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

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Abstract

Toxoplasma gondii is 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 of 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 examined changes in catecholamine expression with infection and explored the underlying alterations in gene expression as a biological mechanism to 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 levels in the brains of *T. gondii*-infected animals was monitored. 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 during *in vitro* infection, we performed infections with catecholaminergic cells. PC12 cells, derived from a pheochromocytoma of the rat adrenal medulla, are fully functional in synthesis and packaging of 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 by Ngo et al 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. Hence, those results are difficult to compare with our approach using neuronal cells that are fully functional in synthesis and release (with potassium activation) of 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 experiments to trigger bradyzoite differentiation. DBH gene expression decreased after three days of infection and further after five days in PC12 cells (30±2-fold), relative to rat GAPDH (p=0.0046) (Figure 2C). Microscopic analysis verified the maintenance of cell numbers and viability during the time course experiments. The level of DBH mRNA in uninfected PC12 cells was unchanged over the course of the experiment (one-way ANOVA, p= 0.58).

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

DBH is the key link between NE and DA, as DBH metabolizes DA into NE. Decreased DBH will decrease synthesis of NE, and simultaneously increase levels of the precursor DA. Suppression of DBH by down-regulated expression of its gene provides a mechanistic explanation for the observed increase in DA in infected PC12 cells above (Figures 1C, 1D) 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. 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 neuronal dysfunction has been observed in infected mice (29). In this study, we found no difference in neurons between infected and uninfected rats based on quantification of a neuron-specific mRNA, that encoding microtubule-associated protein 2 (MAP2) (Figure 3C; $p= 0.57$).

Effect of Sex on Altered Norepinephrine Regulation with Infection

An intriguing observation during these studies was the finding that females did not exhibit the down-regulation of DBH. We noted a large range of DBH mRNA levels in the brains of female animals as an anomaly that could mask an effect by infection. Indeed, infected females

did not exhibit a measurably lower level of DBH (Figure 4A, p=0.45) with infected females possessing higher and lower DBH mRNA levels than vehicle controls (Table 1). A similar finding was observed with infected mice in which CNS levels of DBH mRNA in males were significantly down-regulated (p= 0.0032, n=26) whereas the levels were unchanged in females (p=0.85, n=16) (Supplemental data Fig S2).

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

A range of ESR1 levels was observed in the brains of the female rats, indicative of differences in their estrous cycle (Table 1). Expression of ESR1 was not altered by infection (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 to *T. gondii*, 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 control gene) 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 removed from the home cage, placed in the centre of the open field and allowed to settle for 60 seconds (0-60 seconds), 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). The distance travelled by uninfected mice was one and a half times that of infected animals in the 60-120 ($p<0.0001$) and 120-180 ($p<0.0015$) second intervals. Representative tracking of uninfected and control mice illustrates the decreased locomotor activity during early time points (Figure 6C). Additional, the tracking shown also illustrates 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 180 seconds. In the 5-min intervals between 5 and 15 minutes, 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 investigated for a 1.5 times longer period ($p=0.025$) with a correlation, albeit weak, with DBH mRNA levels (Supplementary Fig S4).

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*, DA (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. In this study down-regulation of DBH was observed in the brains of *T. gondii* infected males but not females (Figures 3 and 4). In females, DBH expression correlated with estrogen levels regardless of infection status (Figure 4). Sex-specific down-regulation of DBH may provide some insight to sex differences in behavioural changes with infection.

286 The down-regulation of DBH expression provides an explanation for the observed decreases
287 in NE in infected brains without a significant increase in DA in brain tissue observed in this
288 and some prior studies (Figure 1). This observation is not surprising given the small
289 proportion of noradrenergic relative to dopaminergic neurons in the brain. Combined with
290 the more severe pathology of *T. gondii* infection in mice with dysfunctional neurons, the small
291 proportion of noradrenergic neurons provides a possible explanation why this and other
292 studies did not detect changes in total brain DA levels with infection (12, 16, 24, 29, 46). In
293 vitro, the down-regulation of DBH found in this study can account, at least in part, for
294 increased DA levels observed in infected PC12 cells observed in earlier studies (9, 19). In
295 those studies, the amount of DA increased with infection while levels of the enzymes in
296 synthesis, tyrosine hydroxylase and dopa decarboxylase (involved in dopamine synthesis)
297 were unchanged, although dopa decarboxylase could be detected in the parasitophorous
298 vacuole. *T. gondii* contains two paralogous genes that encode an aromatic amino acid
299 hydroxlase (TgAAAH), with tyrosine and phenylalanine hydroxylase activities, that is
300 secreted from the parasites into the parasitophorous vacuole (47). Both paralogs were found
301 to be expressed in bradyzoites, whereas only TgAAAH1 was expressed in tachyzoites. The
302 gene products have been found to be involved in oocyst development as proposed at their
303 original discovery (47, 48). The effects of disruption of one of the two paralogs on
304 catecholamine neurotransmission remain inconclusive; hence, collaborative experiments
305 using the recently developed double knockout mutants lacking both genes are ongoing (48).

306 Noradrenergic neurons are principally located in the locus coeruleus (LC) in the brain and
307 project to the thalamus, hippocampus and the frontal and entorhinal cortices (49); efferent
308 noradrenergic neurons originating in the LC were recently found to release DA in the dorsal

hippocampus, thus modulating 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 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 dopa decarboxylase required 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 during *in vivo* infection is similar to that observed for 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

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

Mice were housed five of the same sex per cage, with *ad libitum* access to food pellets and water. Mice were checked for health changes daily and their weight was measured weekly. Any mouse showing severe illness or significant weight loss (25%) was promptly culled. Mice were grouped according to treatment. Mice were infected by intraperitoneal (IP) injection with *T. gondii* type II strain Prugniaud in sterile phosphate-buffered saline (PBS) at 6–14 weeks of age. Infection was monitored by the direct agglutination test (BioMérieux) to detect *Toxoplasma* antibodies, following the manufacturer's instructions, in sera from collected

blood samples. Brains were harvested from euthanized animals and snap frozen. Cryosectioned slices were used for RNA isolation as described for rats below.

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

Growth of pathogens and cultured cells

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

For the induction of parasite conversion to bradyzoite forms, free released tachyzoites were incubated at 37°C in RPMI supplemented with 1% FBS (pH 8.2) for 16-18 hours (hr) in

ambient air then diluted with DMEM (Invitrogen), isolated by centrifugation, and suspended in RPMI (pH 7.4) containing horse serum, FBS and penicillin/streptomycin, as previously described (19). This method was developed because catecholamine-producing cells were found to be sensitive to pH changes severely reducing their production of catecholamines. The parasite number was determined by microscopy and an equal number of treated tachyzoites to cells was used for infections, unless otherwise stated. The viability and differentiation of parasites in PC12 and BE(2)-M17 cultures was monitored by qRT-PCR (as described below) with *T. gondii* gene markers for GAPDH, tachyzoites (SAG1), and 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(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 primers 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 ESR1 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 open field arena had an internal 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 tripod-mounted webcam above the center of the arena.

Mice were individually placed at the center 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 then recorded for 14 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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735

Status	Gender	DBH ($\Delta\Delta C_T$)	ESR ($\Delta\Delta C_T$)	p value DBH (uninfected vs infected) and correlation coefficient (r)
uninfected	m	-1	N/A	
uninfected	m	-0.5	N/A	
uninfected	m	-0.72	N/A	
uninfected	m	-0.14	N/A	p=0.0023 r=0.91 for DBH and cyst number
infected	m	-7.4	N/A	
infected	m	-4.7	N/A	
infected	m	-3	N/A	
infected	m	-3	N/A	
infected	m	-7.2	N/A	
uninfected	f	3.6	3	
uninfected	f	-5.6	-1.5	
uninfected	f	1.9	-1.4	p=0.45 r=0.17 for DBH and cyst number r=0.86 for DBH and ESR
infected	f	3.8	0.35	
infected	f	-2.7	0.13	
infected	f	-1.7	-3.2	
infected	f	-1.6	-0.35	
infected	f	-13	-10	

736

737 Table 1. Sex specific effect of chronic *T. gondii* infection on DBH levels

738 Figure legends

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

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

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

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

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

778 Figure 5: Dopamine ß-hydroxylase suppression is pathogen-specific. A) Plot of DBH gene
779 expression over a time course of 48 hours. Uninfected (grey) and human cytomegalovirus
780 (CMV) infected (black) human BE(2)-M17 neuronal cell line, shown as a percentage of the
781 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) Mean 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

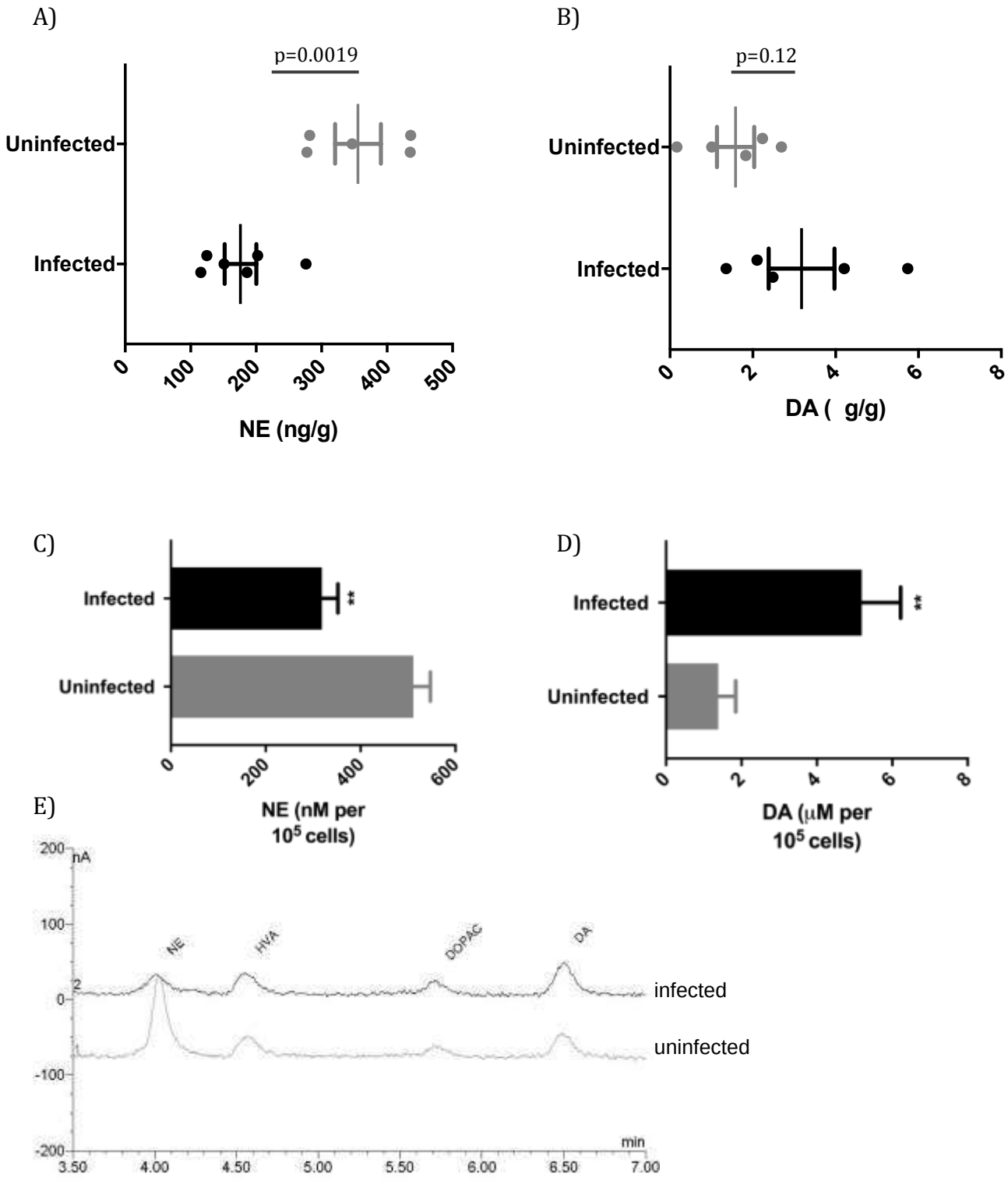
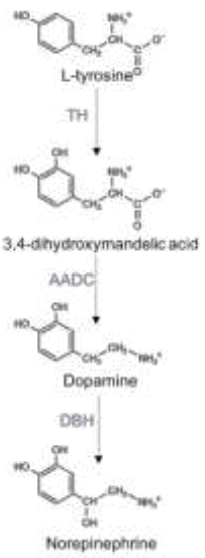
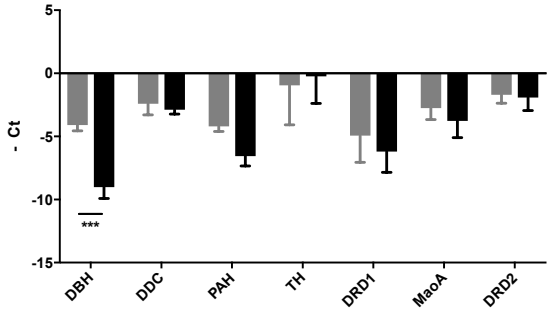

A)
p=0.0019
Uninfected
Infected
0
100
200
300
400
500
NE (ng/g)
B)
p=0.12
Uninfected
Infected
0
2
4
6
8
DA (g/g)
C)
Infected
Uninfected
**
0
200
400
600
NE (nM per
10^5 cells)
D)
Infected
Uninfected
**
0
2
4
6
8
DA (μM per
10^5 cells)
E)
200
nA
100
0
-100
-200
NE
HVA
DOPAC
DA
2
1
infected
uninfected
min
3.50
4.00
4.50
5.00
5.50
6.00
6.50
7.00

Figure 2

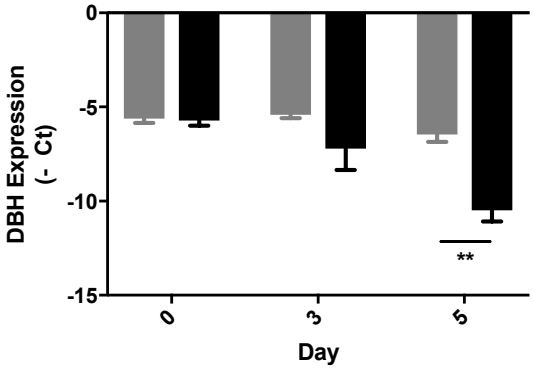
A)



B)



C)



D)

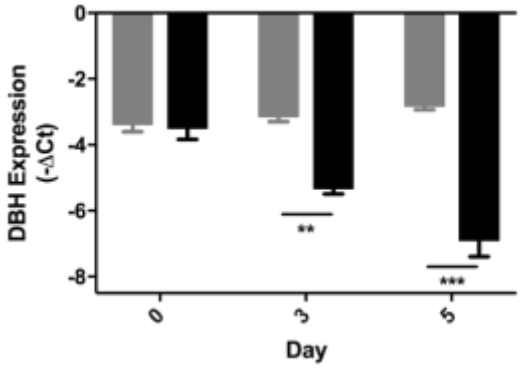


Figure 3

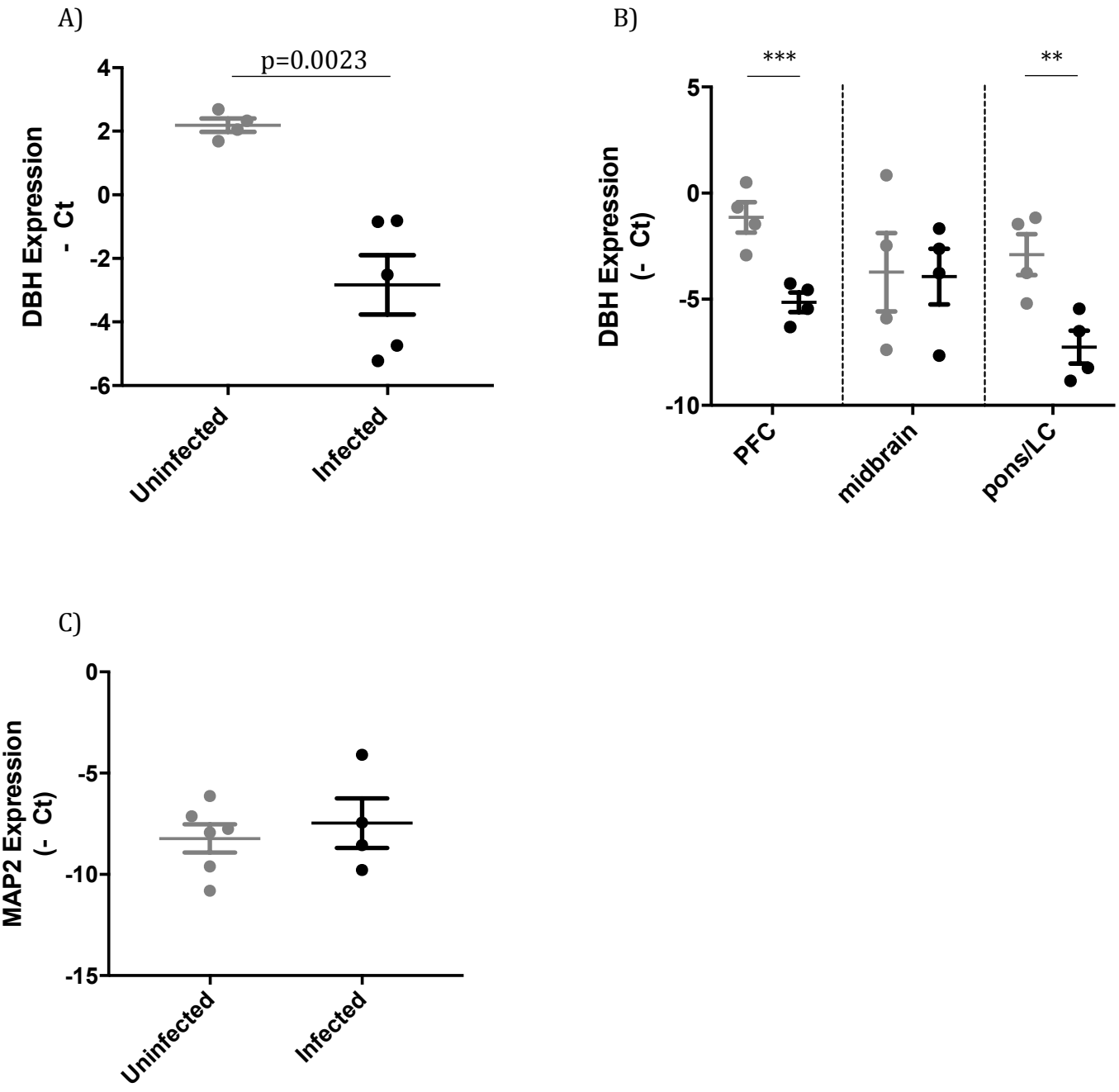
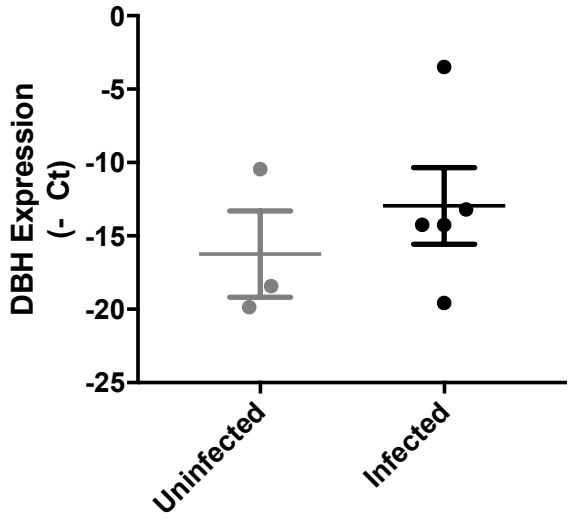
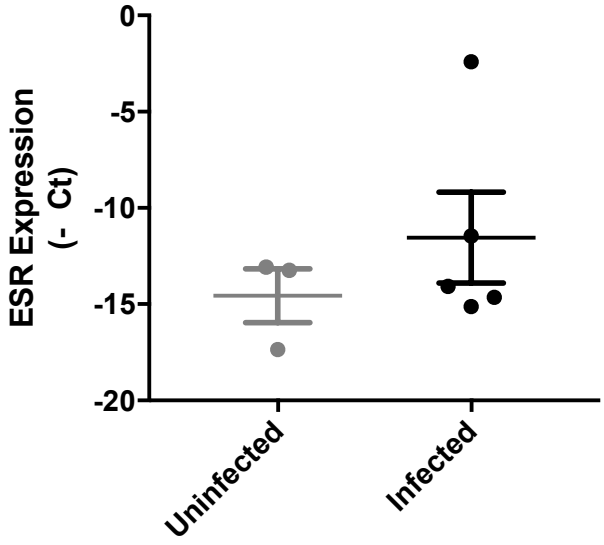

A)
p=0.0023
DBH Expression
- Ct
4
2
0
-2
-4
-6
Uninfected
Infected
B)
***
**
DBH Expression
(- Ct)
5
0
-5
-10
PFC
midbrain
pons/LC
C)
MAP2 Expression
(- Ct)
0
-5
-10
-15
Uninfected
Infected

Figure 4

A)


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

B)


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

C)

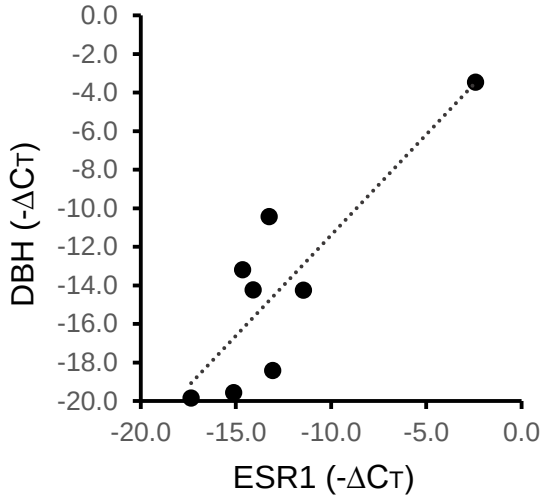

DBH (-ΔCT)
0.0
-2.0
-4.0
-6.0
-8.0
-10.0
-12.0
-14.0
-16.0
-18.0
-20.0
-20.0
-15.0
-10.0
-5.0
0.0
ESR1 (-ΔCT)

Figure 5

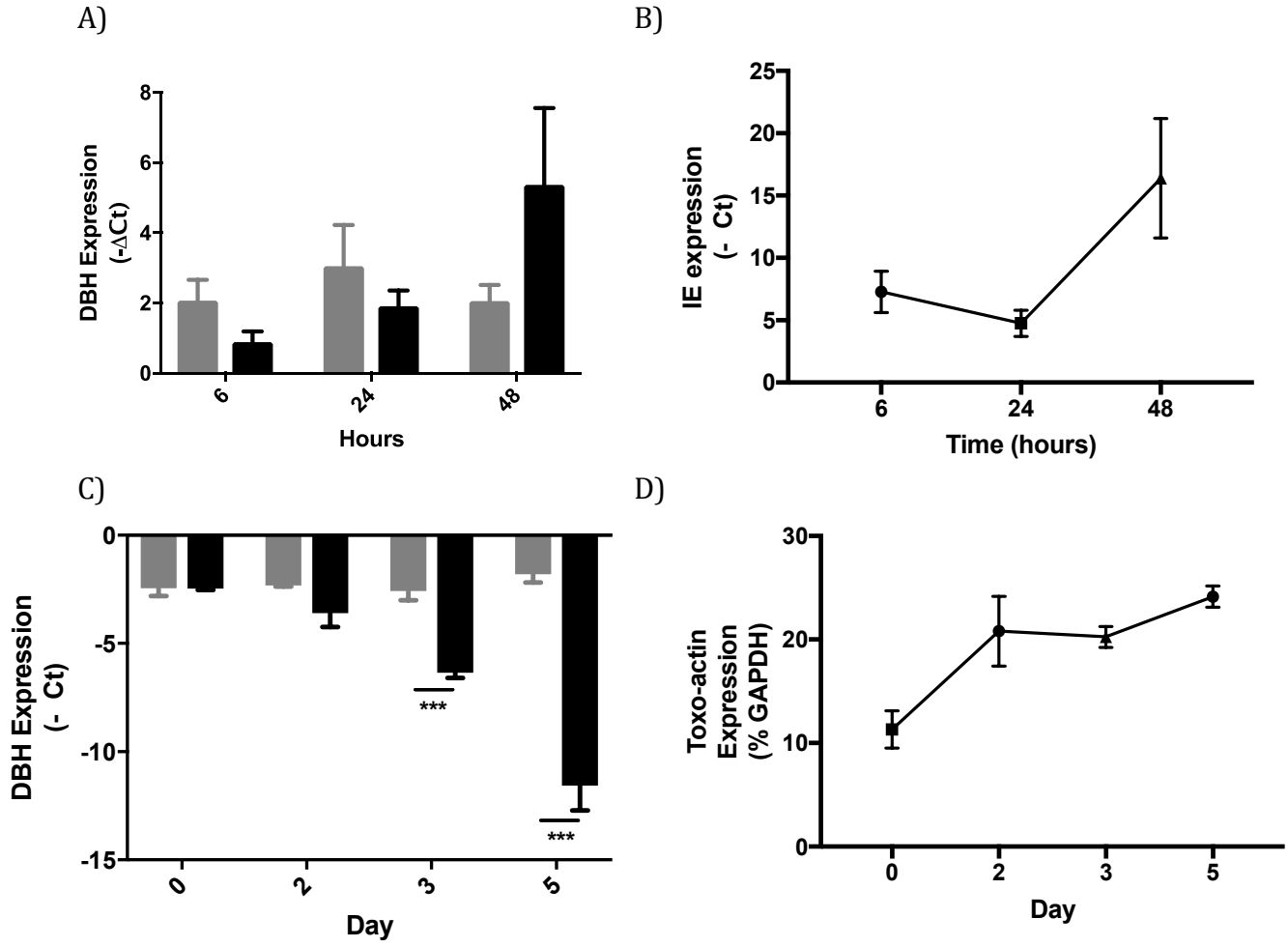
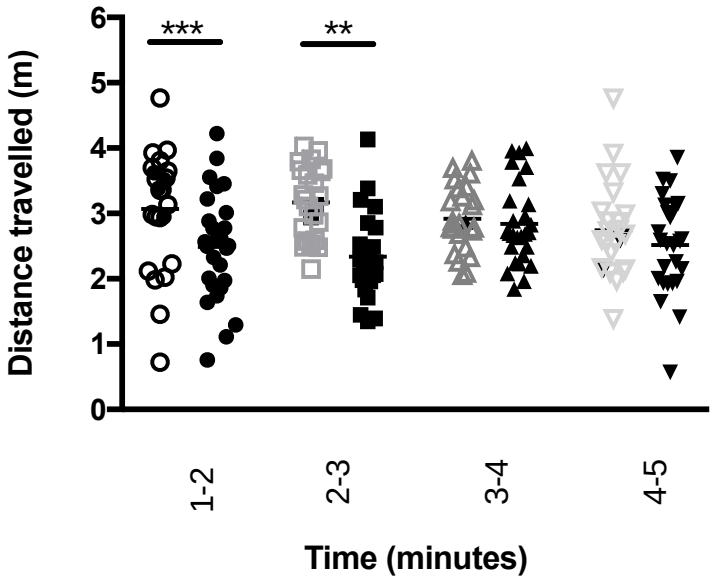
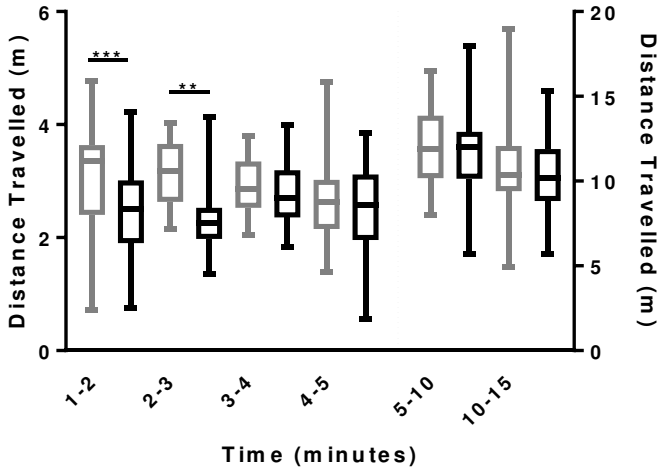

A)
DBH Expression
(-ΔCt)
8
6
4
2
0
6
24
48
Hours
B)
IE expression
(- Ct)
25
20
15
10
5
0
6
24
48
Time (hours)
C)
DBH Expression
(- Ct)
0
-5
-10
-15
***
***
0
2
3
5
Day
D)
Toxo-actin
Expression
(% GAPDH)
30
20
10
0
0
2
3
5
Day

Figure 6

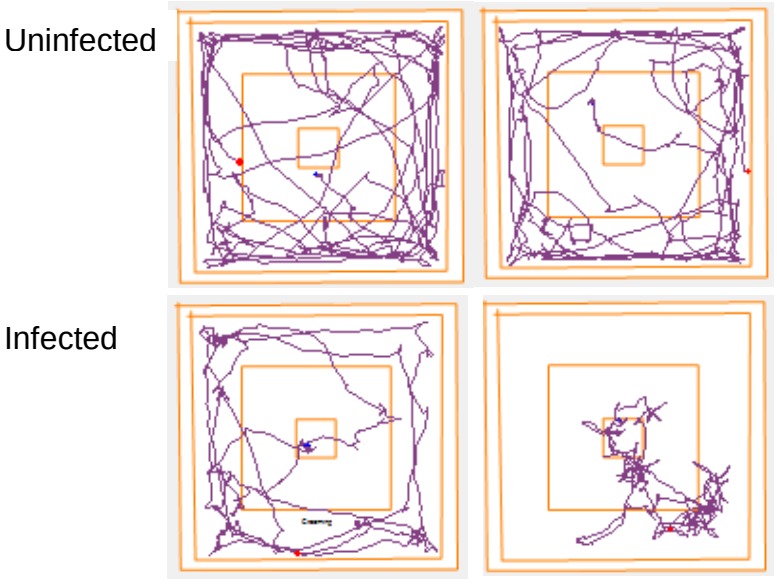
A)


Distance travelled (m)
***
**
1-2
2-3
3-4
4-5
Time (minutes)

B)


Distance Travelled (m)
***
**
Distance Travelled (m)
1-2
2-3
3-4
4-5
5-10
10-15
Time (minutes)

C)


Uninfected
Infected