Downregulation of the central noradrenergic system by *Toxoplasma gondii* infection

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Abstract

The parasitic protozoan *Toxoplasma gondii* becomes encysted in brain and muscle tissue during chronic infection, a stage that was previously thought to be dormant but has been found to be active and associated with physiological effects in the host. Dysregulation of catecholamines in the central nervous system has previously been observed in chronically-infected animals. In the study described here, the noradrenergic system was found to be suppressed with decreased levels of norepinephrine (NE) in brains of infected animals and in infected human and rat neural cells *in vitro*. The mechanism responsible for the NE suppression was found to be down-regulation of dopamine β-hydroxylase (DBH) gene expression, encoding the enzyme that synthesizes norepinephrine from dopamine with down-regulation observed *in vitro* and in infected brain tissue, particularly in the dorsal locus coeruleus/pons region. The down-regulation was sex-specific with males expressing reduced DBH mRNA levels whereas females were unchanged. Rather, DBH expression correlated with estrogen receptor in the female rat brains for this estrogen-regulated gene. DBH silencing was not a general response of neurons to infection as human cytomegalovirus (CMV) did not down-regulate DBH expression. The noradrenergic-linked behaviors of sociability and arousal were altered in chronically-infected animals, with a high correlation between DBH expression and infection intensity. A decrease in DBH

expression in noradrenergic neurons can elevate dopamine levels which provides a possible explanation for mixed observations to changes in this neurotransmitter with infection. Decreased NE is consistent with the loss of coordination and motor impairments associated with toxoplasmosis. Further, the altered norepinephrine synthesis observed here may, in part, explain behavioural effects of infection and associations with mental illness.

Introduction

T. gondii infects warm-blooded animals and is characterised by a transient acute infection wherein vegetative tachyzoite forms rapidly replicate in tissues followed by conversion of some tachyzoites to slowly-replicating bradyzoites generating a persistent chronic infection. Chronic infection can persist for years and potentially the lifetime of the host with the bradyzoite-stage parasites encysted in cells within immunoprivileged tissues, including muscle, eyes, and neurons in the brain. Several reports have published host behavioral changes with infection. A selective loss of aversion to feline urine and increased motor activity has been observed in rodents, specifically manipulating behavior that will enhance the probability of parasite transmission (1, 2).

Toxoplasmosis can be a severe disease in immunocompromised individuals and *in utero*. Infection can cause retinochoroiditis and congenital hydrocephalus and cerebral calcifications. *T. gondii* was recently ranked the second most important food-borne parasite in Europe and is classified as a Neglected Parasitic Infection (CDC, Atlanta) (3). It has also been linked by epidemiological studies to cognitive impairment and major mental illnesses. Severe cases are associated with psychoses, seizures and loss of coordination. Yet there are

currently no available cures for infection. Sensorimotor defects, tremors and headshaking have also been observed in chronically-infected mice (4, 5).

In the brain, encysted bradyzoite-stage parasites are restricted to neurons, and recent work has found that neurons are the primary target cell for *T. gondii* during central nervous system (CNS) infection (6, 7). Recently, a large 'omics' study found canonical pathways in movement disorders, epilepsy, cancer, and Alzheimer's disease associated with altered gene expression in neural stem cells expressing a mixture of astrocyte and neuronal markers after eighteen hours of tachyzoite infection (8). As chronic infection is restricted to neurons in the CNS, this study investigated changes in gene expression in neuron-like cells that express neurotransmitters and can form synapses.

Early studies found changes in dopaminergic neurotransmission associated with infection, with high levels of dopamine (DA) in brain tissue cysts of chronically infected rodents and abrogation of infection-induced behavior changes when animals were treated with dopamine antagonists, haloperidol and GBR-12909 (9–11). Perturbations in catecholaminergic signalling with chronic infection have been observed, with elevated DA metabolites in the cortex and decreased NE in the cortex and amygdala and loss of amphetamine-induced locomotor activity (12, 13). There are discrepancies in observations of changes in dopamine levels in the brain with *T. gondii* infection (14–18). Increased levels of dopamine in infected cells have been found when catecholaminergic cells are maintained at a physiological pH (19). Hence, in this study, we focussed on neuronal neurotransmission as affected during persistent infection examining gene expression changes for a biological

mechanism that can explain observed changes in NE and DA neurotransmission during CNS infection.

Results

Norepinephrine regulation in the brain during *T. gondii* infection

Initially, the effect of chronic infection on CNS NE and DA was monitored; measuring levels in the brains of *T. gondii*-infected animals. The level of NE was significantly decreased in infected animals ($p=0.0019$) with a reduction of $50\pm14\%$ in the brains (Figure 1A). This experiment and those that follow were performed with the Prugniaud strain unless otherwise stated. Decreased NE in *T. gondii*-infected mice has been observed in other studies (12, 14). The suppression observed with infection (Figure 1A) is analogous to decreases in CNS NE levels observed with high affinity DBH inhibitors (20). High doses of disulfiram and nepicastat, that have been used clinically, reduce brain NE levels by 36-45% (21, 22). Although NE was reduced with infection, the rats displayed no obvious signs of pathology. Rats with chronic *T. gondii* infections do not usually exhibit symptoms of illness (23). The median level of DA in the brains of infected rats was increased to double the uninfected level in this cohort, but this was not statistically significant (Figure 1B, $p=0.12$). These observations fit with other investigations, in which high DA levels were observed in cysts but brain tissue levels of DA were unchanged (16, 19, 24).

To assess whether the change in level of NE could also be observed in *in vitro* infections, , we performed infections with catecholaminergic cells. PC12 cells are fully functional to synthesize and package DA and NE for vesicle-mediated release upon stimulation, form dendritic extensions, and express dopamine receptors as a classic cell line model of

catecholaminergic neurons. We shocked Pruniaud tachyzoites with high pH to induce bradyzoite development prior to infection of cells as in previous studies (9, 19). As catecholamine synthesis by PC12 cells is sensitive to pH, this technique was used to maintain the full catecholamine biosynthetic capacity of the cells (25, 26).

NE and DA levels were measured in PC12 cells five days after parasite infection. NE levels were decreased in infected cultures to 62±6.1% ($p=0.0024$) of uninfected cell level (Figure 1C, 1E). The reduction in NE cannot be due to cell lysis as values are expressed relative to cell number. DA levels in infected PC12 cells were greater than uninfected cells ($p=0.0043$) in the same samples that exhibited suppression of NE (Figure 1D). The 3.8±0.74-fold increase is similar to that found in our previously published work with infected PC12 cells (9, 19). *In vitro* infection of catecholamine-producing cells reduced NE whilst elevating dopamine levels.

Regulation of the levels of NE and DA may be due to changes in synthesis, transport and storage, or degradation. Further, the mechanism(s) responsible for the opposing decrease in NE and increase in DA in catecholaminergic cells was unclear from these observations. Therefore, we examined the effects of the parasite on proteins expressed by the host neuronal cells.

Down-regulation of a key enzyme for norepinephrine synthesis during infection

The biological mechanism(s) responsible for the decreased NE with infection was investigated. Preliminary experiments with a genome scan of infected rat catecholaminergic cells for gene expression levels, identified that the most significantly altered expression was down-regulation of the dopamine β-hydroxylase (DBH) gene ($p= 7.2x10^{-13}$) (data not

shown). Although the results were preliminary, rat housekeeping gene expression (GAPDH, ribosomal proteins, tRNA ligases, tubulin) was unchanged whilst *T. gondii* bradyzoite genes (BAG1, LDH2, MAG1) were up-regulated (Table S1). We validated our preliminary data from the transcriptome scan with qRT-PCR of a collection of catecholamine biosynthesis and metabolism genes. The norepinephrine biosynthetic pathway is shown (Figure 2A). The only gene altered in expression in this set was down-regulation of DBH (Figure 2B). Although expression of the phenylalanine hydroxylase gene (PAH) appears reduced, this was not significant ($p=0.06$). Levels of mRNA for tyrosine hydroxylase, dopamine decarboxylase, monoamine oxidase A, and dopamine receptors D1 and D2 were unchanged with infection. The lack of change in rat tyrosine hydroxylase and dopamine decarboxylase gene expression with *T. gondii* infection corresponds with previously published data (9). Hence, DBH expression was specifically down-regulated in infected cells. This might not have been identified in transcriptomic studies published of whole infected brain tissue, that principally identified changes in expression of host immune response genes, with the mixture of cell types in the brain (27, 28). A recent transcriptomic study identified differentially expressed genes after only eighteen hours of infection (ie. during vegetative replication stages) in neural stem cells that expressed a range of markers for structural proteins found amongst different types of neurons and astrocytes (ref??). Hence, those results are difficult to compare with our approach using neuronal cells that are fully functional to synthesize and release (with potassium activation) DA and NE to investigate changes in expression of neuronal genes.

The change in DBH mRNA levels was observed over a time course of infection. Parasites were shocked with alkaline conditions in these (as described in the Methods) and the above

155 experiments to trigger bradyzoite differentiation. DBH gene expression decreased after
156 three days of infection and further after five days in PC12 cells (30±2-fold), relative to rat
157 GAPDH (p=0.0046) (Figure 2C). Microscopic analysis verified the maintenance of cell
158 numbers and viability during the time course experiments. The level of DBH mRNA in
159 uninfected PC12 cells was unchanged over the course of the experiment (one-way ANOVA,
160 p= 0.58).

161 To examine whether the silencing of DBH expression is a general response to *T. gondii*
162 infection, we investigated the effect of infection on a human neuronal cell line. The BE(2)-
163 M17 cell line was derived from a human neuroblastoma and possesses catecholaminergic
164 properties and neuritic processes. These cells were infected with Prugniaud strain *T. gondii*
165 in a similar fashion to the PC12 cells and samples taken after three and five days of infection.
166 Expression of the DBH gene was down-regulated 5.7±1.1-fold by day 3 of infection
167 (p=0.00032) and 17±1.4-fold by day 5 of infection (p=0.0010) (Figure 2D) relative to a
168 housekeeping gene. DBH levels were consistent in uninfected BE(2)-M17 cells throughout
169 the experiment (one-way ANOVA, p=0.97). We also found down-regulation of DBH present
170 using the the *T. gondii* ME49 strain in BE(2)-M17 cells (Supplemental Figure S1).

171 DBH is the key link between NE and DA, with DBH metabolizing DA into NE. Decreased DBH
172 will decrease synthesis of NE, and simultaneously increase levels of the precursor DA.
173 Suppression of DBH by down-regulated expression of its gene provides a mechanistic
174 explanation for the observed increase in DA in infected PC12 cells above (Figures 1C, 1D)
175 coincident with decreased levels of NE. DA was not significantly increased in infected rat

brains (Figure 1B), as might have been expected with the disproportionately smaller number of noradrenergic compared to dopaminergic neurons.

Dopamine β-hydroxylase expression is down-regulated in the brain with infection

We examined whether the down-regulation of DBH gene expression in neuronal cells was detectable during *in vivo* infection. The level of DBH expression in the infected brain was examined. DBH mRNA was quantified in the brains of chronically-infected male rats. Gene expression was down-regulated in infected animals by a median of 32±2.1-fold relative to uninfected animals (Figure 3A; p=0.0023). We examined the relationship between the intensity of brain infection and DBH expression. A strong negative correlation was observed in infected animals between DBH mRNA and cyst density (tissue cysts can contain thousands of bradyzoites), with a correlation coefficient of -0.90 (Table 1). The coefficient of determination (R^2) of 0.82 is a good fit for the linear regression.

DBH is expressed in noradrenergic neurons in the CNS, principally in the locus coeruleus (LC) with efferents extending to most brain regions. Therefore, we examined DBH gene expression in different brain regions in infected animals. DBH mRNA levels were lower (p=0.0034 and 0.012, respectively) in the frontal lobe (prefrontal cortex (PFC)) and the dorsal region (containing the LC, cerebellum, pons, and surrounding tissue) in infected animals. DBH expression was unchanged in the midbrain region containing the hippocampus, thalamus and hypothalamus (p=0.93) (Figure 3B). Hence, the posterior area and the PFC had 2.5-fold and 4.5-fold, respectively, lower DBH mRNA in infected rats.

One plausible alternative explanation for the decrease in NE in the infected rat brains could be poor neuronal health or neuronal death. *T. gondii* can lyse neurons and synaptic loss and

198 neuronal dysfunction has been observed in infected mice (29). In this study, we found no
199 difference in neurons between infected and uninfected rats based on quantification of a
200 neuron-specific mRNA, that encoding microtubule-associated protein 2 (MAP2) (Figure 3C;
201 p= 0.57).

202 **Effect of Sex on Altered Norepinephrine Regulation with Infection**

203 An intriguing observation during these studies was the finding that females did not exhibit
204 the down-regulation of DBH. We noted a large range of DBH mRNA levels in the brains of
205 female animals as an anomaly that could mask an effect by infection. Indeed, infected
206 females did not exhibit a measurably lower level of DBH (Figure 4A, p=0.45) with infected
207 females possessing higher and lower DBH mRNA levels than vehicle controls (Table 1). A
208 similar finding was observed with infected mice in which CNS levels of DBH mRNA in males
209 were significantly down-regulated (p= 0.0032, n=26) whereas the levels were unchanged in
210 females (p=0.85, n=16) (Supplemental data Fig S2).

211 We investigated the reasons for this difference. DBH gene expression is regulated by
212 estrogen, with the estrogen receptor binding to ER-response elements (ERE) at the 5'
213 flanking region of the DBH gene and activating transcription (30, 31). Estrogen, estrogen
214 receptor and DBH mRNA levels fluctuate during the estrous cycle (32). Hence, we measured
215 the levels of estrogen receptor 1 (ESR1) mRNA in the brains of the female rats used in this
216 study.

217 A range of ESR1 levels were observed in the brains of the female rats, indicative of
218 differences in their estrous cycle (Table 1). Expression of ESR1 was not altered by infection
219 (Figure 4B, p=0.40). ESR1 mRNA levels, however, strongly correlated with DBH mRNA

(Figure 4C), with a correlation coefficient of 0.86 ($p=0.0064$), as expected (32). Together, the findings show that DBH expression correlated with ESR1 expression but not infection in females.

These findings provide a biological basis for previously observed sex-specific differences in the effect of *T. gondii* infection on mouse behavior and estrous-dependence of aversive behaviors in female rats (33, 34).

Dopamine β-hydroxylase expression in cytomegalovirus infected human neuronal cells

To test whether DBH down-regulation is a general response to chronic CNS infection or whether it is specific, changes in DBH gene expression in human neuronal cells infected with human cytomegalovirus (HCMV) were measured. DBH mRNA levels were not significantly changed over a time course of HCMV infection in BE(2)-M17 cells ($p>0.13$), with a trend for increased expression at 48 hours (Figure 5A). At this point, HCMV is entering the late stages of viral replication (as indicated by the immediate-early UL123 gene expression in Figure 5B) and yet the data clearly show HCMV infection does not decrease DBH expression. In comparison, DBH gene expression was down-regulated (relative to the marker) in the same cells infected with *T. gondii*, with DBH decreasing over the time course of the experiment (Figure 5C) and a small increase in *T. gondii* (Figure 5D). Hence, DBH down-regulation is specific for *T. gondii* infection.

Suppressed dopamine β-hydroxylase alters norepinephrine-linked behaviors

A decrease in CNS NE, as observed with *T. gondii* infection (Figure 1A), may have specific effects on behavior. Arousal and sociability are associated with CNS noradrenergic signalling (35, 36). Rodents with NE deficiency exhibit lower arousal and increased sociability.

Arousal is measured as a response to evoked or elicited activity and has been quantified in rodents by locomotion in a novel environment, such as an open field, at early time points in the experiment (37). Locomotion was recorded over 1-min intervals for the initial five minutes for chronically-infected and uninfected mice in an open field apparatus, then over 5-min intervals to 15 minutes. The mice were individually placed in the open field and allowed to settle for 60 seconds (minute 1), while the experimenter withdrew from the apparatus, before readings were taken. *T. gondii* infected mice exhibited decreased locomotor activity in the open field at early time points but not at later times (Figure 6). Uninfected mice travelled one and a half times the distance in the 60-120 and 120-180 second intervals, compared to infected animals travelled. This represented a significant difference ($p<0.0001$ and 0.0015, respectively, for each reading). Representative tracking of uninfected and control mice illustrates the decreased locomotor activity during early time points (Figure 6C). The tracking in the figure also replicates the loss of fear of open spaces found in prior studies of *T. gondii*-infected rodents (38). In contrast to early timepoints, infected and control groups showed similar levels of activity in the open field after the three minute timepoints. In the 5-min intervals from minutes 5-15 ambulation was not different, matching prior studies of locomotion in *T. gondii*-infected rodents monitored over longer periods (circa 30 minutes) (38–40). Changes in initial behavioral response or arousal would not have been observed in these earlier studies that did report mobility in 1-minute

intervals. The DBH mRNA levels in the mice exhibited a correlation with early locomotor activity (Supplemental Fig S3). Published studies of *Dbh*-/- knockout mice have described attenuated arousal and decreased locomotion, similar to that observed here, in ambulation in an open field at early time points (35, 36).

Cerebral NE levels have been associated with social interest and male aggression (22). Aggressive behavior is decreased and social memory altered in *Dbh*-/- knockout mice (35). In this study, the three-chambered social approach test was used to measure sociability in uninfected and *T. gondii*-infected mice. This test is a well-established sensitive model for measuring social interactions in mouse models of autism (41). In the first phase of the social approach test, which measures sociability, preference for exploring a cylinder containing a stranger mouse rather than an empty cylinder was measured (42). Chronically-infected mice explored the novel mouse for nearly one and half times longer than the uninfected mice (Supplemental Fig S4). Infection has previously been associated with social interaction, with *T. gondii*-infected rats exhibiting a longer duration of social interaction than controls (43). In Phase 2 of the social approach test, which measures preference for social novelty, mice encountered the Stranger 1 mouse (the now familiar mouse) as well as a novel mouse (Stranger 2) in the formerly empty cylinder. Both uninfected and infected mice investigated the novel stranger, but the infected mice significantly one and a half times longer in contact with the novel stranger with a correlation, albeit weak, with DBH mRNA levels (Supplementary Fig S4; $p=0.025$).

Discussion

In contrast to prior studies of the effect of *T. gondii* on neurotransmission in neurons, this study identified DBH gene regulation as the mechanism responsible for observed changes in norepinephrine and, in vitro, dopamine (9, 12–14, 19). Changes in GABA and glutamate metabolism in the CNS of chronically-infected animals have previously been observed with the distribution of the GABA-associated protein GAD67 altered and (44) and GLT-1 expression in astrocytes reduced to half (45). The change in DBH expression observed in this study may provide a mechanism to explain, at least in part, diverse observations of CNS catecholamines with infection and behaviours associated with infection.

The sex-specific down-regulation of DBH (Figures 3 and 4) may provide some insight to gender differences in behavioural changes with infection. DBH expression is regulated by estrogen in females, as found in study (Figure 4).

The down-regulation of DBH expression provides an explanation for the observed decreases in NE in infected brains without a significant increase in DA in brain tissue observed in this and some prior studies (Figure 1). This observation is not surprising given the small proportion of noradrenergic relative to dopaminergic neurons in the brain. This, combined with the more severe pathology of *T. gondii* infection in mice with dysfunctional neurons, provide a possible explanation why this and other studies did not detect changes in total brain DA levels with infection (12, 16, 24, 29, 46). In vitro, the down-regulation of DBH found in this study can account, at least in part, for increased DA levels observed in infected PC12 cells observed in earlier studies (9, 19). In those studies, the amount of DA increased with infection while levels of the enzymes in synthesis, tyrosine hydroxylase and

dopa decarboxylase were unchanged, although dopa decarboxylase could be detected in the parasitophorous vacuole. *T. gondii* contains two paralogous genes that encode an aromatic amino acid hydroxlase (TgAAAH), with tyrosine and phenylalanine hydroxylase activities, that is secreted from the parasites into the parasitophorous vacuole (47). Both paralogs were found to be expressed in bradyzoites, whereas only TgAAAH1 was expressed in tachyzoites. The gene products have been found to be involved in oocyst development as proposed in their original discovery (47, 48). The effects of disruption of one of the two paralogs on catecholamine neurotransmission remain inconclusive; hence, collaborative experiments using the recently developed double knockout mutants lacking both genes are ongoing (48).

Noradrenergic neurons are principally located in the locus coeruleus (LC) in the brain and project to the thalamus, hippocampus and the frontal and entorhinal cortices (49), and combined with recent findings of efferent noradrenergic neurons originating in the LC releasing DA in the dorsal hippocampus can modulate a wide range of behaviors (50, 51). *T. gondii* cysts have been observed in these brain regions (52, 53). In this study, changes were observed in noradrenaline-related behaviors of arousal and social interactions (Figures 6 and supplemental data). Previously, down-regulation of the noradrenergic system has been observed to change social behavior with DBH knockout mice displaying increased sociability with lower aggression and social memory as well as reduced anxiety (35). Chronic *T. gondii* infection has also been found to impair long-term fear memory, a process that NE enhances (12, 54). Although one could attempt to reverse the parasite-induced effects on noradrenaline-related behaviors with noradrenergic inhibitors, antipsychotic drugs have antiparasitic effects (24, 55, 56), and L-threo-3,4-

dihydroxyphenylserine cannot be used because the required dopa decarboxylase for activation is altered by *T. gondii* infection (9, 57).

There is a link between NE levels, *T. gondii* infection and movement and coordination of the host. Both *Dbh*-/- knockout in mice and noradrenergic neuron loss in the LC (in rats) lead to motor impairments and development of dyskinesia (58, 59). Further, mice lacking NE are susceptible to seizures (60, 61). Chronic infection with *T. gondii* in mice has also been associated with coordination difficulties (62), and loss of coordination is a common symptom of human toxoplasmosis. Severe toxoplasmosis can cause seizures, with documented cases of patients exhibiting Parkinsonian traits such as bradykinesia (63, 64). Effects of altered GABA metabolism with *T. gondii* infection (observed in an earlier study) in promoting seizures would be compounded by a lack of anticonvulsant effect promulgated by NE (44).

DBH gene expression correlated with the intensity of infection but the low number of neurons that are infected *in vivo* is difficult to reconcile with the large decrease in DBH expression (65). This global effect in *in vivo* infection is similar to that observed in GAD67 (glutamic acid decarboxylase) distribution in the brains of *T. gondii*-infected mice (44). The neuroimmune response may be involved although DBH was down-regulated in infected PC12 cells *in vitro*. Global changes could be mediated by injection of parasite proteins into cells without infecting the cells, as has been observed with neurons in infected mice (7, 66). The mechanism responsible for the global changes is the subject of ongoing studies.

In summary, infection of the CNS influences brain neurophysiology with *T. gondii* infection decreasing NE levels through down-regulating DBH gene expression. The regulation of DBH

by estrogen may explain sex specific effects of infection as indeed DBH was not down-regulated in infected females. Down-regulation of DBH whilst suppressing NE can elevate DA in the same neurons. The consequential effects on neurological signalling of these alterations will be the subject of future studies as they depend upon the location of the noradrenergic neurons and dopamine receptors. The mechanism(s) whereby the parasite down-regulates DBH expression needs clarification. This may be via a parasite mechanism similar to *T. gondii* ROP18 altering JAK/STAT signaling pathways or via the regulation of vasopressin receptor by epigenetic changes (67, 68). The neurophysiological changes observed may provide insights into the mechanisms responsible for behavioral effects of *T. gondii* infection (69).

Materials and Methods

Ethics

All procedures were approved by the University of Leeds Animal Ethical and Welfare Review Board and performed under United Kingdom Home Office Project and Personal Licences in accordance with the Animals (Scientific Procedures) Act, 1986. Rat brain sections were from infections conducted at the School of Public Health, Imperial College London (ICL) and procedures were approved by the ICL Animal Care and Use Committee and following the same Home Office, HSE, regulations and guidelines. Considerations of replacement, reduction, and refinement were taken in the use of animals for research.

Rodent and rodent infections

372 The (BALB/cAnNCrl x C57BL/6NCrl)F_1 mice used in this study were bred by crossing
373 C57BL/6NCrl males to BALB/cAnNCrl females (Charles River Laboratories). The C57BL/6
374 inbred strain has been used as the genetic background in prior behavioral studies of *Dbh*-/-
375 knockout mice, while the BALC/c inbred strain possesses genetic resistance to control *T.*
376 *gondii* brain infection and develops a latent chronic infection (22). In pilot studies, purebred
377 C57BL/6NCrl mice infected with *T. gondii* showed severe toxoplasmic encephalitis.

378 Mice were housed five of the same sex per cage, with *ad libitum* access to food pellets and
379 water. Mice were checked for health changes daily and their weight was measured weekly.
380 Any mouse showing severe illness or significant weight loss (25%) was promptly culled.
381 Mice were grouped according to treatment. Mice were infected by intraperitoneal (IP)
382 injection with *T. gondii* type II strain Prugniaud in sterile phosphate-buffered saline (PBS)
383 at 6–14 weeks of age. Infection was monitored by the direct agglutination test (BioMérieux)
384 to detect *Toxoplasma* antibodies, following the manufacturer's instructions, in sera from
385 collected blood samples. Brains were harvested from euthanized animals and snap frozen.
386 Cryosectioned slices were used for RNA isolation as described for rats below.

387 Rat samples were from Lister Hooded rats (Harlan UK Ltd), males and females housed
388 separately and provided food and water *ad libitum*, that were infected at approximately 3
389 months of age via IP injection of 1 x 10^6 tachyzoites in sterile PBS. Uninfected control rats
390 were IP injected with sterile PBS and sacrificed 5-6 months post-infection, with brains
391 quick-frozen for cryosectioning. Sagittal slices were processed for RNA by dissolution with
392 Trizol™ (Thermo Fisher) for processing following manufacturer's instructions.

393 **Growth of pathogens and cultured cells**

394 The *T. gondii* Prugniaud strain was maintained in human foreskin fibroblast cell line Hs27
395 (ECACC 94041901), as previously described (47). Rat adrenal phaeochromocytoma (PC-12)
396 cells (kind gift from C. Peers; ECACC 88022401) were maintained in RPMI (Invitrogen,
397 Paisley, UK), supplemented with 10% horse serum (Invitrogen), 5% fetal bovine serum
398 (FBS; Invitrogen), and 100 units/ml penicillin/streptomycin (Sigma, Poole, UK). PC-12 cells
399 were passaged by triturating, centrifuging 800 rpm for 10 min in a table top centrifuge,
400 resuspending in fresh media and incubating at 37°C in an atmosphere of 5% CO_2. The
401 BE(2)-M17 cells (kind gift from R. Wade-Martins, Oxford University) were maintained in a
402 1:1 ratio of F12 Hams to OptiMEM (GIBCO, USA) media supplemented with 10% horse
403 serum (GIBCO, USA), 5% FBS (GIBCO, USA) and 100units/mL penicillin streptomycin
404 (Sigma, USA) and incubation in 5% carbon dioxide and 37°C.

405 For the induction of parasite conversion to bradyzoite forms, free released tachyzoites were
406 incubated at 37°C in RPMI supplemented with 1% FBS (pH 8.2) for 16-18 hours (hr) in
407 ambient air then diluted with DMEM (Invitrogen), isolated by centrifugation, and
408 suspended in RPMI (pH 7.4) containing horse serum, FBS and penicillin/streptomycin, as
409 previously described (19). This method was developed because catecholamine-producing
410 cells were found to be sensitive to pH changes severely reducing their production of
411 catecholamines. The parasite number was determined by microscopy and an equal number
412 of treated tachyzoites to cells was used for infections, unless otherwise stated. The viability
413 and differentiation of parasites in PC12 and BE(2)-M17 cultures was monitored by qRT-
414 PCR (as described below) with *T. gondii* markers for GAPDH, tachyzoites (SAG1), and
415 bradyzoites (SAG4 and BAG1) (Supplementary Figure S5).

For HCMV studies, cells were infected with wild type Merlin HCMV strain for 1 hour then washed and incubated with fresh media. RNA was harvested at the times shown. Cells were confirmed permissive for HCMV by IE antigen staining, which demonstrated similar susceptibility for infection as the neuronal cell line U-373, an established permissive HCMV cell line.

Transcriptome analysis

A transcriptome screen was conducted to assert genes that are potentially differtially expressed with infection. PC-12 cells were cultured in poly-D-lysine-coated 6-well plates (Sigma). Following 24 hours of incubation, 6 x 10^4 cells were changed to medium with 1% horse serum, 0.5% FBS. After a further 24 hr, 100 ng/ml of Nerve Growth Factor (NGF; Sigma) was added. The addition of NGF was repeated once every 24 hr throughout the length of the experiment. Control experiments found no effect of NGF on growth or bradyzoite conversion of *T. gondii* (data not shown). After 72 hr from the initial addition of NGF, dendritic extensions were visible from differentiated cells. At this point, induced Prugniaud tachyzoites were transferred to each well, maintaining a parasite density of 2.5 x 10^4 cells/ml. Cells were harvested immediately following infection (day 0) and after three and six days of infection for RNA extraction. The cultures were monitored daily by light microscopy. At day 6 of infection, the parasitaemia level was 60-70%, with little observable cell lysis (data not shown).

Cells were detached from the surfaces by manual removal with a scraper and several parallel biological repeats were pooled. The suspended cells were pelleted by centrifugation at 800xg for 10 minutes and lysed with TRI Reagent solution (Invitrogen) followed by

centrifugation at 12,000xg for 10 minutes at 4°C. RNA was purified following manufacturer's instructions. RNA samples were stored at -80°C.

mRNA was enriched using a Poly(A)Purist™ MAG Kit (Ambion) followed by further enrichment using RiboMinus™(Ambion), following manufacturer's instructions. Following quality control analysis using a Bioanalyzer (Agilent), cDNA libraries were prepared from RNA using the Epicentre ScriptSeq v2 RNA-Seq Library Preparation Kit and sequenced using the Illumina Hiseq 2000 at the University of Liverpool Centre for Genomic Research. Two libraries for each pool of biological repeats of infected and uninfected cells at the three timepoints were sequenced. RNA sequencing generated 353m paired-end reads, with a total of 26,405 *Rattus norvegicus* genes identified.

The Illumina reads from the RNA sequencing were separately mapped to *Rattus norvegicus* and *Toxoplasma gondii* reference genomes using Tophat 2.0.8b (70). Differential expression analyses were performed using edgeR package version 3.0.4 (71) for the reads aligned to the rat genome. The reads that aligned with the *T. gondii* genome were analysed for bradyzoite markers (Table S1). A gene was considered as differentially expressed (DE) if the fold change was greater than two ($-1 > \log2(\text{fold change}) > 1$) and the False discovery rate < 0.01 (maximum false positive genes are 1% of the genes). The resultant 488 genes form a set of DE genes that exhibit down- or up-regulation. The enriched GO (Biological Process) and KEGG pathway terms for up- and down-regulated gene sets were computed using DAVID and are tabulated in Table S2 (72).

Reverse transcriptase PCR and quantitative PCR

For RT-qPCR assays, cultures of 2.5 x 10^4 PC12 or BE(2)-M17 cells in multiwell plates were infected with induced *T. gondii* tachyzoites. Cells were recovered by centrifugation and the cell pellet frozen (-80°C) for RNA extraction and HPLC-ED analysis.

RNA was purified using Direct-zol™ (Zymo) and reverse transcribed to cDNA using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher), following manufacturer's instructions. RT-qPCR was performed on RNA, as described previously, using SYBR® Green Real-Time PCR Master Mix (Thermo Fisher) using rat GAPDH primers (Qiagen), DDC primers 5'-CGGAGAAGAGGGAAGGAGATGGT-3′ and 5'-GCCGTGGGGAAGTAAGCGAAG-3′, TH primers 5'-CCCAAAGTCTCCATCCCCTTC-3' and 5'- GGTTGAGAAGCAGTGTTGGGA-3', MoaA primers 5'-GTGTGG GAGGCAGGACTTAC-3' and 5'-CTGGCGAATCACCCTTCC-3'; PAH 5'-CTGGGGAACGGTGTTCAGGA-3' and 5'-TCTTCACGGAAACCGCAGTA-3'; DRD1 primers 5'-CAAGTCCCCGGAAGTGTG-3' and 5'-CAGGTGTCGAAACCGGATG-3', DBH primers 5'-CCACAATCCGGAATATA-3' and 5'-GATGCCTGCCTCATTGGG-3', and ESR primers 5'-CTACGCTGTACGCGACAC-3' and 5'-CCATTCTGGCGTCGATTG-3'.

HPLC for monoamines

The catecholamines DA and NE were measured by HPLC-ED, adapting a previously published method (19). Briefly, cultures were harvested by scraping cells, recovered by centrifugation, and an aliquot taken for cell counting and normalization. The remaining cells were recovered again and resuspended in 350 µL of perchloric acid, followed by sonication. The mixture was centrifuged at 14,000 rpm for 15 minutes at 4°C to remove particulates, and an aliquot was taken for HPLC analysis. NE was detected at 4.5 minutes and DA at 8

minutes (flow rate 0.4ml/min) by HPLC-ED on a Dionex UltiMate 3000 system (Thermo Fisher).

Mouse Behavioral Testing

After establishment of chronic infection (4-5 weeks), mice were tested in a battery of behavioral tests in the following order, with an interval of 2 days between each test: open field > marble burying > social approach. Prior to testing, mice were habituated to handling for 5 minutes per day for 7 days. Ethanol (70%) was used to clean the arena between mice. The arena was left to dry for 3-4 minutes before commencing the next subject.

Open Field Test

The internal open field arena had a diameter of 40 x 40 cm with a semi-transparent Perspex wall. The arena floor was white plastic. To prevent the mice from seeing the surrounding room, a cylinder of white card was placed around the arena 30 cm away from its walls. The ambulation of the mice was recorded using a webcam that was placed on a tripod above the arena.

Mice were individually placed at the centre of the arena facing the same wall. Readings began after the initial 60 seconds because of disturbances involved in the experimenter removing mice from their cages, placing them in the open field and withdrawing to a computer to manually start the recording. Distance travelled was recorded for 15 minutes without interruptions or intervals. using AnyMaze tracking software (Stoelting Co.).

Social Approach

Sociability was assessed using a three-chambered arena (60 x 40 cm) that had two openings (7 x 8 cm) to allow the mouse access to the left and right chambers from the central chamber (each chamber measured 40 x 20 cm). The test involved using two unfamiliar mice that had been habituated to stainless steel cylinders (10 cm W x 10.5 cm H) prior to the test. The cylinders were made of vertical metal bars separated by 9 mm, which allowed air exchange and increased the possibility of contact between the test and stranger mice.

Following a previously published protocol (41), a test mouse was placed into the central chamber of the three-chambered arena. The 'habituation' stage was carried out for 15 minutes; at the end of this time, the test mouse was moved to the central chamber and the openings to the side chambers were blocked by guillotine doors. A cylinder was placed in both the right and the left chamber. A stranger mouse ('stranger 1', a young male C57BL/6NCrl) was placed in the cylinder in either the left or right chamber (balanced between treatment groups). Following this, the doors were removed and 'phase 1' was initiated, lasting 10 minutes.

Social approach was scored when the test mouse's nose poked through the bars of either the cylinder containing stranger 1 or the empty cylinder. At the end of phase 1, the test mouse was placed in the central chamber and the doors were shut. Then, a new unfamiliar mouse ('stranger 2') was placed in the formerly empty cylinder. At this point, phase 2 was initiated, again lasting for 10 minutes. Social approach was scored when the test mouse's nose poked through the bars of either the cylinder containing stranger 1 or the cylinder

containing stranger 2. The cylinders and floor were then wiped clean with 70% ethanol. The experimenter wore nitrile gloves throughout the procedure.

Statistical Analysis

GraphPad Prism (Version 7) was used for statistical analyses. Unless otherwise stated datasets were compared using Student's T-test with p value calculated. All data are plotted as mean ± SEM.

Competing financial interests statement

There are no competing financial interests for the authors.

Authors' contributions

The main manuscript text was written by I.A., E.T. and G.M., with input from all authors. I.A. and E.T. contributed equally to this study. Experiments were performed and figures and tables prepared by I.A., E.T., M.A., G.B. and M.S.V. I.A., G.M. and J.W. contributed to the conceptualization and experimental planning. M.R. is supported by MRC Fellowship G:0900466. The Stanley Medical Research Institute supported early components of this study.

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Figure legends

Figure 1: Catecholamine levels with *T. gondii infection* in the brain and catecholaminergic cells. A) A graph of the norepinephrine concentration in the brains of uninfected and infected rats with each point representing one animal and bar showing the mean and +/- SEM (p=0.0019, Student's t-test; n=6 infected and 6 mock-infected animals). B) Dopamine levels in the brains of the same uninfected and infected rats shown graphically (p=0.12, Student's t-test t). C) Norepinephrine levels in uninfected and infected catecholaminergic PC12 cells at day 5 of infection; p=0.0024, n=3 biological replicates. D) Levels of dopamine in the same infected PC12 cells plotted as above. p=0.0043, n= 3 biological replicates with triplicate readings. E) Overlay of chromatograms from HPLC-ED of uninfected and infected PC12 cells.

Figure 2: Norepinephrine biosynthesis in catecholaminergic cells with *T. gondii* infection. A) Dopamine and norepinephrine biosynthetic pathway showing synthesis from tyrosine. DBH, dopamine β-hydroxylase; AADC, aromatic amino acid decarboxylase (also DDC); TH, tyrosine hydroxylase. Reactions in which dopamine and/or norepinephrine are bound (e.g. receptors dopamine receptor D1 (DRD1), dopamine receptor D2 (DRD2)) or degraded (e.g. monoamine oxidase A (MaoA)) are not included in this schematic. B) Expression of the set of catecholaminergic genes during infection (black) or uninfected (grey). Only the DBH gene expression was significantly altered by infection (n=3 biological replicates with triplicate readings, ***, p=0.008). The abbreviations are as above as well as PAH, phenylalanine hydroxylase. Error bars are ±SEM. C) Dopamine ß-hydroxylase mRNA levels during a time course of infection (black) relative to uninfected (grey) PC12 catecholaminergic cells

773 relative to a rat housekeeping gene. **, p=0.0046, n=3 biological replicates. D) Plot of the
774 level of DBH mRNA in a human BE(2)-M17 neuronal cells over a time course of infection
775 relative to a human GAPDH showing that *T. gondii* induces DBH down-regulation in rat and
776 human neuronal cells. **, p=0.0010; ***, p=0.00032; n=3 biological replicates.

777 Figure 3: Infection down-regulates dopamine ß-hydroxylase gene expression in the brain. A)
778 DBH gene expression in the brains of uninfected (grey) and chronically-infected (black)
779 male rats plotted relative to GAPDH (p=0.0023, n=4 uninfected and 5 infected animals). B)
780 Brain region specific DBH gene expression in uninfected and infected rats. PFC, prefrontal
781 cortex; LC, locus coeruleus. Error bars are ±SEM. *, p=0.012; **, p=0.0034, n=4 uninfected
782 and infected animals. C) Plot showing expression of the neuronal MAP2 gene (as a
783 percentage of GAPDH) in uninfected (grey) and chronically-infected (black) brains for the
784 animals in A (p=0.57).

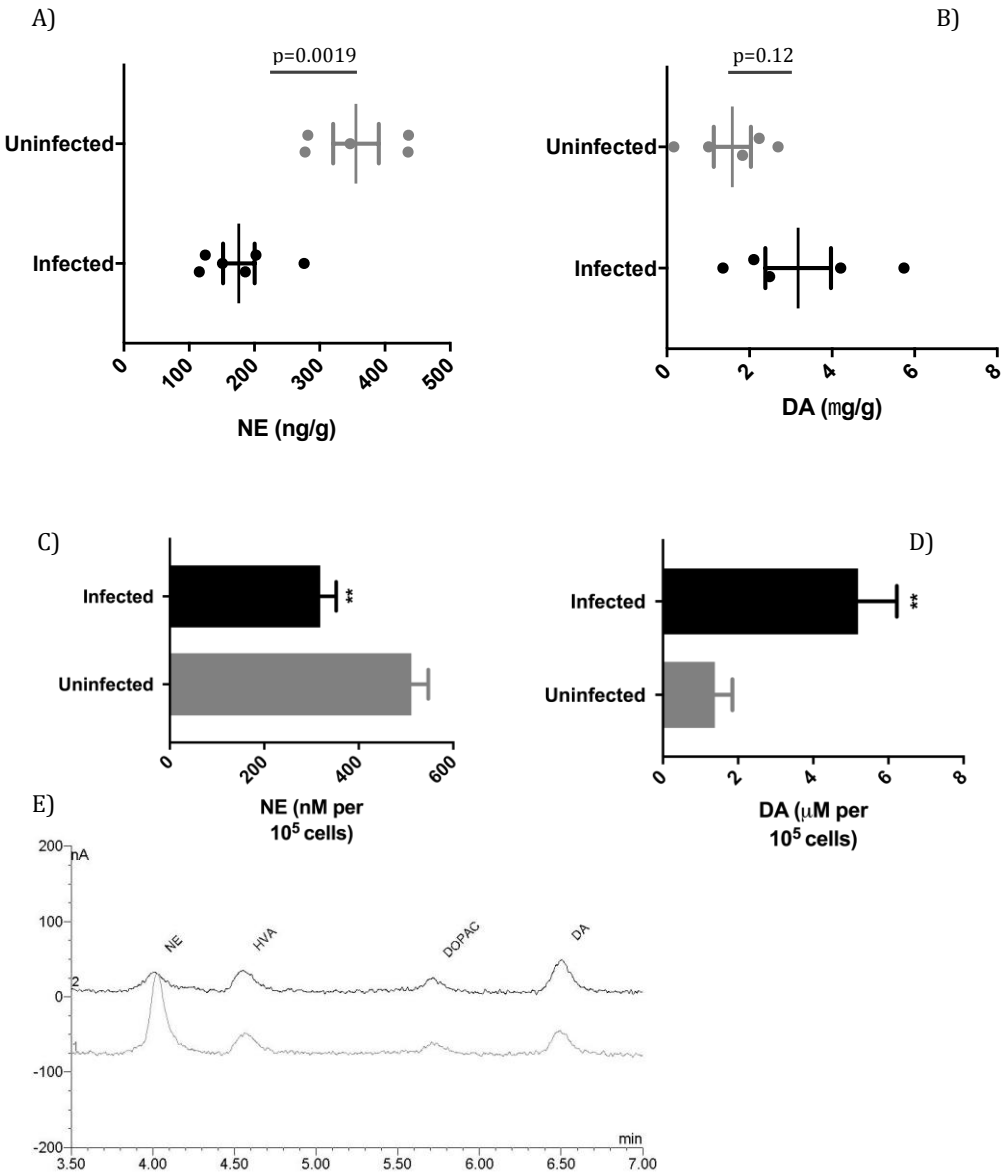
785 Figure 4: Dopamine ß-hydroxylase expression was not suppressed in infected females. A) A
786 plot of DBH mRNA in the brains of uninfected (grey) and chronically infected (black) female
787 rats is plotted (±SEM; n=3 uninfected and 5 infected animals; p=0.45). B) The expression of
788 estradiol receptor 1 (ESR1) gene in brains the same female rats shown graphically (±SEM;
789 p=0.40) and correlation of DBH versus ESR1 gene expression in the brains (Pearson's
790 correlation coefficient = 0.86).

791 Figure 5: Dopamine ß-hydroxylase suppression is pathogen-specific. A) Plot of DBH gene
792 expression over a time course of 48 hours. Uninfected (grey) and human cytomegalovirus
793 (CMV) infected (black) human BE(2)-M17 neuronal cell line, shown as a percentage of the
794 housekeeping gene; n=2 biological repeats of triplicate measures. B) Accumulation of HCMV

UL123 immediate-early (IE) as percent gene expression (normalized to GAPDH) over a time course. C) Plot shows DBH expression over a similar time course for uninfected (grey) and *T. gondii* infected (black) human neuronal cells, as a percentage of the housekeeping gene. ***, p=0.0015 and 0.0012, respectively, n=3 biological repeats with triplicate measures; error bars indicate SEM. D) The intensity of *T. gondii* infection over the time course based on levels of *T. gondii* actin plotted as a percentage of host GAPDH.

Figure 6: Locomotion and anxiety-related behaviour are altered in infected animals. A) Ambulation of uninfected (grey) and infected mice (black) in the open field at single minute timepoints with the mean. **, p=0.0015; ***, p=0.000097; n= 24 uninfected and 27 infected mice. B) Graph of distance moved for each mouse over the 15 minute time course of the experiment plotted as a box plot with whiskers representing min and max at single minute timepoints followed by five minute timepoints,. **, p=0.0015; ***, p=0.000097. C) Tracking in the open field for representative uninfected (top) and infected (bottom) mice from 0-180 seconds of the trial.

Figure 1

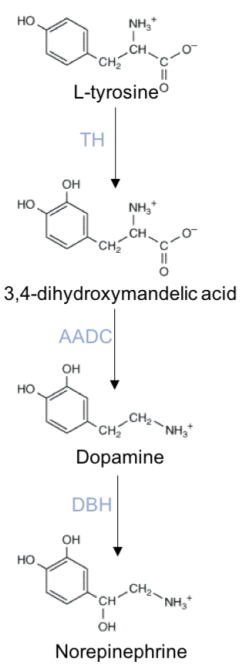


infected

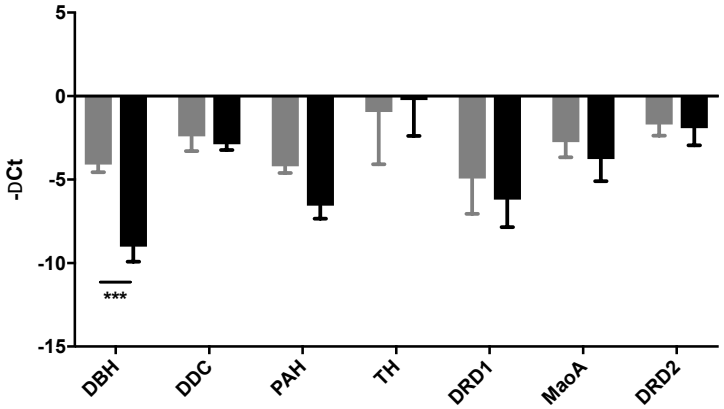
uninfected

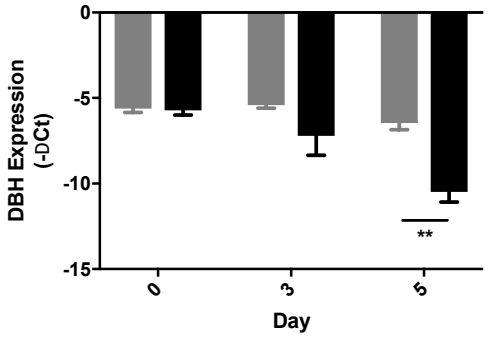
Figure 2

A)


L-tyrosine
TH
3,4-dihydroxymandelic acid
AADC
Dopamine
DBH
Norepinephrine

B)


5
0
-5
-10
-15
-DCt
***
DBH
DDC
PAH
TH
DRD1
MaoA
DRD2


0
-5
-10
-15
DBH Expression
(-DCt)
**
0
3
5
Day

C)

D)

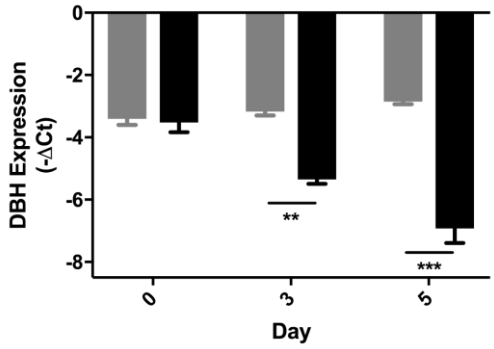
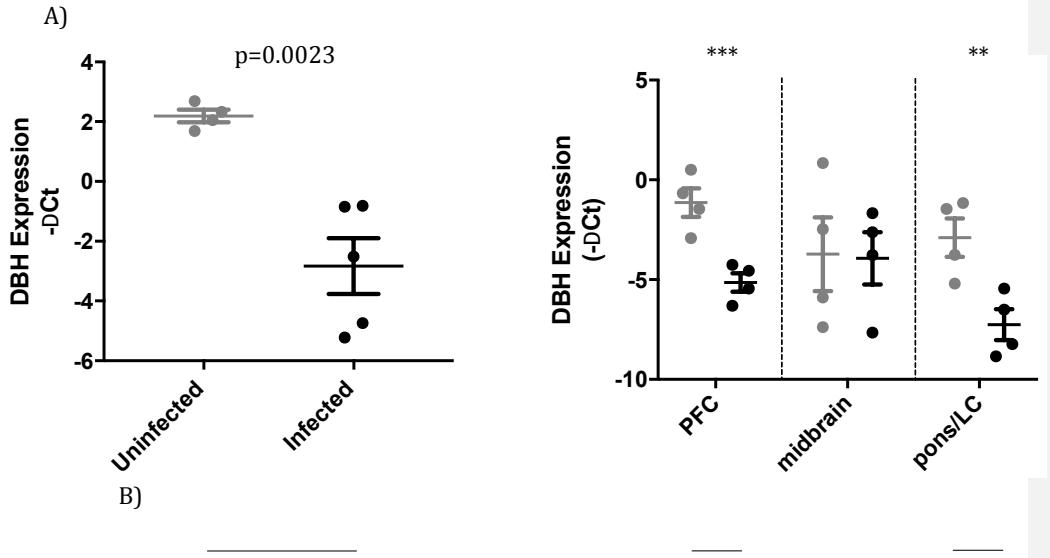
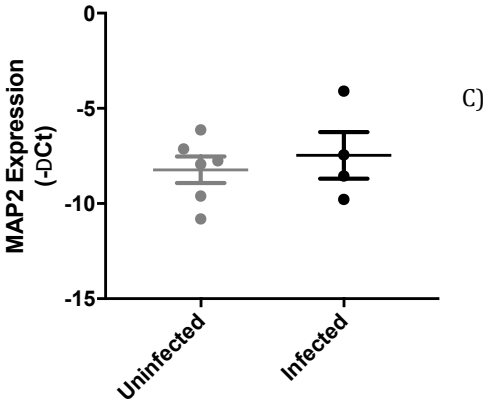

0
-2
-4
-6
-8
DBH Expression
(-ΔCt)
**
***
0
3
5
Day

Figure 3

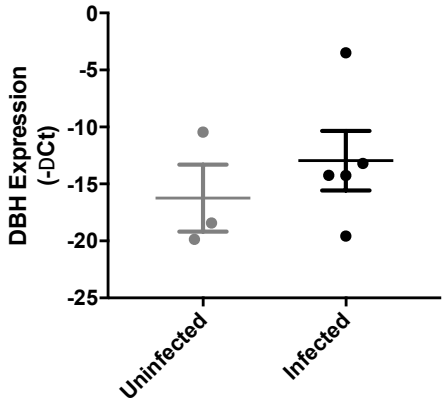

A)
p=0.0023
DBH Expression
-DCt
4
2
0
-2
-4
-6
Uninfected
Infected
***
**
DBH Expression
(-DCt)
5
0
-5
-10
PFC
midbrain
pons/LC
B)


MAP2 Expression
(-DCt)
0
-5
-10
-15
Uninfected
Infected

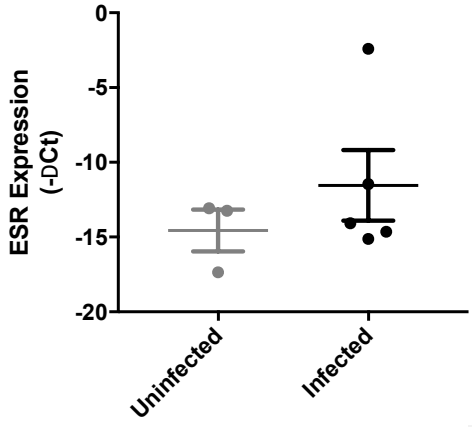
C)

Figure 4

A)


DBH Expression
(-DCt)
0
-5
-10
-15
-20
-25
Uninfected
Infected

B)


ESR Expression
(-DCt)
0
-5
-10
-15
-20
Uninfected
Infected

C)

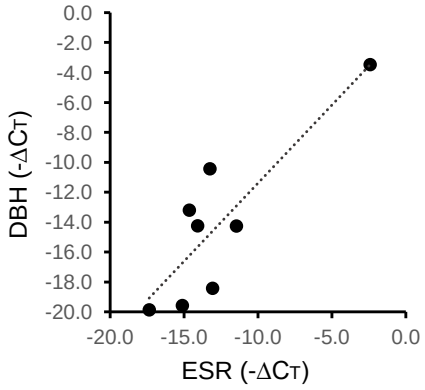
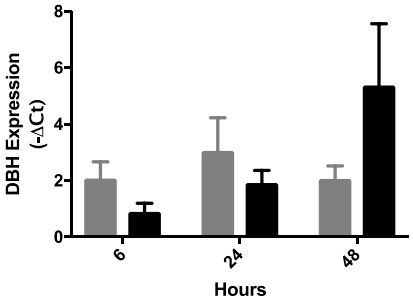
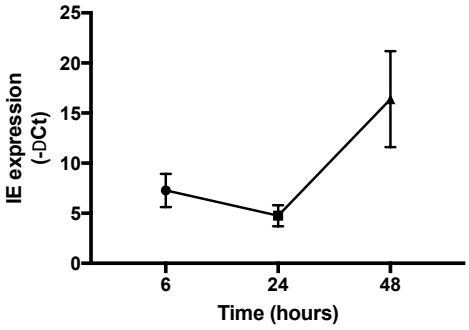

DBH (-ΔCT)
0.0
-2.0
-4.0
-6.0
-8.0
-10.0
-12.0
-14.0
-16.0
-18.0
-20.0
-20.0
-15.0
-10.0
-5.0
0.0
ESR (-ΔCT)

Figure 5



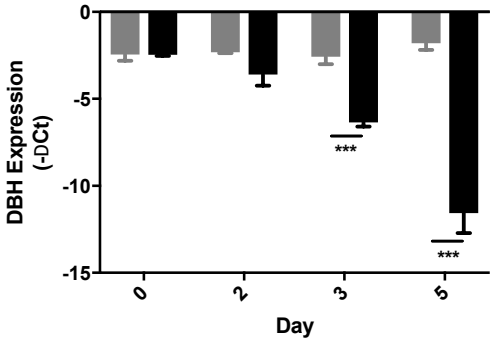
A)



B)

C)

D)



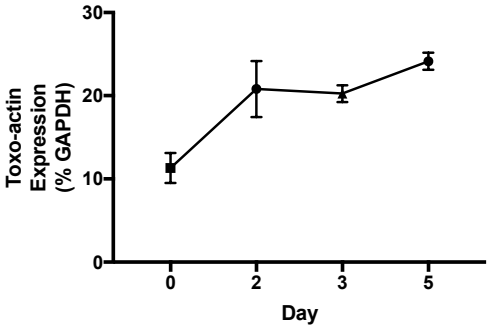
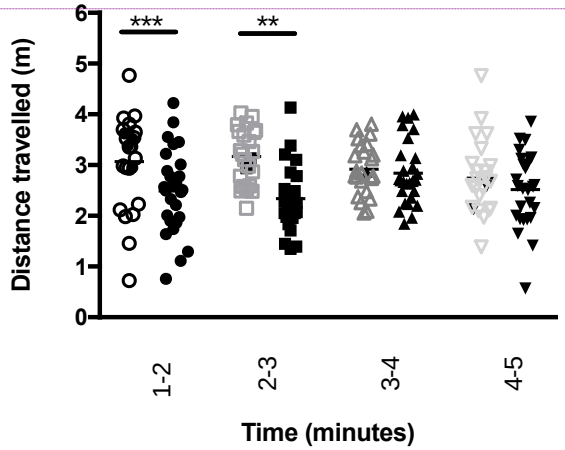
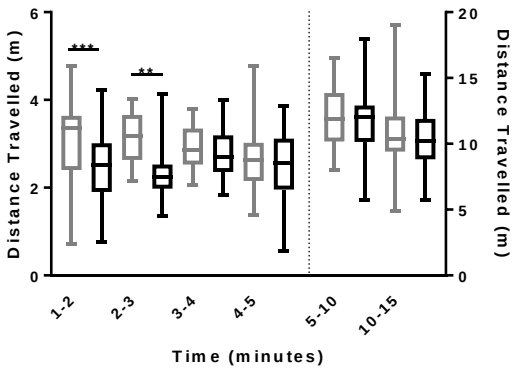


Figure 6

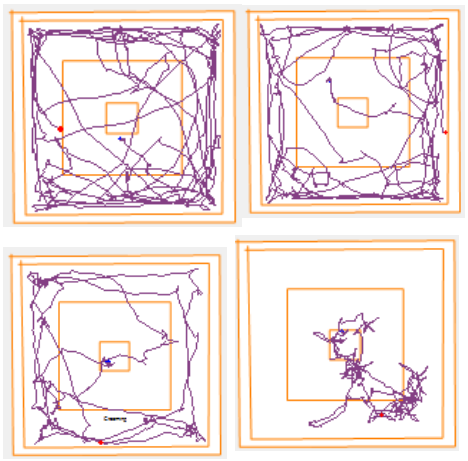
A)



B)



C)



Comment [GM1]: Make symbols smaller

Downregulation of the central noradrenergic system by *Toxoplasma gondii* infection
Supplemental Data

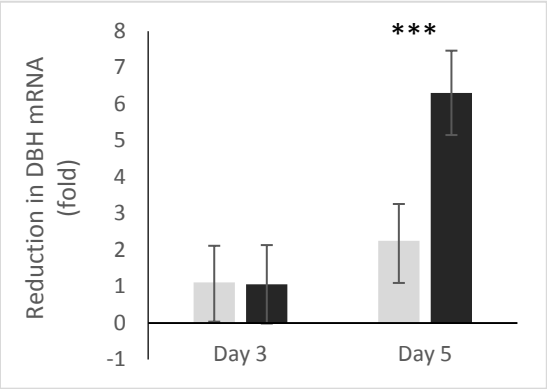
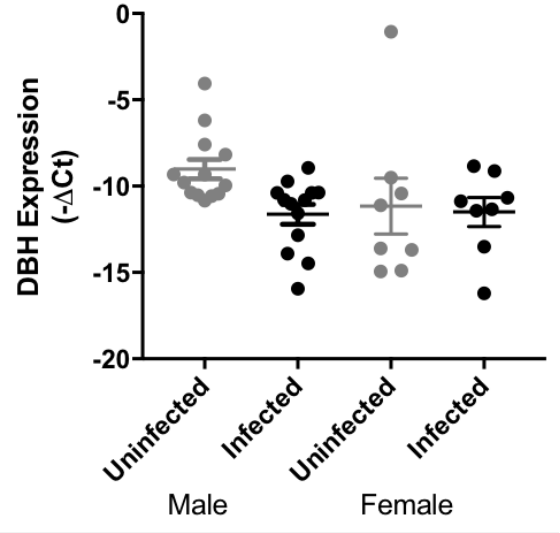


Figure S1: Expression of DBH mRNA in the human neuronal cells infected with T. gondii strain ME49. BE(2)-M17 cells were infected (black) and uninfected (grey) (***, p= 0.00076 for infected vs uninfected on Day 5), three biological replicates). Error bars are ±SEM.



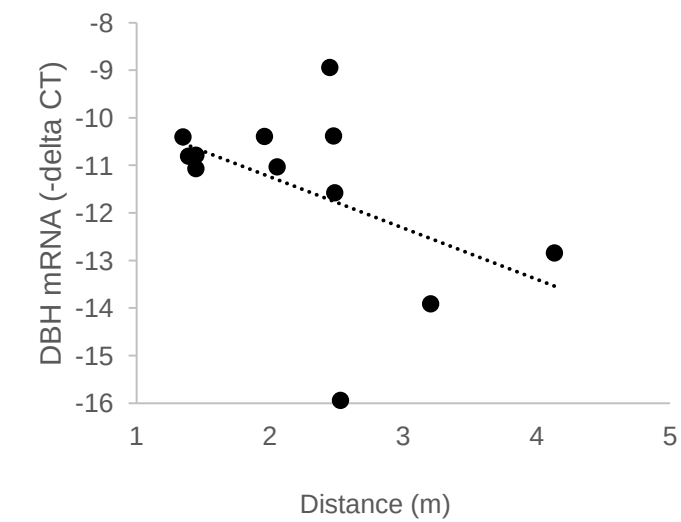
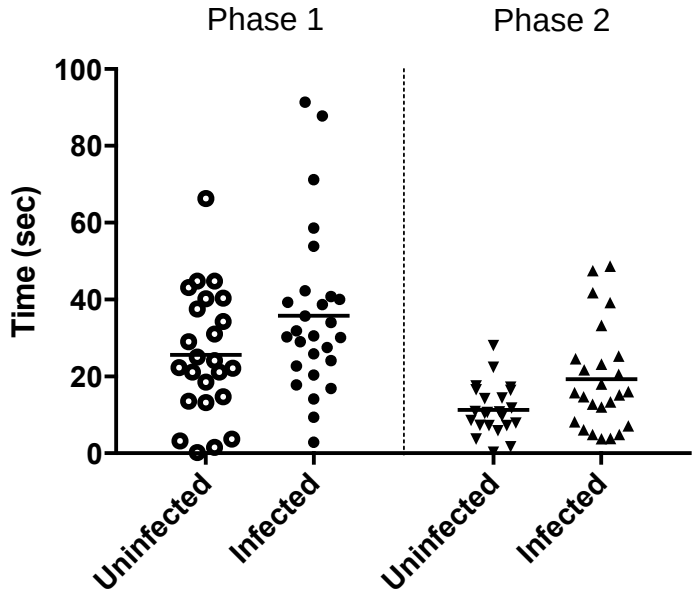


Figure S2: Expression of DBH mRNA in the brains of infected (black) and uninfected (grey) male and female mice (p= 0.0032, n=26 and p=0.85, n=16, respectively). Error bars are ±SEM.

Figure S3: Correlation graph of locomotion in *T. gondii* infected mice with DBH gene expression. Expression of the DBH gene in brains of male mice (ΔC_T relative to GAPDH) with the distance travelled over 120-180 seconds of the open field trial. Correlation coefficient, -0.48.

A)



B)

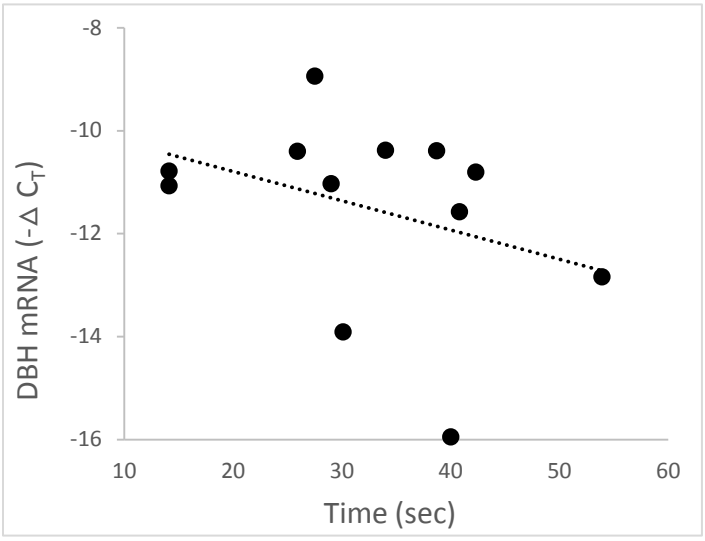


Figure S4: Social approach and dopamine ß-hydroxylase gene expression with *T. gondii* infection. A) A combined plot showing time spent (seconds) investigating a novel mouse (Stranger 1) in preference to an empty container in phase 1 of the test of uninfected (grey) and infected animals (black). Time spent investigating a second novel mouse (Stranger 2) in preference to the first stranger mouse was measured in phase 2. For the two phases, the p values are 0.063 and 0.025, respectively. B) Correlation graph of DBH level and social approach in infected animals. Plot of the level of DBH versus the time spent investigating a stranger mouse (Pearson's correlation coefficient = -0.35).

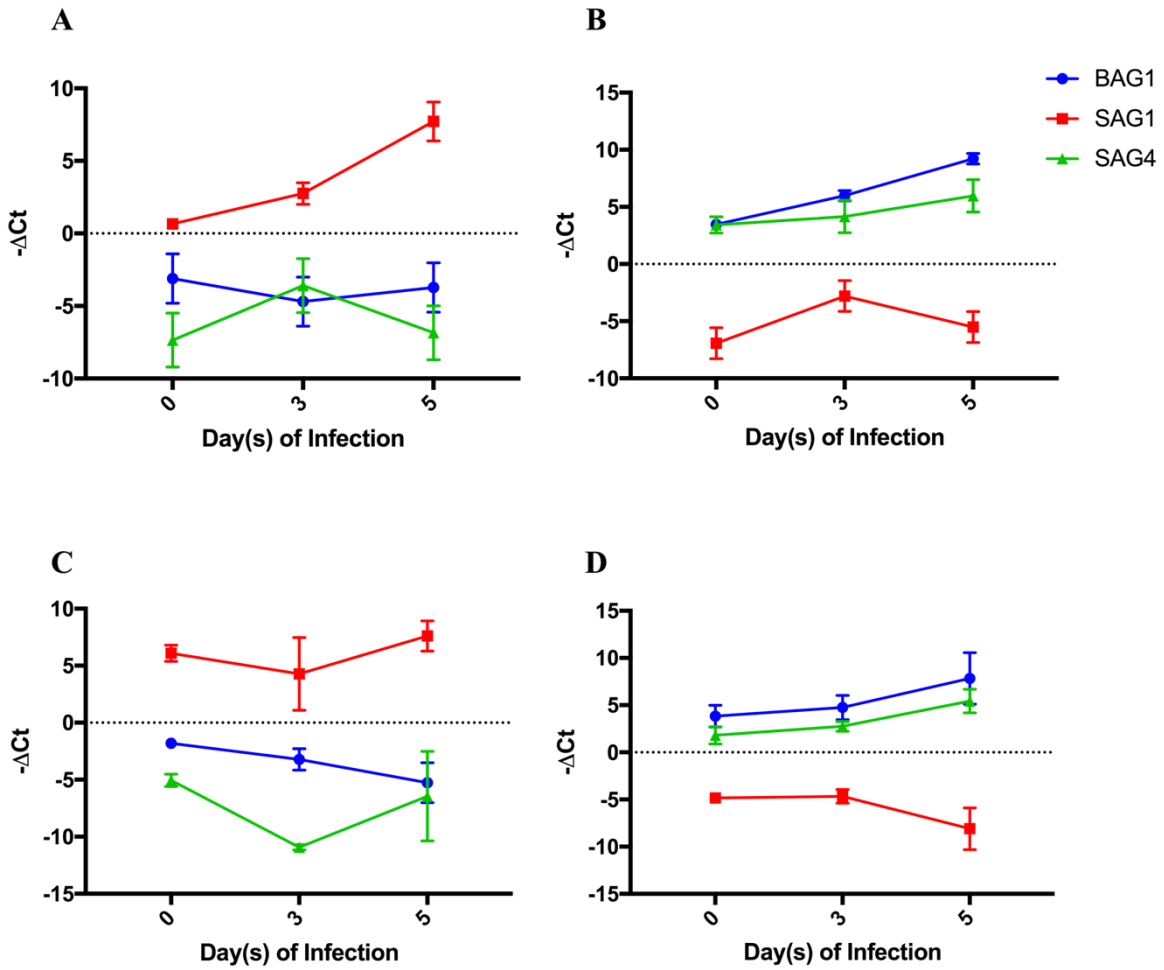


Figure S5. pH shocking of liberated *T. gondii* induces a bradyzoite-like phenotype. All plots show expression of the bradyzoite markers BAG1 (blue) and SAG4 (green), as well as the tachyzoite marker SAG1 (red) expressed in relation to the housekeeping gene GAPDH over a time course of 5 days of infection. A) RNA was collected over a time course of 5 days from tachyzoite infected rat catecholaminergic PC12 cells. ±SEM shown, n=2 biological repeats. B) Rat catecholaminergic PC12 cells were infected with pH shocked *T. gondii* and cultured for 5 days. RNA was collected on day(s) 0, 3 and 5; RT-qPCR was then performed. ±SEM shown, n=2 biological repeats. C) Human neuronal M17 cells were infected with *T. gondii* tachyzoites. RNA was collected over a time course of 5 days from uninfected and tachyzoite infected human neuronal M17 cells. ±SEM shown, n=2 biological repeats. D) Human neuronal M17 cells were infected with pH shocked *T. gondii* and cultured for 5 days. RNA was collected on day(s) 0, 3 and 5; RT-qPCR was then performed ±SEM shown, n=2 biological repeats.

Tables

Description	Gene	Fold increase
Bradyzoite antigen 1	BAG1	147.2
L-lactate dehydrogenase 2	LDH2	14.3
Cyst matrix protein	MAG1	5.1
Bradyzoite surface protein	SAG4	3.1
Enolase 1	ENO1	4.0

Table S1. Expression of *T. gondii* bradyzoite marker genes in six day infected shocked PC12 cells expressed as fold increase from day 0.

Top ten up-regulated GO terms with *T. gondii* infection

GO Term	P Value	Fold Enrichment	FDR*
GO:0030968 endoplasmic reticulum unfolded protein response	4.45E-07	12.87	8.11E-04
GO:0034620 cellular response to unfolded protein	5.01E-07	12.67	9.15E-04
GO:0002385 mucosal immune response	8.18E-07	20.98	1.49E-03
GO:0002251 organ or tissue specific immune response	9.98E-07	20.33	1.82E-03
GO:0002227 innate immune response in mucosa	1.81E-06	27.87	3.31E-03
GO:0035967 cellular response to topologically incorrect protein	2.95E-06	10.08	5.38E-03
GO:0006986 response to unfolded protein	3.23E-06	9.96	5.89E-03
GO:0050830 defense response to Gram-positive bacterium	6.41E-06	9.09	1.17E-02
GO:0035966 response to topologically incorrect protein	1.48E-05	8.12	2.70E-02
GO:0035556 intracellular signal transduction	1.75E-05	1.87	3.19E-02

*FDR is false discovery rate for maximum false positive genes in this GO set

Top ten down-regulated GO terms with *T. gondii* infection

Term	P Value	Fold Enrichment	FDR*
GO:0048666 neuron development	2.29E-06	2.71	4.16E-03
GO:0022008 neurogenesis	2.48E-06	2.30	4.52E-03
GO:0007399 nervous system development	2.50E-06	2.04	4.56E-03
GO:0031175 neuron projection development	3.79E-06	2.85	6.90E-03
GO:0051270 regulation of cellular component movement	5.68E-06	2.96	1.03E-02
GO:0030182 neuron differentiation	8.08E-06	2.38	1.47E-02
GO:0006928 movement of cell or subcellular component	8.28E-06	2.22	1.51E-02
GO:0010243 response to organonitrogen compound	9.61E-06	2.63	1.75E-02
GO:0007409 axonogenesis	1.85E-05	3.84	3.36E-02
GO:0048699 generation of neurons	2.36E-05	2.22	4.30E-02

*FDR is false discovery rate for maximum false positive genes in this GO set

Table S2. Top up- and down-regulated rat genes in six day infected shocked PC12 cells.