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Patterns of *Toxoplasma gondii* cyst distribution in the forebrain associate with individual variation in predator odor avoidance and anxiety-related behavior in male Long-Evans rats

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Abstract

Toxoplasma gondii (*T. gondii*) is one of the world's most successful brain parasites. *T. gondii* engages in parasite manipulation of host behavior and infection has been epidemiologically linked to numerous psychiatric disorders. Mechanisms by which *T. gondii* alters host behavior are not well understood, but neuroanatomical cyst presence and the localized host immune response to cysts are potential candidates. The aim of these studies was to test the hypothesis that *T. gondii* manipulation of specific host behaviors is dependent on neuroanatomical location of cysts in a time-dependent function post-infection. We examined neuroanatomical cyst distribution (53 forebrain regions) in infected rats after predator odor aversion behavior and anxiety-related behavior in the elevated plus maze and open field arena, across a 6-week time course. In addition, we examined evidence for microglial response to the parasite across the time course. Our findings demonstrate that while cysts are randomly distributed throughout the forebrain, individual variation in cyst localization, beginning 3 weeks post-infection, can explain individual variation in the effects of *T. gondii* on behavior. Additionally, not all infected rats develop cysts in the forebrain, and attenuation of predator odor aversion and changes in anxiety-related behavior are linked with cyst presence in specific forebrain areas. Finally, the immune response to cysts is striking. These data provide the foundation for testing hypotheses about proximate mechanisms by which *T. gondii* alters behavior in specific brain regions, including consequences of establishment of a homeostasis between *T. gondii* and the host immune response.

1. Introduction

Toxoplasma gondii, one of the most successful parasites worldwide, is capable of infecting nearly all warm-blooded animals (Dubey, 2010) and chronically infects around 35% of the human population, with exposure rates in the United States, Northern Europe and East Asia estimated to be between 10–20%, and exposure rates in central-southern Europe, Southeast Asia, Africa and South America estimated to be as high as 40–60% (Pappas *et al.*, 2009). An intracellular parasite, *T. gondii* can persist chronically in the brain with a remarkably high

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host survival rate (Montoya and Liesenfeld, 2004). While chronic infection is considered “non-pathogenic” in immunocompetent human hosts, evidence suggests that *T. gondii* engages in manipulation of host biology and behavior (Flegr, 2013; Kaushik *et al.*, 2012), and has been epidemiologically linked to a growing list of psychiatric disorders in humans (Groer *et al.*, 2011; Miman *et al.*, 2010; Pearce *et al.*, 2012; Pedersen *et al.*, 2012; Torrey *et al.*, 2007).

Work in rodents has revealed numerous behavioral consequences of *T. gondii* infection, one of the most prominent being a disruption of predator odor avoidance behavior (Berdoy *et al.*, 2000; Vyas *et al.*, 2007a). While there is debate over the extent and specificity of induced behavioral changes, the ability to detect what may be subtle behavioral changes is highly sensitive to stimulus parameters (Vyas *et al.*, 2007b), host parameters (species, strain, and sex), as well as infection parameters (dose, strain, time course). Despite this challenge, many independent groups have identified conditions under which disruption of predator odor avoidance behavior is observed in *T. gondii*-infected rats and mice (Berdoy *et al.*, 2000; Haroon *et al.*, 2012; Vyas *et al.*, 2007a; Xiao *et al.*, 2012). Effects of *T. gondii* on predator odor avoidance behavior are particularly relevant as *T. gondii* has an indirect life cycle with transmission occurring through multiple hosts (including rodents), but with sexual reproduction occurring solely in feline predators. Evolutionary pressures in the context of this reproductive restriction may have led to the emergence of parasite-induced biological and behavioral changes that increase the rates of feline predation of infected rodents, and therefore the reproductive success of the parasite. Such changes appear to include (but are perhaps not limited to) a disruption of predator odor avoidance behavior.

Mechanisms by which *T. gondii* alters host behavior are not well understood. Distributed throughout functional neural circuits in the brain, *T. gondii* cysts and the host immune response to these cysts represent likely candidates for the underlying causes of behavioral changes. Cyst distributions in mice have been reported to include neural circuits known to regulate fear- or anxiety-related behavior (Berenreiterova *et al.*, 2011; Haroon *et al.*, 2012). Changes in mRNA expression in behaviorally-relevant regions have also been reported in the brains of infected mice (Xiao *et al.*, 2012). Nevertheless, only one study (in mice) has shown that behavior is linked to differences in *T. gondii* cyst presence and location (Afonso *et al.*, 2012). In that study, mice with specific combinations of cyst localization had altered exploratory, risk-related, and fear-related behavior. However, while studies in infected mice can be confounded with a high mortality rate and sickness behavior, rats and humans rarely demonstrate sickness behavior following *T. gondii* infection (Dubey and Frenkel, 1998; Innes, 1997). While immune response has been demonstrated around cysts in chronically infected mice (Kim and Boothroyd, 2005), less is known about the neuroimmune response to cysts in rats over time. Studies in rats have suggested that subtle tropisms in cyst distribution for the amygdala (Vyas *et al.*, 2007a) or more generally medial brain structures (i.e. nucleus accumbens, hypothalamus, prefrontal cortex, ventral tegmental area) (Gonzalez *et al.*, 2007) may explain effects on behavior. However, no studies have examined a direct link between cyst distribution and behavior in rats over time.

This study was designed to test the hypothesis that *T. gondii* manipulation of specific host behaviors is dependent on the location of cysts within the brain and that cyst distribution and behavioral changes are time-dependent functions post-infection. We infected male Long-Evans rats and examined neural distribution of cysts and evidence for microglial activation weekly across a 6 week time course in conjunction with behavioral testing in a predator odor approach-avoidance task, elevated plus maze, and open field arena. Our findings demonstrate that not all *T. gondii*-infected rats have cysts in the forebrain and, in those that do, while cysts are randomly distributed throughout the forebrain, individual variation in

cyst localization, beginning 3 weeks post-infection, may explain individual variation in the effects of *T. gondii* on behavior.

2. Methods

2.1 Toxoplasma gondii preparation

A Prugnault type II strain of *T. gondii*, genetically modified to constitutively express green fluorescent protein (GFP) under GRA2 promoter and firefly luciferase under tubulin promoter (provided by J. Boothroyd Laboratory), was maintained as tachyzoites by passage through human foreskin fibroblast monolayers. Infected fibroblasts were washed with phosphate buffered saline (PBS), resuspended in PBS and syringe-lysed to release tachyzoites. Tachyzoites were counted by haemocytometer for infection dosage.

2.2 Subjects and T. gondii-infection

Male Long-Evans rats (10 weeks at start of experiment; Charles River Laboratories) were housed in groups of 3 by treatment and handled weekly for weight monitoring after infection and 2 minutes daily for one week prior to the start of behavioral testing. Health status was monitored throughout the experiment. Rats were randomly assigned to 1 of 6 time course treatment groups (1, 2, 3, 4, 5, and 6 weeks post-infection [wpi]; n=18; N=108). For each treatment, 18 rats were each injected with 10 million *T. gondii* tachyzoites (intraperitoneally; i.p.) in approximately 0.5 mL sterile PBS on a single day in the appropriate week prior to brain and blood (tissue) collection. An uninfected control group (n=18) was mock-infected with sterile PBS (i.p.) 4 weeks prior to tissue collection. All procedures related to animal maintenance and experimentation were approved by the Stanford University Administrative Panel for Laboratory Animal Care and conformed to the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of rats used and their suffering.

2.3 Experimental design

Starting 9 days prior to tissue collection, rats were tested, one trial each, in the open field (OF), elevated plus maze (EPM), and bobcat urine approach-avoidance (BCAA) task. Due to the large sample sizes, each behavioral test, and tissue collection, was blocked across three consecutive days (see Figure 1 for diagram of experimental design). Timing for each behavioral test and for tissue collection had a 3-day window post-infection (e.g. for 3 wpi: OF occurred 12–14 days post-infection (dpi); EPM, 15–17 dpi; BCAA, 18–20 dpi; and tissue collection, 21–23 dpi). Importantly, each individual subject had 3 days between each behavioral test, as well as prior to tissue collection. Furthermore, rats in the 1 week time point were included for anatomical distribution of forebrain cysts, but were not included in behavioral testing as testing would have overlapped with infection. Testing and tissue collection occurred from 8 AM to 4 PM each day with each treatment equally balanced across and within each day of behavioral testing and tissue collection (14:10 hour light-dark cycle with lights on at 6 AM). All behavior was video-recorded and analyzed by an experimenter blind to the treatment groups.

2.4 Behavioral Testing

Open Field (OF)—Each rat (9 days prior to tissue collection) was placed at one edge of a square testing arena made of black acrylic (75 × 75 × 30 cm), and exploration across a 4×4 grid was video-recorded for 5 minutes to determine time spent in the center, number of line crossings into the center, and number of line crossing around the edge of the arena. Criterion for line crossing was 2/3 of the body crossing over a dividing line, a conservative measure intended to eliminate nose pokes and indecisive investigation. Data was analyzed for the 5

minute duration and a secondary analysis was also performed on the first 2 minutes to determine if an anxiety-like behavioral phenotype may occur early in the test. Indirect fluorescent lighting provided 20 lux in corners of the open field and 60 lux at the center of the arena. Rats were returned to their home cage after behavioral testing and placed back in the holding room. Arena was thoroughly cleaned and wiped with 70% ethanol between each test.

Elevated Plus Maze (EPM)—Each rat (6 days prior to tissue collection) was placed in the center crux of an elevated plus maze (15 × 60 cm arms, 80 cm elevated, 15 cm closed arm walls; black acrylic) and exploration of open and closed arms was video-recorded for 5 minutes to determine time spent in open arms, time spent in closed arms, time spent in the middle crux, open arm entries, and closed arm entries. Criteria for line crossing was 2/3 of the body crossing over a dividing line. Indirect fluorescent lighting provided 15 lux at the ends of each closed arm, 120 lux at the ends of the open arms and 80 lux in the center of the arena. Rats were returned to their home cage after behavioral testing and placed back in the holding room. Arena was thoroughly cleaned and wiped with 70% ethanol between each test.

Bobcat urine approach-avoidance (BCAA)—Each rat (3 days prior to tissue collection) was placed in the center of a rectangular testing arena (70 × 30 × 33 cm; clear plastic), with bobcat urine (PredatorPee.com, Hermon, Maine) and rabbit urine (Foggy Mountain, Lexington Outdoors, Inc., Lincoln, Maine) on opposite ends, and exploration was video-recorded for 15 minutes. One mL bobcat urine and one mL rabbit urine were applied to gauze pads placed at opposite ends of the arena behind plastic grates so the rats could smell but not physically interact with the pads (left and right balanced presentation of each stimulus). Due to the presence of preceding behavioral testing, rats were not habituated to BCAA arena prior to testing. However, approach to an aversive stimulus due to novelty and risk assessment behavior was a concern. Therefore, in order to minimize influence of initial bouts of exploratory and risk-assessment behavior that occur during the beginning of the trial (unpublished data), time spent in the bobcat bisect was determined for the last 5 minutes of a 15 minute trial and expressed as bobcat bisect time divided by 5 minutes (bobcat occupancy ratio; or predator odor aversion behavior). As above, criteria for crossing into a bisect was 2/3 of the body crossing over the dividing line. Rats were returned to their home cage after behavioral testing and placed back in the holding room. The arena was thoroughly cleaned and wiped with 70% ethanol between each test.

2.5 Brain and blood collection for histology and antibody detection for seroconversion

Three days following exposure to the BCAA paradigm, rats were deeply anaesthetized with Euthasol (200 mg/mL sodium pentobarbital and 25.6 mg/mL phenytoin, i.p.). Prior to perfusion, 1 mL of blood was withdrawn from the right ventricle and kept on ice for later antibody determination. The right atrium was opened and rats were transcardially perfused through the left ventricle with ice-cold 0.05 M sodium phosphate buffer (PB) with saline (PBS) followed by 4% paraformaldehyde in 0.1 M PB containing 1.5% sucrose (pH 7.4). Brains were collected and stored in the same fixative at 4 °C overnight after which they were transferred to 0.1 M PB for two 12-hour rinses. Brains were cryoprotected for 72 hours (until sunk) in 30% sucrose in 0.1 M PB and were blocked in a coronal plane (−5.0 mm Bregma) into forebrain and hindbrain halves using a stainless steel rat brain matrix (BSRS005-1, Zivic Instruments, Pittsburgh, PA). Brains were then rapidly frozen in isopentane on dry ice and stored at −80 °C. Forebrains were sectioned (40 micrometer, at −18 °C using a Microm HM505E cryostat) in six alternate series, each containing sections at 240 micrometer intervals. Series were stored in cryoprotectant storage buffer (30% ethylene glycol (v/v), 20% glycerol (v/v) in 0.05M PB; pH 7.4) at −20 °C. Within 8 hours on ice,

blood was centrifuged at 3000 rpm for 10 minutes at room temperature and serum was collected and stored at -20°C for later confirmation of infection. Seroconversion was confirmed by an indirect immunofluorescence antibody assay and serum IgG was quantified by ELISA (VIR-ELISA anti-toxo-IgG, Viro-immun Labor Diagnostika, GmbH, Oberusel, Germany; modified to use peroxidase-conjugated AffinitiPure goat anti-rat IgG, Fc_{γ} fragment specific antibody, 112-035-008, Jackson ImmunoResearch Laboratories, West Grove, PA). For anti-*T. gondii* IgG quantification, serum from all treatments were analyzed at dilutions of 1:30,000. Serum from 1 and 2 wpi was rerun at 1:2000 dilution in order to achieve lower levels of detection at 1 wpi and to be able to calibrate those values using values obtained for 2 wpi at both 1:2000 and 1:30,000. Lack of antibody presence was confirmed in uninfected controls using serum dilutions of 1:100.

2.6 Immunohistochemical staining

With GFP-expressing *T. gondii*, 1 of 6 brain series was processed for immunohistochemical identification of the microglial marker, IBA-1, and the nucleic acid stain, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Free-floating sections were incubated at room temperature in 12-well tissue culture plates gently shaken on an orbital shaker throughout the procedure. All rinses were 15 minutes unless stated otherwise. Sections were rinsed three times in 0.05 M PBS, and then preincubated 1 hour in PBS containing 0.3% Triton X-100 (PBST) and 3% bovine serum albumin. Sections from Series 1 were then incubated 18 hours with rabbit anti-IBA-1 primary antibody (019-19741, WACO Chemicals USA, Richmond, VA) diluted 1:1000 in 0.1% PBST and 1% bovine serum albumin, followed by 3 PBS rinses and a 2 hour incubation in AlexaFluor 594 goat anti-rabbit IgG secondary antibody (A11012, Invitrogen, Eugene, OR) diluted 1:200 in PBS. Sections were then rinsed 2 times prior to incubation for 30 minutes with DAPI (D9542, Sigma-Aldrich, St. Louis, MO) diluted 1:1000 in 0.1% PBST. Free-floating sections were then rinsed 3 times in PBS, rinsed briefly in 0.15% gelatin in water, mounted on clean glass slides and allowed to air-dry to affix sections to slides prior to coverslipping with polyvinyl alcohol mounting medium with DABCO antifade (10981, Sigma-Aldrich, St. Louis, MO).

2.7 Determination and localization of *T. gondii* and immune response to *T. gondii*

T. gondii was identified by GFP emission and cysts were localized and counted under 20X magnification in sequential 40 micrometer serial sections from a complete 1 of 6 forebrain series (6.12 to -4.36 mm Bregma, 40 micrometer section every 240 micrometers) using an Olympus IX70 fluorescent microscope. Criteria for cysts were 3-dimensional spheres embedded in the tissue with dense “grape cluster” label (see Figure 4C, inset), ranging in diameter from approximately 10 to 50 micrometers, visible in the 488 (GFP) and 405 (DAPI) channels, but absent from the 594 (red; IBA-1) channel. For familiarization with cyst criteria, observers were trained with independent tissue samples from *T. gondii*-infected rats in which cysts were labeled with rhodamine dolichos biflorus agglutinin (RL-1032, Vector Laboratories, Burlingame, CA) to provide a cyst wall marker in the red fluorescent channel for confirmation of GFP cyst label. IBA-1 label, (constitutively expressed in resting microglia and macrophages and upregulated with activation (Sogn *et al.*, 2013)) served in a parallel analysis to identify immune-related response to *T. gondii*. In addition to identifying ongoing microglial response to cyst presence in chronically infected rat brains, IBA-1 label both helped to confirm cyst presence and also helped to identify sites of small clusters of individual parasites that could have otherwise been missed in the signal to noise ratio for GFP. Importantly, IBA-1 label was used to supplement cyst distribution analysis as cysts (versus individual parasites) were readily apparent with GFP emission and numerous instances of cyst presence in the absence of microglial activation were observed. Host cell characteristics were not examined. Rat brain sections were compared to a stereotaxic rat brain atlas (Paxinos and Watson, 2005) for localization of *T. gondii*. Representative atlas

plates, one within each section (every 240 microns), were used to map cyst location from 1 complete forebrain series for each rat. Cyst burden was determined within each region in which cysts were identified. The areas of each complete brain region in which cysts were identified were then quantified across the representative (240 micron spaced) plates. Region area was quantified by measuring pixels in Adobe Photoshop CS4 and converted to square millimeters. Photomicrographs were acquired from both an Olympus IX70 fluorescent microscope and a Zeiss LSM 510 Meta confocal scanning laser microscope and were arranged in CorelDraw version X6.

2.8 Data Analysis and Statistics

All statistical analyses used IBM Statistical Package for the Social Sciences (SPSS) version 19.0. All data for ANOVA meets the assumption of equality of variance. All reported mean values are provided with standard error of the mean (SEM). All post-hoc comparisons were made with Fisher's protected least significant difference (LSD) or Dunnett's tests when appropriate. Two-tailed significance was accepted at $p < 0.05$.

2.8.1 Cyst Presence—Chi-square analysis was used to determine if the percentage of rats with cysts in the brain differed across the time course. Cyst presence in each region (number of rats with cysts in each region) was expressed relative to the size of the region (area) and normalized to a mean of 1. Chi-square analysis was used to determine if the observed normalized relative frequency of cysts in each brain region differed from the expected frequency if cysts were randomly distributed.

2.8.2 Cyst Burden—One-way analysis of variance (ANOVA) was used to determine if the mean relative cyst burden in the forebrain differed across the time course (means calculated only from those rats with cysts). Mean cyst burden in each region was expressed relative to the size of the region. One-way ANOVA was used to evaluate variance in cyst burden that could be attributed to region.

2.8.3 Seroconversion and body weight—Two-way ANOVA was used to examine effects of wpi and cyst presence on serum IgG. Two-way ANOVA was used to examine effects of wpi and cyst presence on body weight from weekly measurements throughout the course of the experiment. Fisher's protected LSD were used for post-hoc comparison of means.

2.8.4 Behavior—Two-way ANOVA was used to examine effects of wpi and cyst presence on OF, EPM and BCAA behavioral endpoints in *T. gondii*-infected rats. For BCAA behavior, two-way ANOVA using change-point analysis was used to determine if and when, post-infection, cyst presence became predictive of attenuation of predator odor aversion behavior. This involved a series of 4 planned comparisons starting with 2 wpi versus behavioral data from 3,4,5,6 wpi pooled, and then sequentially examining 2 and 3 wpi pooled versus 4,5,6 wpi pooled; 2,3,4 wpi pooled versus 5,6 wpi pooled; and finally 2,3,4,5 wpi versus 6 wpi. This series of planned comparisons was used to determine the grouping scheme resulting in the most significant interaction between group and cyst presence on behavior, indicating the time point at which cyst presence resulted in a change in behavior.

2.8.5 Cyst presence in specific regions relative to behavior—In order to generate hypotheses about in which of the 39 forebrain regions cyst presence was most likely to be contributing to differences in a specific behavior (BCAA, EPM or OF), mean behavior of *T. gondii*-infected rats with cysts in each specific region was plotted relative to the effect size when compared to behavior of *T. gondii*-infected rats without cysts. Effect size was calculated using Cohen's d ($(\text{mean}_1 - \text{mean}_2) / \sqrt{[(s.d._1^2 + s.d._2^2) / 2]}$). This analysis allowed for

the ranking of brain regions by degree of association between cyst presence and behavioral change in order to determine in which of the 39 brain regions cysts were most likely to be contributing to the difference in behavior observed in *T. gondii*-infected rats with cysts relative to those without cysts.

3. Results

There was no mortality or overt signs of sickness at any point due to *T. gondii* infection. *T. gondii*-infected rats did not differ in body weight from PBS-injected uninfected controls at any time point following infection. Six of the 108 brains from *T. gondii*-infected rats and 2 brains from uninfected rats were excluded from the data set as technical difficulties with cryostat sectioning prevented distribution analysis of a complete series in those six brains. Therefore, cyst distribution analyses represent the following final sample sizes from an initial $n=18$: wpi[n]; 1[17], 2[16], 3[17], 4[18], 5[17], and 6[17]; overall $N = 102$.

3.1 Not all *T. gondii*-infected rats displayed evidence of *T. gondii* cysts in the forebrain

Forty-five of 102 *T. gondii*-infected rats (44%) displayed evidence of parasites in the forebrain (see section 2.7 for criteria). The forebrains of the other 57 *T. gondii*-infected and 16 uninfected rats contained neither cysts nor evidence of parasites or chronic infection (see discussion of IBA-1 label below for evidence of chronic infection). Chi-square analysis (Chi-square = 2.61; $p > 0.05$) revealed no difference in the percentage of rats with cysts at each time point (ranging from 35–58%; Figure 2A). The mean cyst burden in forebrains of rats with cysts also was not different across the 2–6 week time course (one-way ANOVA; $F_{(4,34)} = 0.531$; $p = 0.714$; Figure 2B; 1 wpi burden was not quantified). Rats that developed cysts in the forebrain, versus those that did not, neither differed in body weight prior to nor after infection.

While not all rats showed evidence for *T. gondii* cysts in the forebrain, all *T. gondii*-infected rats tested positive for presence of *T. gondii* IgG antibodies in the serum at the time of tissue collection. All uninfected rats tested negative for presence of *T. gondii* IgG antibodies in the serum at the time of tissue collection. Two-way ANOVA revealed an interaction between wpi and cyst presence on ($F_{(5,82)} = 5.852$; $p < 0.001$; Figure 3). Post-hoc comparison of means revealed that IgG increased weekly through 6 wpi in *T. gondii*-infected rats with cysts, whereas *T. gondii*-infected rats without cysts showed an initial increase at 2 wpi but no further increases at later timepoints. IgG was significantly higher in *T. gondii*-infected rats with cysts compared to those without at 3, 5 and 6 wpi (4 wpi is also higher in rats with cysts, but falls short of significance; Figure 3).

T. gondii parasites and cysts were present as early as 1 wpi. The majority of parasite presence at this time point took the form of large areas or fields of individual parasites intermixed with numerous small cysts (see Figure 4, panels A and B). Due to the difficulty in differentiating clusters of individual parasites and small cysts at this time point, we report location, but not quantification of the cyst burden at 1 wpi. The locations in which parasites were observed at this early time point are indicated in Table 1, which may provide some insight into sites of initial entry into the brain. While smaller fields of individual parasites were also present from 2–6 wpi, *T. gondii* presence was mainly contained in cysts at these later time points. Cysts existed both independently and in clusters of multiple cysts from 1–6 wpi (see Figure 4 for photomicrograph plates depicting examples of fields of parasites, individual cysts and cyst clusters at specific time points).

Local areas of distinct increased IBA-1 label were observed in all *T. gondii*-infected rats with cysts but not in *T. gondii*-infected rats without cysts or uninfected rats. Increases in IBA-1 label formed 3-dimensional spheres around cysts, preceding and following individual

cysts, clusters of cysts, and parasites (see Figure 4). While some brains had cysts that were not associated with local increases in IBA-1 label, all brains that had cysts had evidence of areas of clustered microglia with morphology characteristic of an activated state. In this way, characteristic IBA-1 label served as a biomarker for brains that contained cysts but, importantly, was not a biomarker for all individual or clusters of cysts. It was common to observe IBA-1 label preceding the presence of cysts which, examining serial 40 micrometer sections 240 micrometers apart, presented the possibility of finding IBA-1 label in one section that did not contain cysts, but was associated with cysts in the subsequent section. This also raised the possibility that IBA-1 label could identify cysts present in the 240 micron gap between sampled sections (confirmed in some rats). In this way, using IBA-1 label across serial sections is an efficient aid to locating cysts, as the IBA-1 label was larger and spanned more three-dimensional area than did individual cysts or clusters of cysts. However, there were clear instances of cysts with baseline/unactivated IBA-1-labelled microglia in the vicinity (see Figure 4).

3.2 Cysts are randomly distributed throughout the forebrain without regional tropism

Cysts were identified in 53 forebrain regions across the 45 *T. gondii*-infected rats with cysts (1–6 wpi). Thirty-nine of those 53 regions contained cysts in at least 2 rats (Table 1). Pooling rats from 3–6 wpi (based on rationale from section 3.4 indicating that cyst presence is associated with behavior after 3 wpi), Chi-square analysis revealed no difference in the percentage of rats with cysts in each brain region as compared to the expected percentage if cysts were randomly distributed (Chi-square = 24.66; $p = 0.95$; Figure 5A). Even though there was no significant difference in cyst presence in specific forebrain areas, it is worth noting that the PVN and POA stood out as above 2X normalized presence and AO, Pir, S1, CPu, ic, and CxA/AA stood out as below 0.5X (see Table 1 for abbreviations). Again with data from rats pooled across 3–6 wpi, one-way ANOVA of regional differences in cyst burden (relative to area) revealed no difference in mean cyst burden per region ($F_{(38,1170)} = 1.305$; $p = 0.104$; Figure 5B).

3.3 *T. gondii* infection, alone, did not alter any behavioral measures

One-way ANOVA examining effect of *T. gondii* infection on behavior revealed no effect of wpi on BCAA (bobcat occupancy ratio, $F_{(5,101)} = 1.255$; $p = 0.289$), EPM (time spent in open arms, $F_{(5,101)} = 0.197$; $p = 0.963$; time spent in closed arms, $F_{(5,101)} = .416$; $p = 0.837$; open arm entries, $F_{(5,101)} = 0.095$; $p = 0.993$; or closed arm entries, $F_{(5,101)} = 0.453$; $p = 0.810$), or OF behavior (time spent in the center, $F_{(5,100)} = 1.160$; $p = 0.332$; number of entries into the center, $F_{(5,100)} = 1.534$; $p = 0.186$; or number of line crossings around the edge of the arena $F_{(5,100)} = 1.587$; $p = 0.171$). Data for the first 2 minutes of the OF similarly revealed no effect of wpi on any behavior (data not shown). Given that not all infected rats had evidence for *T. gondii* in the forebrain, we next examined whether there was an effect of cyst presence on behavior in *T. gondii*-infected rats.

3.4 *T. gondii*-infected rats with cysts in the forebrain showed attenuation of predator odor aversion and increased anxiety-related behavior from 3–6 wpi

Two-way ANOVA examining effects of wpi and cyst presence on bobcat occupancy ratio revealed an interaction between wpi and cyst presence that only approached significance ($F_{(4,71)} = 2.334$; $p = 0.064$; Figure 6A). In order to test the specific hypothesis that the relationship between cyst presence and attenuation of predator odor aversion behavior develops over time, and may not be present at early time points, a more sensitive change-point analysis (as described in 2.8.4) revealed a relationship between cyst presence and attenuation of predator odor aversion behavior at and after but not before 3 wpi (see Figure 6A). Therefore, behavioral data from 3–6 wpi were pooled for further analysis. *T. gondii*-

infected rats with cysts in the forebrain (3–6 wpi) showed less predator odor aversion behavior than *T. gondii*-infected rats without cysts (no cysts, bobcat occupancy ratio = 0.334 ± 0.024 ; cysts, bobcat occupancy ratio = 0.429 ± 0.029 ; $t_{(63)} = -2.554$, $p = 0.013$; Figure 6B). We then examined EPM and OF behavior (3–6 wpi) to determine if there were effects of cyst presence on anxiety-related, exploratory or locomotor behaviors that coincided with effects on predator odor aversion behavior.

Effect of cyst presence on EPM—*T. gondii*-infected rats with cysts (3–6 wpi) spent more time in the closed arms of the EPM than rats without cysts (no cysts, 150.3 ± 5.2 seconds; cysts, 169.3 ± 7.4 seconds; $t_{(63)} = -2.095$, $p = 0.041$) and conversely less time in the middle and open arms, although neither middle or open arm endpoints were significantly altered by cyst presence.

Effect of cyst presence on OF—*T. gondii*-infected rats with cysts (3–6 wpi) had no differences in any behaviors in the open field relative to *T. gondii*-infected rats without cysts (time spent in the center; no cysts, 8.9 ± 1.5 seconds; cysts, 6.2 ± 1.0 seconds; $t_{(62)} = 1.456$, $p = 0.151$). Analysis of the first two minutes of the OF also revealed no effect of cyst presence on open field behavior (data not shown).

3.5 *T. gondii*-infected rats with cysts in specific forebrain regions show more attenuation of predator odor avoidance and anxiety-related behavior

Having demonstrated that *T. gondii*-infected rats with cysts (3–6 wpi) showed less aversion to predator odor relative to *T. gondii*-infected rats without cysts, the degree of association between cyst presence in specific regions and changes in specific behaviors (BCAA, EPM and OF; 3–6 wpi) was assessed. Mean behavior of *T. gondii*-infected rats with cysts in each of 39 specific forebrain regions were assessed relative to *T. gondii*-infected rats with no cysts. It is important to note that, in rats with cysts, overall cyst burden was not correlated with differences in any behavioral endpoints (BCAA, bobcat ratio, $p = 0.577$; EPM, open time, $p = 0.717$, close time, $p = 0.993$, open entries, $p = 0.416$, close entries, $p = 0.643$; OF, center time, $p = 0.984$, center entries, $p = 0.779$, locomotion, $p = 0.832$) suggesting that overall cyst burden does not explain changes in behavior and emphasizing the potential importance of regional cyst location.

Bobcat Odor Approach-Avoidance—Comparison of means between *T. gondii*-infected rats with cysts in each region, relative to those without cysts revealed a number of regions in which cyst presence was more likely to be contributing to attenuation of predator odor aversion in *T. gondii*-infected rats with cysts (Figure 7). In descending order, the regions in which cyst presence resulted in a difference in BCAA behavior with the greatest effect size relative to *T. gondii*-infected rats with no cysts (bobcat ratio = 0.334 ± 0.024) were as follows: the sublenticular extended amygdala (EA, bobcat ratio = 0.685 ± 0.129 , $d = 1.859$), parietal association cortex (PtA, bobcat ratio = 0.653 ± 0.144 , $d = 1.560$), substantia innominata/diagonal band (SI/DB, bobcat ratio = 0.487 ± 0.023 , $d = 1.427$), dentate gyrus (DG, bobcat ratio = 0.495 ± 0.038 , $d = 1.381$), frontal cortex area 3 (Fr3, bobcat ratio = 0.620 ± 0.163 , $d = 1.272$), globus pallidus (GP, bobcat ratio = 0.160 ± 0.164 , $d = 1.248$), internal capsule (ic, bobcat ratio = 0.473 ± 0.034 , $d = 1.247$), preoptic area/magnocellular preoptic nucleus (POA, bobcat ratio = 0.465 ± 0.016 , $d = 1.223$), prelimbic and infralimbic cortices (PL/IL, bobcat ratio = 0.596 ± 0.135 , $d = 1.207$), cingulate cortex area 1 (Cg1, bobcat ratio = 0.486 ± 0.045 , $d = 1.174$), retrosplenial cortex (RS, bobcat ratio = 0.552 ± 0.092 , $d = 1.147$), accessory olfactory bulb (AO, bobcat ratio = 0.454 ± 0.035 , $d = 1.070$), lateral hypothalamus (LH, bobcat ratio = 0.501 ± 0.054 , $d = 1.021$), and lateral septum (LS, bobcat ratio = 0.531 ± 0.108 , $d = 0.987$). A complete listing of regional effects on behavior

and effect size can be found in Figure 7. See Table 1 for sample sizes of rats with cysts in each region.

Elevated Plus Maze—Comparison of means between *T. gondii*-infected rats with cysts in each region, relative to those without cysts, revealed a number of regions in which cyst presence was more likely to be contributing to the increase in time spent in the EPM closed arms in *T. gondii*-infected rats with cysts (Figure 8). In descending order, the regions in which cyst presence resulted in a difference in EPM closed arm time with the greatest effect size relative to *T. gondii*-infected rats with no cysts (EPM closed arm time = 150.3 ± 5.3 seconds) were as follows: the frontal association cortex (FrA, EPM closed time = 206.4 ± 16.1 seconds, $d = 1.888$), globus pallidus (GP, EPM closed time = 190.9 ± 22.0 seconds, $d = 1.160$), cingulate cortex area 2 (Cg2, EPM closed time = 193.2 ± 22.3 seconds, $d = 1.112$), ventral pallidum (VP, EPM closed time = 188.5 ± 17.0 seconds, $d = 1.095$), olfactory tubercle (Tu, EPM closed time = 186.5 ± 19.3 seconds, $d = 1.028$), nucleus accumbens (Acb, EPM closed time = 182.0 ± 13.4 seconds, $d = 0.986$). A complete listing of regional effects on behavior and effect size can be found in Figure 8. See Table 1 for sample sizes of rats with cysts in each region.

Open field—Comparison of means between *T. gondii*-infected rats with cysts in each region, relative to those without cysts, revealed a number of regions in which cyst presence was more likely to be contributing to a difference in time spent in the center of the OF in *T. gondii*-infected rats with cysts (Figure 9). In descending order, the regions in which cyst presence resulted in a difference in OF center time with the greatest effect size relative to *T. gondii*-infected rats with no cysts (OF center time = 8.6 ± 1.4 seconds) were as follows: the parietal association cortex (PtA, OF center time = 2.0 ± 1.5 seconds, $d = 1.038$) and sublenticular extended amygdala (EA, OF center time = 2.8 ± 2.4 seconds, $d = 0.859$). A complete listing of regional behavior and effect size can be found in Figure 9. In a measure of general locomotion in the open field (perimeter line crossings) comparison of means between *T. gondii*-infected rats with cysts in each region, relative to those without cysts revealed less regional influence of cyst location on this behavior (Figure 10). Nevertheless, two regions in which cyst presence resulted in a difference in locomotion with the greatest effect size relative to *T. gondii*-infected rats with no cysts (line crossings = 28.4 ± 1.4) were the auditory cortex (Au, line crossings = 22.3 ± 2.9 , $d = 0.879$) and frontal association cortex (Fr3, line crossings = 24.2 ± 0.9 , $d = 0.696$). A complete listing of regional behavior and effect size can be found in Figure 10. See Table 1 for sample sizes of rats with cysts in each region.

4. Discussion

We demonstrated that only a subpopulation of *T. gondii*-infected rats developed chronic infection marked with *T. gondii* in the forebrain. *T. gondii*, intracellular cysts and activated microglia were present in forebrains by 7–9 dpi and persisted until at least 6 wpi. Importantly, attenuation of predator odor aversion did not develop until 3 wpi and occurred in only the subpopulation of infected rats with cysts. Cysts in specific forebrain regions were associated with attenuation of predator odor aversion and/or increased anxiety in the elevated plus maze. We observe that neuroanatomical cyst location may determine the nature of *T. gondii*-induced behavioral changes.

4.1 Seropositivity for anti-*T. gondii* IgG was not predictive of chronic cyst presence

While not all *T. gondii*-infected rats developed chronic infection with cysts in the forebrain, all *T. gondii*-infected rats tested positive for serum anti-*T. gondii* IgG. This concurs with studies showing that IgG seropositivity reflects exposure, but not chronic infection (Dubey

and Frenkel, 1998; Jones and Dubey, 2012). Interestingly, 50% of *T. gondii*-seropositive HIV/AIDS patients who become immunocompromised show no symptoms of toxoplasmic encephalitis (Luft and Remington, 1992), suggesting that humans may also carry *T. gondii* antibodies without harboring parasites in the brain. We demonstrated that rats with forebrain cysts had a higher serum IgG from 3–6 wpi relative to infected rats without forebrain cysts. Notably, HIV/AIDS patients with higher IgG titers have more risk for developing toxoplasmic encephalitis (Derouin *et al.*, 1996; Hellerbrand *et al.*, 1996).

Prior reports have shown resistance to *T. gondii* in rats, relative to species such as mice (Dubey and Frenkel, 1998; Innes, 1997; Li *et al.*, 2012; McCabe and Remington, 1986). Resistance refers not only to the likelihood of avoiding chronic infection despite exposure, but also to avoiding sickness behavior despite chronic infection. This makes a rat model particularly attractive for studying chronic infection. Resistance in mice is variable and highly dependent on host sex and strain and *T. gondii* strain (Pung and Luster, 1986; Roberts *et al.*, 1995; Suzuki, 2002). A recent report demonstrates cysts as well as sickness in only a subpopulation of infected female C57BL/6J mice (Afonso *et al.*, 2012). Factors influencing resistance are numerous and include activity of inducible nitric oxide synthase and arginase-1 in peritoneal macrophages of rats (Li *et al.*, 2012), glucocorticoid dependent T-cell responses (Kugler *et al.*, 2013), toll-like receptors (Andrade *et al.*, 2013) and immunity related GTPases (Fleckenstein *et al.*, 2012).

4.2 Cysts were evenly distributed throughout the forebrain of *T. gondii*-infected rats

Parasites were present in the forebrain as early as 7–9 dpi and, throughout the time course. The lack of regional tropism is consistent with the idea that *T. gondii* enters the brain through microvasculature, and does so via infected immune cells (Feustel *et al.*, 2012). Indeed, *T. gondii* increases migration of infected circulating immune cells (Lambert *et al.*, 2006), perhaps increasing dissemination. A widespread distribution is consistent with most of the rodent literature (Afonso *et al.*, 2012; Berenreiterova *et al.*, 2011; Haroon *et al.*, 2012) and even in studies reporting tropisms in rats (Gonzalez *et al.*, 2007; Vyas *et al.*, 2007a), there was a wide distribution of parasites in the brain.

4.3 Evidence for changes in behavior were associated with cyst presence at and after 3 weeks post-infection

Cyst presence at the earliest time point (2 wpi) was not associated with attenuation of predator odor aversion, suggesting that physical cyst presence alone cannot explain changes in behavior, but that the mechanism(s) through which cyst presence is related to behavior may require time to develop. This delay concurs with studies in rats in which this behavior is assessed 4–8 wpi (Lamberton *et al.*, 2008; Vyas *et al.*, 2007b).

4.4 Individual variation in neuroanatomical cyst location was associated with individual variation in predator odor approach-avoidance behavior and anxiety-related behavior

This is the first study in rats demonstrating that regional *T. gondii* cyst presence in individual subjects is associated with behavior. More than cyst presence alone, consideration of specific anatomical location of cyst presence increased the association between cyst presence and behavioral changes in the predator odor aversion task, as well as anxiety-related behavior in the elevated plus maze and open field. Examining function attributed to regions may provide insight into mechanisms through which *T. gondii* cyst presence disrupts behavior. However, some potential mechanisms, such as the local immune response, may impact regions adjacent to cysts and be the mediator of altered behavior. Deconstructing cause and effect of regional cyst presence on behavior is therefore particularly challenging, given the variety of convergent mechanisms that could contribute to disruption of predator

urine odor avoidance, including, but not limited to, aspects of novelty-seeking, motivation, risk assessment, olfaction, and spatial memory.

In examining those regions with the strongest association between cyst presence and attenuation of predator odor aversion behavior, one readily sees connections between the behavioral alteration and the known functions of many of these regions. The sublenticular extended amygdala (EA) is involved in emotional processing and detection or attribution of salience for stimuli (Liberzon 2003) integral to decisions regarding approach-avoidance. The retrosplenial cortex (RS) and parietal association cortex (PtA) are involved in spatial navigation and spatial memory (Ranganath and Ritchey, 2012; Torrealba and Valdes, 2008). The medial preoptic area (mPOA) controls appetitive male sexual behavior (Dominguez and Hull, 2005) which is inversely related to predator avoidance (Kavaliers *et al.*, 2001). Prelimbic and infralimbic cortices (PL/IL) control anxiety-related behavior in response to a predator (Adamec *et al.*, 2012). Lateral hypothalamus (LH) is involved in reward-seeking appetitive behaviors (Kelley, 2004). For reasons discussed above, attempting to attribute functional relevance to each implicated region may be less useful than recognition of the fundamental concept that regional cyst presence is important for changes in behavior. Furthermore individual subjects can have cysts in multiple interconnected brain regions that could have synergistic or antagonistic effects on behavior. For these reasons it is also less useful to discuss the functional significance of regions in which cyst presence is associated with behavioral changes in the elevated plus maze or open field.

That regional cyst presence is important for changes in behavior provides the foundation for using what we know about *T. gondii*-host biology to test hypotheses explaining the proximate mechanisms by which cysts in a specific region can alter behavior. Examples of proximate mechanisms include altered neurotransmission due to known parasite metabolic requirements for amino acid precursors glutamine and tryptophan (Macrae *et al.*, 2012; Pfefferkorn *et al.*, 1986), or altered dopamine homeostasis, a speculation prompted by the *T. gondii* genome containing a homolog to the mammalian tyrosine hydroxylase gene (Prandovszky *et al.*, 2011). Alternatively, the neurobiological mechanisms of altered behavior may be more directly related to the immune response to *T. gondii* which would have potential impact on adjacent regions as well. From IBA-1 label in the present study, such an immune response is striking, perhaps being of more significance than cysts themselves. Cysts are often the center of a surrounding sphere of activated microglia (and astrocytes) extending into adjacent regions (see Figure 4). Persistence and non-pathogenicity of *T. gondii* is known to be dependent on establishment of a homeostatic balance with the host neuroimmune response (Aliberti, 2005; Denkers *et al.*, 2012; Miller *et al.*, 2009). The host immune response to cysts involves upregulation of kynurenic acid, an endogenous compound which modulates glutamatergic, cholinergic, monoaminergic and GABAergic signaling (Schwarcz *et al.*, 2012). The ability to examine each of these hypotheses is founded on the present demonstration that regional cyst presence is important for behavior.

4.5 Distribution-specific influences on behavior can explain inconsistencies regarding reported effects of *T. gondii* on behavior

The widespread distribution of cysts within individual subjects has implications for individual differences in behavioral changes. Various behavioral effects of infection, relating to neophobia, learning and memory, anxiety, predator odor aversion and locomotion have been reviewed recently (Kaushik *et al.*, 2012). Our results suggest that if cysts localize in a region in which their presence can influence a behavior (or adjacent to a region in which distal effects of cyst presence can influence a behavior), the subject is more likely to show alterations in that behavior. The ability to detect changes in a specific behavior would be a function of how likely it is for a cyst to end up within (or adjacent to) nodes of a network

influencing that behavior in enough individual subjects to influence the mean. Effect of infection on predator odor aversion in rats has been reported by two groups, only in males, and always when evaluating approach-avoidance to predator urine (Berdoy *et al.*, 2000; Lamberton *et al.*, 2008; Vyas *et al.*, 2007a; Vyas *et al.*, 2007b; Webster *et al.*, 2006), with no negative reports. This could be attributed to the nature of this behavioral measure being susceptible to modulation via convergent mechanisms. In the context of *T. gondii* fitness, whether the parasite is disrupting novelty-seeking behavior, anxiety, impulsivity, sexual proactivity, or risk assessment, there are many known behavioral alterations that may converge to increase the proximity of an infected rodent to a cat.

4.6 Conclusions

While it may seem obvious that the subpopulation of rats that develop changes in behavior are, *within* those that seroconvert, those that actually develop cysts in the brain, this is a critical distinction as chronic *T. gondii* infection is often confirmed by seropositivity. Our data suggest that seroconversion indicates exposure, but not chronic infection, and that attenuation of predator odor aversion and changes in anxiety-related behavior are linked with cyst presence in specific brain areas. Future studies will determine mechanisms through which cyst presence in specific regions can disrupt functional neural circuits and behavior.

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Figure 1.

Diagram of experimental design indicates timing of infections relative to behavioral testing and tissue collection. All rats in each treatment were infected on a single day in the appropriate week leading up to 3-day blocks of behavioral testing in the open field (OF), elevated plus maze (EPM), and bobcat urine approach-avoidance (BCAA) task and a 3-day block of brain and blood (tissue) collection. Individual rats had 3 days between each behavioral test and prior to tissue collection. All treatments were equally balanced across and within each day of behavioral testing and tissue collection.

Figure 2.

Not all rats that were infected with *T. gondii* developed chronic infection with evidence of parasites/cysts in the forebrain, but those that did, ended up with similar cyst burdens across the time course. A) Graph depicts percentage of *T. gondii*-infected rats that developed chronic infection with evidence of parasites/cysts in the forebrain across the time course (45 out of 102 rats; 44%). B) Graph depicts mean cyst burden in rats with cysts across the time course. Due to presence of fields of individual parasites and numerous small cyst-like structures unique to the 1-week time point, the burden was not assessed at 1 week. Error bars indicate SEM.

Figure 3.

While not all *T. gondii*-infected rats developed chronic infection with evidence of parasites/ cysts in the forebrain, anti-*T. gondii* IgG antibodies were detectable (above control serum from uninfected rats) in all *T. gondii*-infected rats by 1 week post-infection (wpi). Graph depicts ELISA quantification of anti-*T. gondii* IgG antibodies measured from serum collected from *T. gondii*-infected rats at the time of tissue collection. Anti-*T. gondii* IgG antibodies increased across the time course and were higher in *T. gondii*-infected rats in which forebrain cysts were identified with histology (closed circles), versus those in which cysts were not found (open circles). * indicate significant differences between cyst and no cyst group means at specific time points ($p < 0.05$). Error bars indicate SEM.

Figure 4.

Photomicrographs depict representative examples of *T. gondii* (GFP, green) cysts and individual parasites in brain sections, with microglial IBA-1 immunostaining (Alexa Fluor 594, red) across the time course. DAPI nuclear stain (blue) provides cytoarchitectural detail. Panels are arranged by weeks post-infection (wpi) indicated by the number to the left of each row. Numbers (gray) in each panel indicate rostrocaudal level, millimeters Bregma (Paxinos and Watson, 2005), followed by region of interest depicted in each panel. Abbreviations are indicated in Table 1. Large fields of individual parasites were more common at earlier time points (A, B). Individual cysts (C, H, N, P) and clusters of cysts (F, G, I, K, L) were observed at each time point. Cysts were observed with evidence of activated dense clusters of microglia (F, I, O, Q, R) as well as independent of microglial activation (C, N). Some panels (D, E) indicate evidence for microglial activation independent of evidence for *T. gondii* in the immediate section. A ruptured cyst (M) was observed at 5 wpi; insets z_1 and z_2 indicate different depths of focus through the ruptured

cyst at higher magnification. Scale bars (white) = 50 micrometers for main panels and 10 micrometers for insets.

Figure 5.

Tissue cysts were observed across many brain regions. Bar graphs depict (A) relative cyst presence and (B) relative cyst burden in each region in which cysts were observed in more than one animal (3–6 weeks post-infection; wpi). *T. gondii* cysts were identified by green fluorescent protein emission under 20X magnification in serial 40 micrometer sections, 240 micrometers apart, through the forebrain from 6.12 to –4.36 millimeters from Bregma. A) Regional cyst presence represents the number of rats with cysts in each region divided by the region area and normalized to a mean of 1. B) Regional cyst burden represents the mean number of cysts per region divided by the region area. Regions are arranged along the x-axis in rostrocaudal anatomical order (see Table 1 for abbreviations). Dotted lines represent mean values across regions (including multiplication factors 0.5X, 2X, and 4X in panel A). Error bars represent SEM.

Figure 6.

T. gondii-infected rats with cysts in the brain displayed attenuated predator odor aversion behavior relative to *T. gondii*-infected rats without cysts at and after 3 weeks post-infection (wpi). A) Bars depict, across the time course, the mean bobcat occupancy ratio (time spent in the half of the arena containing bobcat urine relative to total time) in *T. gondii*-infected rats with cysts (filled) and without cysts (open). B) Circles depict the mean bobcat occupancy ratio in *T. gondii*-infected rats with cysts (filled) and without cysts (open) when data was pooled from 3–6 wpi. * indicate significant differences between cyst and no cyst group means ($p < 0.05$). Error bars represent SEM.

Figure 7.

T. gondii-infected rats with cysts in specific forebrain regions (3–6 weeks post-infection) displayed attenuated predator odor aversion behavior. Scatter plot diagram indicates, for *T. gondii*-infected rats with cysts in each brain region, (y-axis) the mean bobcat occupancy ratio (time spent in the half of the arena containing bobcat urine relative to total time; larger ratio is less aversion), and (x-axis) the effect size (Cohen's *d*-value) relative to the behavior of *T. gondii*-infected rats that do not have cysts (dotted line). Open gray circle indicates the overall magnitude and effect size of the mean behavior of *T. gondii*-infected rats that had cysts anywhere in the forebrain relative to *T. gondii*-infected rats that did not have cysts (dotted line). Abbreviations and sample sizes are listed in Table 1.

Figure 8.

T. gondii-infected rats with cysts in the forebrain (3–6 weeks post-infection) spent more time in the closed arms of an elevated plus maze compared those without cysts in the forebrain. The magnitude and effect size of this difference was greater when rats were grouped by presence of cysts in specific forebrain regions. Scatter plot diagram indicates, for *T. gondii*-infected rats with cysts in each brain region, (y-axis) the mean time spent in the closed arms of the elevated plus maze, and (x-axis) the effect size (Cohen's *d*-value) relative to the behavior of *T. gondii*-infected rats that do not have cysts (dotted line). Open gray circle indicates the overall magnitude and effect size of the mean behavior of *T. gondii*-infected rats that had cysts anywhere in the forebrain relative to *T. gondii*-infected rats that did not have cysts (dotted line). Abbreviations and sample sizes are listed in Table 1.

Figure 9.

Cyst presence in specific forebrain regions was more highly associated with changes in anxiety-related behavior in the open field than overall cyst presence. Scatter plot diagram indicates, for *T. gondii*-infected rats with cysts in each forebrain region, (y-axis) the mean time spent in the center of the open field arena and (x-axis) the effect size (Cohen's *d*-value) relative to the behavior of *T. gondii*-infected rats that do not have cysts (dotted line). Open gray circle indicates the overall magnitude and effect size of the mean behavior of *T. gondii*-infected rats that had cysts anywhere in the forebrain relative to *T. gondii*-infected rats that did not have cysts (dotted line). Abbreviations and sample sizes for each region are listed in Table 1.

Figure 10.

Cyst presence in specific forebrain regions was more highly associated with changes in locomotion behavior in the open field than overall cyst presence. Scatter plot diagram indicates, for *T. gondii*-infected rats with cysts in each forebrain region, (y-axis) the mean number of perimeter line crossings in the open field and (x-axis) the effect size (Cohen's *d*-value) relative to the behavior of *T. gondii*-infected rats that do not have cysts (dotted line). Open gray circle indicates the overall magnitude and effect size of the mean behavior of *T. gondii*-infected rats that had cysts anywhere in the forebrain relative to *T. gondii*-infected rats that did not have cysts (dotted line). Abbreviations and sample sizes for each region are listed in Table 1.

Regional cyst distribution

Table identifies regions in which cysts were identified in more than 1 rat at each time point post-infection. For each region, abbreviation, distance from Bregma, and represented area of analysis are listed along with the number of rats in which cysts were identified in each region at each time point.

Table 1

Region	Abbreviation	Bregma Range	Area (mm ²)	weeks post-infection (# rats with cysts)						
				1	2	3	4	5	6	SUM
frontal association cortex	FrA	6.48 to 5.52	31.09	0	0	1	2	1	0	4
accessory olfactory bulb	AO	6.72 to 3.12	41.88	0	2	1	1	0	1	5
orbital cortex	O	5.76 to 2.40	93.33	0	2	5	4	3	2	16
prelimbic and infralimbic cortices	PL/IL	5.28 to 2.40	49.53	1	1	0	2	2	0	6
frontal cortex, area 3	Fr3	4.80 to 2.64	24.67	0	3	1	1	1	0	6
nucleus accumbens	Acb	3.36 to 0.48	50.45	0	1	1	3	1	1	7
olfactory tubercle	Tu	3.36 to -0.24	42.02	1	2	0	2	2	0	7
cingulate cortex area 1	Cg1	4.32 to -1.44	58.22	1	2	3	1	2	0	9
claustrum	Cl	4.32 to -1.92	26.39	0	1	1	1	3	0	6
secondary motor cortex	M2	5.28 to -3.12	139.75	1	3	4	4	3	0	15
ventral pallidum	VP	3.12 to -0.96	30.16	1	0	1	2	2	1	7
primary motor cortex	M1	4.80 to -3.12	146.16	2	3	6	4	8	3	26
agranular insular cortex	AI	4.32 to -2.88	84.38	1	3	3	2	4	1	14
lateral septum	LS	2.16 to -0.72	43.00	1	1	0	2	2	1	7
cingulate cortex area 2	Cg2	2.16 to -1.44	36.42	1	2	1	3	0	0	7
granular/dysgranular insular cortex	GI/DI	3.36 to -2.88	83.02	1	1	2	2	4	0	10
piriform cortex	Pir	4.80 to -4.32	149.55	1	3	2	3	4	2	15
substantia inominata/ diagonal band	SI/DB	1.68 to -1.44	17.70	1	1	1	0	2	1	6
endopiriform nucleus	En	4.32 to -4.32	45.55	1	1	0	3	3	0	8
bed nucleus of stria terminalis	BST	0.72 to -1.44	18.08	0	1	0	1	1	0	3
primary somatosensory cortex	SI	3.36 to -4.32	526.88	4	5	6	7	9	5	36
caudate putamen	Cpu	2.88 to -3.84	282.27	2	1	5	4	7	3	22
preoptic area	POA	0.48 to -1.44	23.44	1	0	3	1	3	1	9
sublenticular extended amygdala	EA	-0.72 to -1.68	10.13	0	0	1	0	2	0	3

Region	Abbreviation	Bregma Range	Area (mm ²)	weeks post-infection (# rats with cysts)					
				1	2	3	4	5	6
secondary somatosensory cortex	S2	1.20 to -3.84	84.88	2	2	2	3	3	2
	PVN	-0.96 to -1.92	3.06	0	0	0	1	2	0
paraventricular hypothalamic nucleus	GP	-0.24 to -3.12	34.95	0	0	1	0	2	0
	AH/DA	-0.96 to -3.12	8.17	0	0	0	1	1	0
anterior/dorsal hypothalamic area	ic/cp	0.00 to -4.32	67.19	3	0	2	0	1	0
	Cx/AA	-0.24 to -4.32	45.67	2	0	1	0	1	1
cortical amygdala transition area/amygdaloid area	thal	-0.96 to -4.32	191.20	3	2	3	5	5	2
	LH	-1.20 to -4.32	47.70	0	1	2	2	5	2
basolateral amygdaloid nucleus	BL	-1.68 to -4.32	20.39	0	0	0	1	1	0
	DG	-1.68 to -4.32	35.77	0	1	0	1	2	1
dentate gyrus	Ca	-1.68 to -4.32	76.16	1	0	2	4	2	1
	Or	-1.68 to -4.32	44.50	0	0	2	2	1	1
oriens layer of the hippocampus	RS	-1.68 to -4.32	46.05	0	0	0	3	3	0
	PtA	-3.12 to -4.08	29.05	2	0	1	0	2	0
retrosplenial granular/dysgranular cortex	Au	-3.12 to -4.32	36.33	1	0	1	0	1	1