", which can help the parasites to go across the epithelium of the small intestine[69]. Inspiringly, under shear force conditions tachyzoites can adhere to the human vascular endothelium to increase the invasive percentage[70]. These lines of evidence prompted us to wonder if *T. gondii* crosses the BBB via the intercellular space. Some scholars named it as 'paracellular entry' [71], but until now, no study presented direct evidence to support it.

We hypothesized that *T. gondii* disrupts the TJ of BBB and allows *T. gondii* to pass into the brain by paracellular entry, in which C3 plays a crucial role. Previous studies reported that C3 disrupts the renal tubules barrier to develop proteinuria [72, 73]. In brains, blood-cerebrospinal fluid (CSF) barrier is as vital as the BBB, where the choroid plexus epithelial (CPEpi) cells represent an essential component of this barrier. Additionally, the CPEpi cells express the C3a receptor (C3aR) of C3, which is a G protein-coupled receptor[74]. Moreover, myosin light-chain kinase (MLCK) is a key regulator of TJ permeability, and by the addition of C3a, it transiently increases its phosphorylation [75]. C3a was proved by immunofluorescence to be able to disorganize the TJ of mouse CPE. This effect of C3a was also found in the renal and pulmonary epithelia[73, 76]. It represents one line of evidence for our hypothesis of the involvement of TJ pathway in KM mouse brain during *T. gondii* infection. Transmembrane proteins compose TJs, including occludins, claudins, and intracellular proteins, which maintain the integrity of the BBB. These intracellular proteins contain ZO-1, ZO-2

and ZO-3 [77] [78]. Moreover, Protein kinase C (PKC) is essential for the normal assembly of TJs. PKC affects the opening of TJs and plays a role in decreasing and increasing the permeability of TJ[79]. Phorbol 12-myristate 13-acetate as an agonist of PKC can lead to the TJs disintegration [80]. Persistent activation of PKCα decreases the expression of TJ proteins and increases the BBB permeability, which means that the barrier function is lost [81]. In this study, the TJ pathway, and majority of HDEPs were down-regulated such as the PKC, however a few HDEPs were up-regulated; TJ protein zonula occludens-2 (ZO-2) was one of them. This up-regulation may be a compensatory effectof the TJ damage caused by *T. gondii* infection, which induces the PKC down-regulation to stimulate the secretion of TJ protein, in order to restore the TJ functionality.

In other contexts, the pharmacologic interventions of complement cascade especially in C3 has been explored such as autoimmune arthritis[82] and reactive airway disease[83]. Our findings elicit lots of significant questions for further studies. Complement C3 manipulation of the BBB integrity might be employed to allow for increased access of *T. gondii* into the CNS. Further preclinical studies could make this hypothesis possible.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgements

This work was supported, in part, by grants from the national key research and development program

of China (2017YFD0501305), Major scientific research projects (characteristic innovation) of Guangdong Province (2017KTSCX018), the Guangzhou Zoo Project (h2016141), the Natural Science Foundation of Guangdong Province (2016A030313396), and Huizhou science and technology projects (2015B040009001). The authors acknowledge the efforts of Prof. Virginia Aragon [IRTA, Centre de Recerca en Sanitat Animal (CReSA-IRTA), Barcelona, Spain] for her contribution in proofreading of this work.

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Table 1: HDEPs of the complement and coagulation cascades pathway in the infected mice's brain.

Protein	Gene symbol	Description	Toxoplasma encephalistis/Control (Fold change)
Plasminogen	Plg	Plasmin is an important enzyme present in blood that degrades many blood plasma proteins, including fibrin clots. The degradation of fibrin is termed fibrinolysis.	5.032887165
Antithrombin-III	Serpinc1	AT III is generally referred to solely as "Antithrombin", which is a small protein molecule that inactivates several enzymes of the coagulation system.	3.018619571
Fibrinogen beta chain	Fgb	Fibrinogen beta chain, also known as FGB, is a gene found in humans and most other vertebrates with a similar system of blood coagulation.The protein encoded by this gene is the beta component of fibrinogen, a blood-borne glycoprotein composed of three pairs of nonidentical polypeptide chains. Following vascular injury, fibrinogen is cleaved by thrombin to form fibrin which is the most abundant component of blood clots.	6.43841143
Fibrinogen alpha chain	Fga	The protein encoded by this gene is the alpha component of fibrinogen.	3.565736133
Coagulation factor XIII A chain	F13a1	Coagulation factor XIII is the last zymogen to become activated in the blood coagulation cascade. Plasma factor XIII is a heterotetramer composed of 2 A subunits and 2 B subunits. The A subunits have catalytic function, and the B subunits do not have enzymatic activity and may serve as plasma carrier molecules.	4.641328363
Alpha-2-macroglobulin-P	A2m	Alpha 2 macroglobulin acts as an antiprotease and is able to inactivate an enormous variety of proteinases. It functions as an inhibitor of fibrinolysis by inhibiting plasmin and kallikrein. It functions as an inhibitor of coagulation by inhibiting thrombin.	10.46278151

Complement C3	**C3**	Complement component 3, often simply called C3, is a protein of the immune system. It plays a central role in the complement system and contributes to innate immunity.	11.06623745
Complement C4-B	**C4b**	Complement component 4, in humans, is a protein involved in the intricate complement system, originating from the human leukocyte antigen (HLA) system. It serves a number of critical functions in immunity, tolerance, and autoimmunity with the other numerous components.	9.375619888
Complement C1q subcomponent subunit A	**C1qa**	The C1q complex is a protein complex involved in the complement system, which is part of the innate immune system. C1q is part of the C1-complex.	5.649369717

Table 2: HDEPs of the Tight junction pathway in the infected mice's brain.

Protein	Gene symbol	Toxoplasma encephalistis/Control (Fold change)	Description
Tight junction protein ZO-2	Tjp2	4.23769481	Tight junction protein ZO-2 plays a role in tight junctions and adherens junctions. It has a role as a scaffold protein which cross-links and anchors Tight Junction (TJ) strand proteins, which are fibril-like structures within the lipid bilayer, to the actin cytoskeleton.
Protein kinase C	Prkca	0.403918909	Protein kinase C modulates membrane structure events.
Protein kinase C gamma type	Prkcg	0.314442886	
Ras-related protein Rab-3B	Rab3b	0.408556377	Ras-related protein Rab-3B plays a role in protein transport.
Alpha-actinin-2	Actn2	0.44870987	As a bundling protein, it is thought to anchor actin to a variety of intracellular structures.
Myosin-9	Myh9	7.139545535	Myosins comprise a superfamily of ATP-dependent motor proteins and are best known for their role in muscle contraction and their involvement in a wide range of other motility processes in eukaryotes. Virtually all eukaryotic cells contain myosin isoforms. During cell spreading, myosin-9 plays an important role in cytoskeleton reorganization, focal contacts formation.
Myosin-10	Myh10	0.355443681	
Myosin-11	Myh11	0.228766571	
Protein Shroom2	Shroom2	0.389755496	Shroom2 is both necessary and sufficient to govern the localization of pigment granules at the apical surface of epithelial cells. It is possible that SHROOM2 mutations may contribute to human visual system disorders.
Catenin alpha-1	Ctnna1	2.592327188	Catenin alpha-1 associates with the cytoplasmic domain of a variety of cadherins. The association of catenins to cadherins produces a complex which is linked to the actin filament network, and which seems to be of primary importance for cadherins cell-adhesion properties.

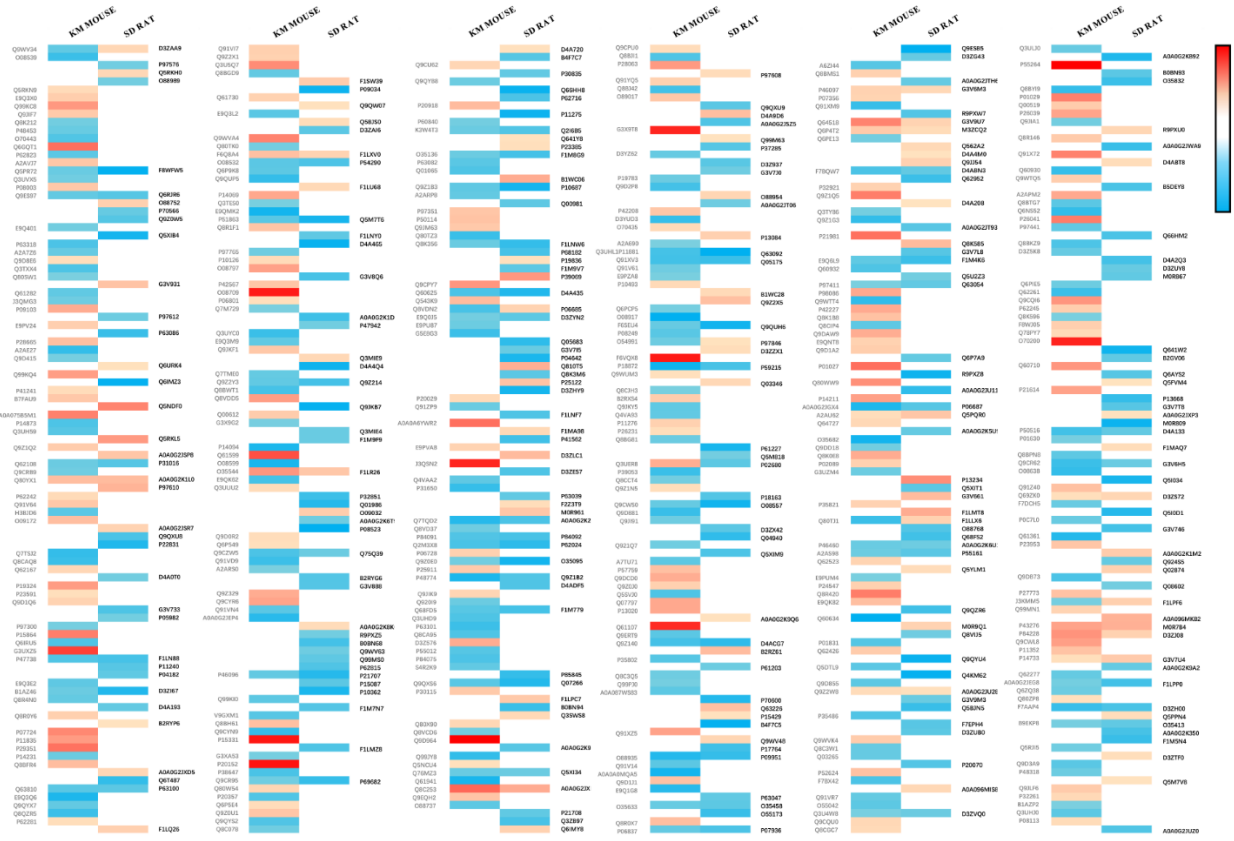

Fig. 1. Heat map of HDEPs. The HDEPs with a fold change of ≥ 2-fold or ≤ 2-fold and p ≤ 0.05 are shown in the heat map, which include 381 proteins in rats and 269 in KM mice. Upregulation is displayed by orange color and downregulation is displayed by blue color.

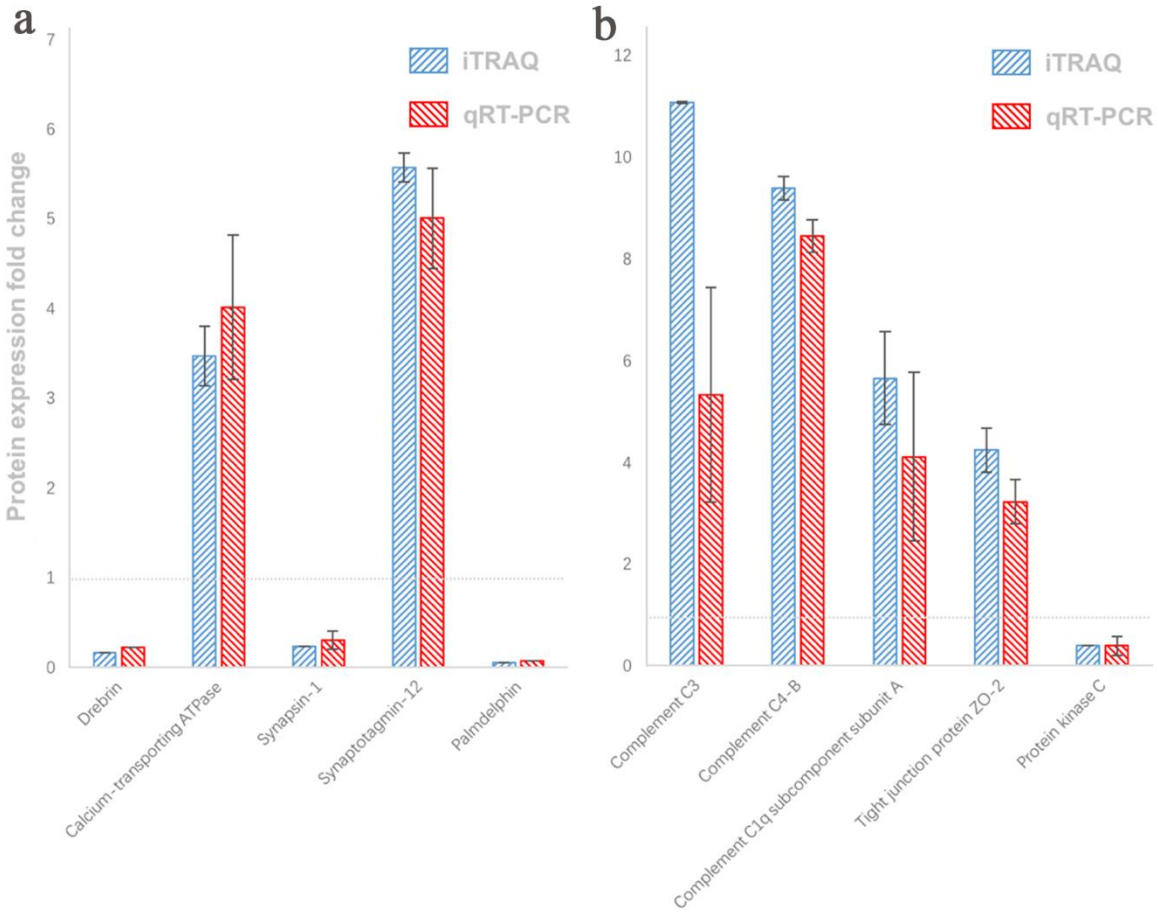

Fig. 2. The fold change in mRNA expression, detected by qRT-PCR. The clustered columns show that the expression patterns of 10 selected proteins detected by qRT-PCR were consistent with those obtained by iTRAQ. (a) The clustered column shows the expression patterns of the SD rats' selected proteins. (b) The clustered column shows the expression patterns of the KM mice's selected proteins. Error bars represent SEM.

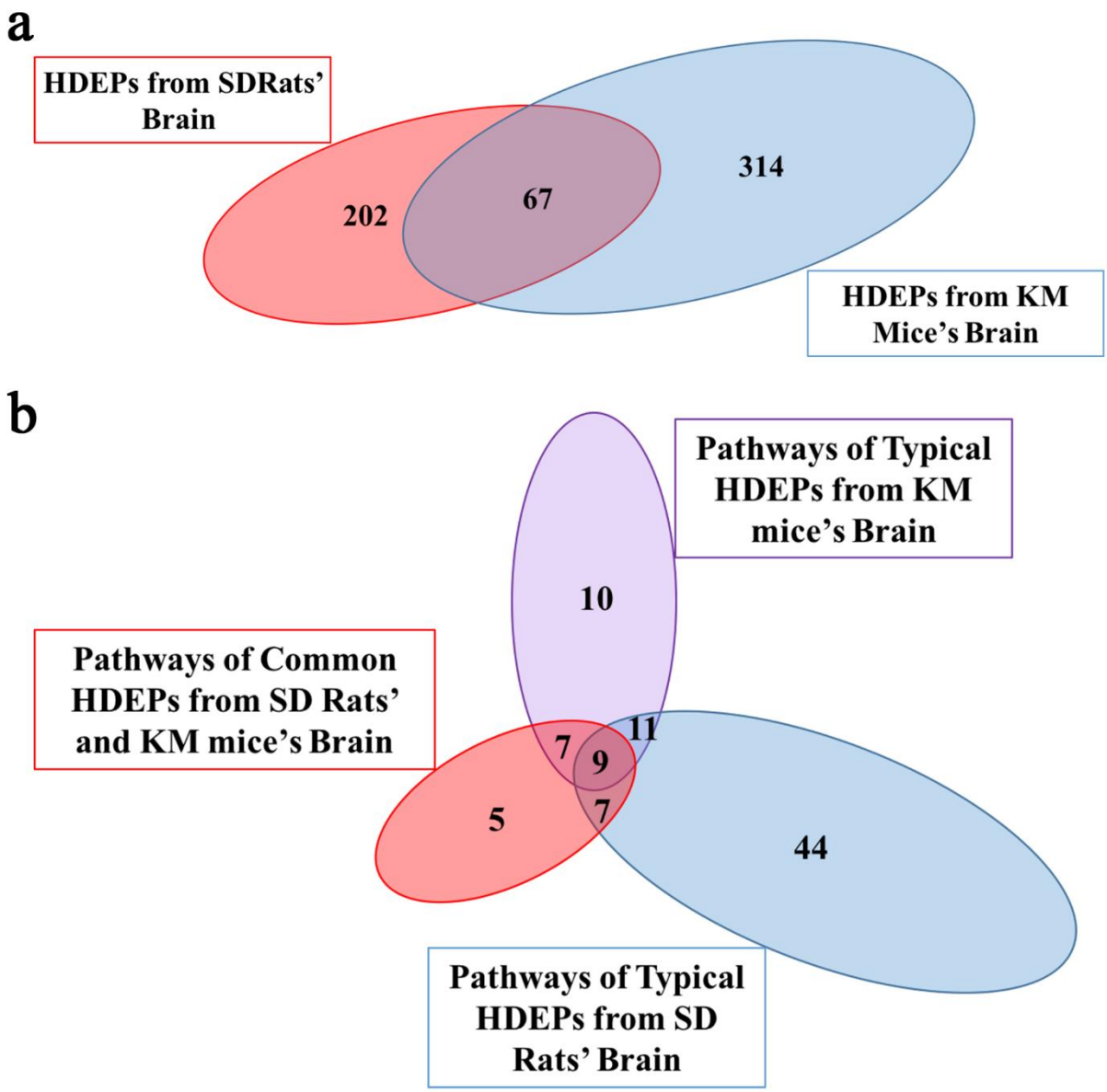

Fig. 3. Comparison of the HDEPs. (a) Venn diagrams of HDEPs between SD rats' and KM mice' brain. Proteins with base mean fold change ≥ 2 or ≤ 2, and $p < 0.05$ were collected for analysis.

(b) By using the KEGG database, we investigated biological functions of these HDEPs and mapped them to 93 pathways in total. Venn diagrams of pathways of HDEPs between SD rats' and KM mice' brain; $p < 0.01$ were collected for each pathway.

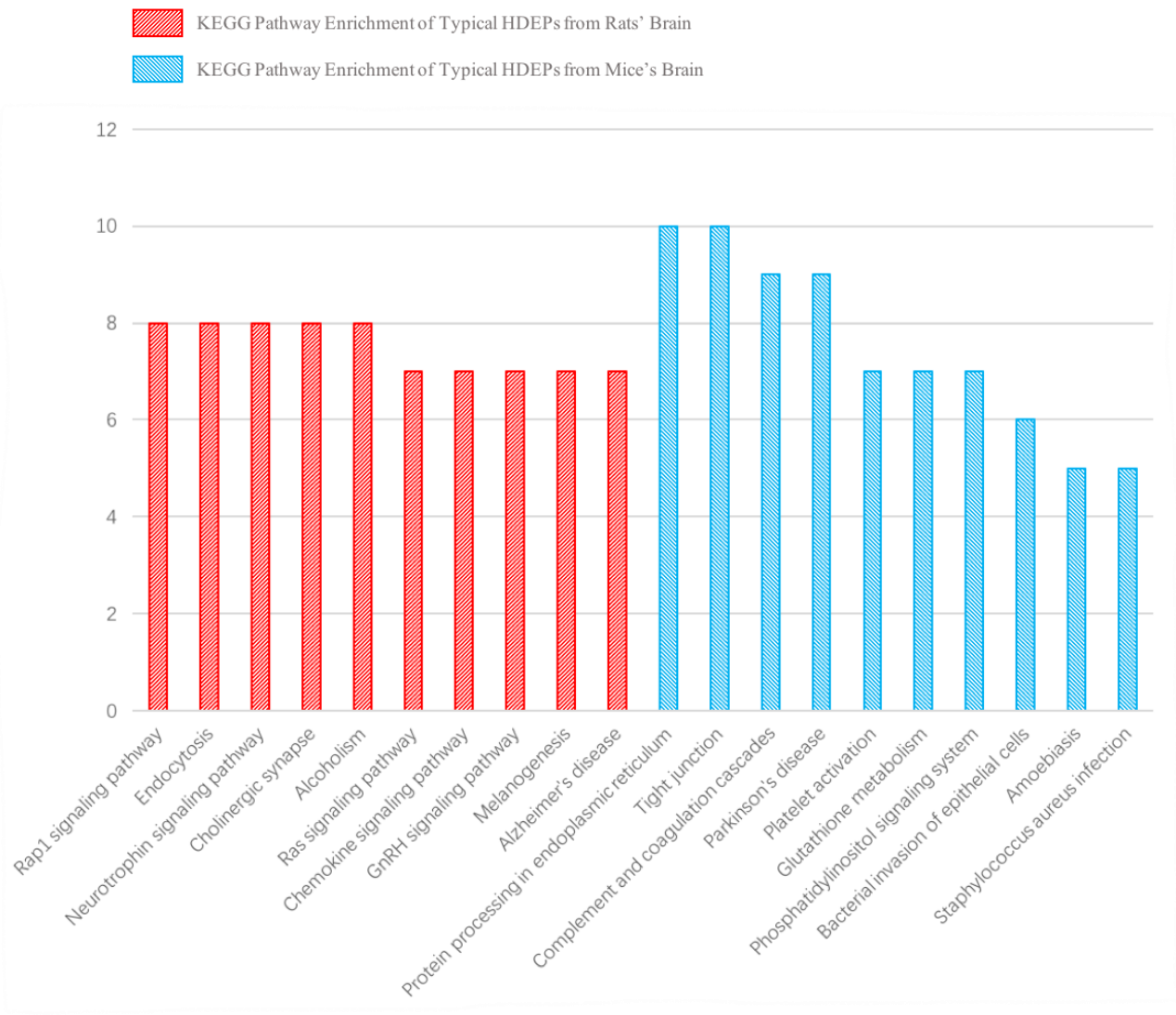

Fig. 4. The most enriched pathways of HDEPs. The clustered column shows the top 10 enriched pathways of typical HDEPs from KM mice and the other 10 of typical HDEPs from SD rats.

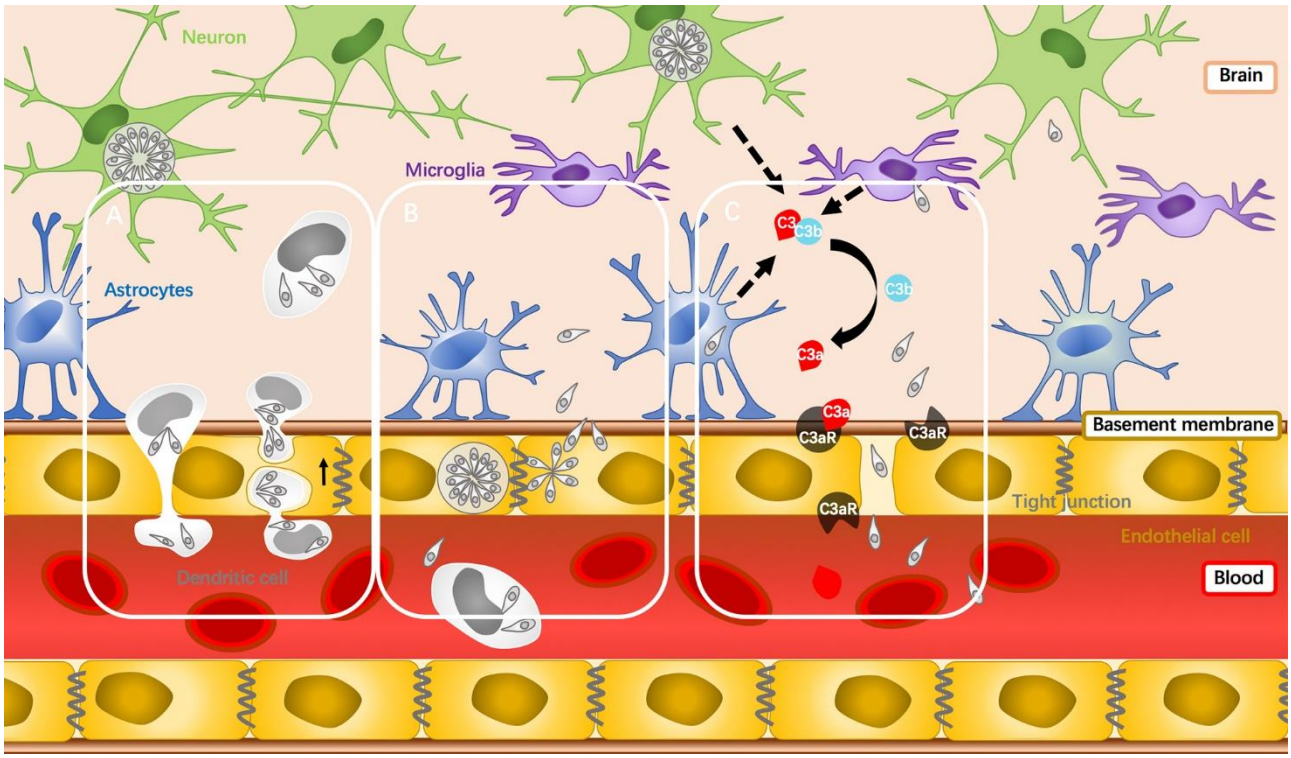

Fig. 5. The routes of *T. gondii*'s brain infection. (A) Trojan-horse like mechanism: The infected immune cells bring the intracellular parasites through the BBB. (B) Transcellular crossing mechanism: The bloodstream tachyzoites adhere to, invade in, replicate at, and pass from the endothelial cells of CNS to enter the CNS parenchyma. (C) Paracellular entry mechanism: Cooperating with C3 the bloodstream tachyzoites may directly cross the BBB through the brain TJ.