TOXOPLASMA GONDII REDUCES INNATE FEAR TO PREDATOR URINE BY ALTERING THE BRAIN OF ITS RODENT HOST

A DISSERTATION
SUBMITTED TO THE DEPARTMENT OF NEUROSCIENCE AND THE COMMITTEE ON GRADUATE STUDIES OF STANFORD UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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d. Abstract

The protozoan parasite *Toxoplasma gondii* is known to dampen fear responses to cat urine in infected rodent hosts. Such manipulation is likely advantageous to the parasite in the wild, possibly increasing predation by the definitive host of *Toxoplasma*, the cat. Here we report that in infected rats, *Toxoplasma* modifies limbic structures responsive to appetitive, sexual stimuli, converting ensembles of neural activity in regions of sexual attraction to respond to cat urine. This argues that the loss-of-aversion phenotype might be, in part, a gain-of-attraction to cat odors. Understanding how *Toxoplasma* is inspiring these behavioral changes in the host brain likely requires knowledge of the precise anatomical location of the parasite in the host brain. For this reason, we used a genetically-engineered *Toxoplasma* in a mouse that allowed us to track, at single-neuron resolution, the location of the parasite and the location of any neuron it invaded or tried to invade (“*Toxoplasma*-interacted neurons”). We report high individual variation in loss-of-aversion to cat urine in infected mice and that numbers of *Toxoplasma*-interacted neurons in the total brain did not explain this variation. However, variation in number of *Toxoplasma*-interacted neurons in the corpus striatum predicted response to the dopamine D2 receptor antagonist haloperidol, providing evidence that *Toxoplasma* is interacting with dopamine in the infected host brain.
e. Acknowledgements

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Chapter I. Introduction

Toxoplasma gondii is a coccidian protozoan parasite, the cause of clinical Toxoplasmosis. Sexual reproduction of Toxoplasma occurs exclusively in cats, after which the parasites are shed via the feces to form environmentally hardy cysts which can remain viable for months to years (Dubey, 2008). Intermediate hosts, including humans, acquire Toxoplasma via carnivorism of an already infected host or consumption of cysts in soil or water (Dubey, 2008).

It is estimated that up to 35% of humans have active or have had a past Toxoplasma infection. Infection is usually subclinical in immunocompetent humans, but can become opportunistic if the host is immunocompromised, as in AIDS, where it can lead to fatal encephalopathy. In the rare case of symptomatic Toxoplasmosis in an immune competent human, the disease can present as swollen lymph nodes, fever, myalgia, abdominal pain or chorioretinitis, an infection of the eye. Congenital Toxoplasmosis passed from infected mother to her fetus can cause blindness, mental retardation or fetal death in some cases (Lehmann et al., 2006).

Though Toxoplasma can infect a broad range of vertebrate non-feline hosts, special attention in recent years has been paid to the impact of infection on rats and mice, based on early observations that chronic infection altered exploratory behaviors (Hutchinson et al., 1980; Hay et al., 1983a; Hay et al., 1983b; Hay et al., 1984; Hay et al., 1985; Webster et al., 1994) and aversion to cat odors (Berdoy et al., 2000a; Webster, 2001; Webster et al., 2006b). These findings compelled the idea that Toxoplasma in the infected rodent could induce a loss-of-aversion phenotype to its definitive host, perhaps via an increase in activity, a decrease in neophobia or a lessened fear response to the cat
odors themselves. If these behavioral changes in intermediate host behavior increase rates of predation by felines, this could drive selection pressure for the parasite. Other parasitically-induced host manipulations have been found in multiple non-mammalian hosts (Biron et al., 2005; Kuris et al., 2007; Libersat et al., 2009; Adamo, 2013; Knight, 2013), though it is thought to be less common in mammals given the complexities of the mammalian nervous system and the efficacy of the blood-brain barrier to keep pathogens out. It should be noted that increased predation rates in *Toxoplasma*-infected rodents have never been confirmed nor tested in either natural or laboratory settings, so the ecological validity of predation-rate based hypotheses remains an open question.

The innate rodent behavioral fear response to cat odors is a well-studied (Dielenberg et al., 2001; Canteras, 2002; Choi et al., 2005; Takahashi et al., 2007), reliable phenotype, and exists in laboratory rodents generations removed from exposure to any feline or non-feline predator. Any mechanism by which *Toxoplasma* is perturbing the innate fear response in rodents is of great relevance to animal models of anxiety and fear and their associated disorders in humans, which affect about 18% of the U.S. population in a given year and can manifest as panic, phobia, obsessive-compulsive disorder or post-traumatic stress disorder. An understanding of the impact and mechanisms of *Toxoplasma* on the host brain in rodents as a model of mammalian anxiety and fear is therefore of pressing relevance given its potential medical, zoonotic and epidemiological importance.
Behavioral effects in rodents

*Loss-of-aversion to cat urine.* Cat odors induce rapid, innate and stereotyped defensive behaviors in rats and mice at first exposure, a likely response to the evolutionary pressures of predation. A common behavioral measure of aversion is time spent near the aversive stimulus, with uninfected animals preferring to avoid areas with cat urine. Since the first observation of loss-of-aversion to cat urine in *Toxoplasma*-infected rats (Berdoy et al., 2000a), multiple studies have confirmed similar loss-of-aversion in infected rats (Webster et al., 2006b; Vyas et al., 2007; House et al., 2011) and mice (Vyas et al., 2007; Haroon et al., 2012b; Xiao et al., 2012; Ingram et al., 2013). Infected rodents spend significantly more time closer to the normally aversive cat urine, though these differences can be arguably interpreted as a loss of aversion, a gain of attraction, or both (House et al., 2011). Some studies in rats have argued for specificity of *Toxoplasma* manipulations toward cat urine, observing that infected rats do not lose aversion to non-feline predator odors nor display changes in multiple other fear or anxiety related behaviors (Vyas et al., 2007; Lamberton et al., 2008). More recent studies in different strains of rats and with different strains of *Toxoplasma* have challenged these results, observing *Toxoplasma*-induced alterations in anxiety measures unrelated to feline odors (Gonzalez et al., 2007; Evans et al., 2014), though it is unclear whether the reported differences are from individual variation in *Toxoplasma* infection or from intended experimental variability. Regardless, the claim of specificity is likely too strong, as the burden of proof for such a claim would demand repeated and reliable testing against an exhaustive list of odors, conditions, strains and behaviors.

Surprisingly, similar loss-of-aversion to cat urine has been observed in both
infected rats and infected mice, despite large differences in sickness behavior, lethality and dose of *Toxoplasma* in rats and mice. For example, laboratory infections in rats using large doses of as many as $10^7$ *Toxoplasma* tachyzoites caused no obvious sickness behavior or lethality, and only half of rats appear to develop brain cysts (Afonso et al., 2012; Evans et al., 2014). In contrast, laboratory infection in mice is highly pathogenic at a range of smaller doses, from 400 to $10^4$ tachyzoites, and can cause sickness or death in a high percentage of mice during the acute stages of parasite proliferation, depending on mouse and parasite strain. It is possible that rat-specific immune factors account for this species-dependent pathogenicity, and these immune differences are likely to influence the outcome of infection as *Toxoplasma* settles into chronic tissue or brain cysts. Nonetheless, multiple studies have shown loss-of-aversion in infected mice to cat urine, in both females (Vyas et al., 2007; Haroon et al., 2012b; Xiao et al., 2012) and males (Ingram et al., 2013), with one study observing loss-of-aversion in females but not males (Xiao et al., 2012), arguing that *Toxoplasma* has a generalized ability to influence fear responses in both rats and mice.

*Non-aversion behaviors.* Studies in mice looking at non-aversion behaviors have found multiple motor and cognitive effects of chronic *Toxoplasma* infection, including deficits in memory and learning (Hodkova et al., 2007; Wang et al., 2013), exploration (Hermes et al., 2008; Gatkowska et al., 2012), and motor coordination (Gulinello et al., 2010; Wang and Bao, 2013). Intriguingly, two studies found that chronic *Toxoplasma* infection is protective against stroke (Arsenijevic et al., 2007) after middle-cerebral artery occlusion and against progression of Alzheimer’s-like memory deficits in Alzheimer’s
model mice (Jung et al., 2012), possibly due to a *Toxoplasma*-related immune anti-inflammatory response in the brain (Aliberti, 2005). The evidence thus supports the idea that *Toxoplasma* infection in mice is not inducing specific changes to cat odor aversion, but is instead a multifactorial insult with a broad range of effects on the mouse host including sickness, death, motor and cognitive dysfunction and induced immunological states.

*Variables of infection.* Studies in mice looking at *Toxoplasma*-induced changes in behavior have differed in the age, strain and sex of the mouse, the strain and dose of the parasite, the route of infection, the measured behavioral outcome and the days post-infection of behavioral testing (Table 1). Though this could arguably be evidence for a robustness of the *Toxoplasma* phenotype, it nonetheless makes it difficult to draw conclusions across studies or cross-validate results given such variable study by study methodology. For example, recent evidence indicates that laboratory infections in rats result in chronic brain cysts in only a subset of infected rats (Evans et al., 2014) and while cysts tend to be found in all infected mouse brains, the numbers vary widely depending on parasite strain, mouse strain and time post-infection (Kim et al., 2007; Berenreiterová et al., 2011; Buchholz et al., 2013). Given the heterogeneity of *Toxoplasma* infection protocols, it is difficult to conclude if these observations are a generalized property of *Toxoplasma* infection or a likely consequence of, for example, parasite dose, infection route, or an interaction between parasite strain, mouse strain, and timing post-infection. In order to assess whether behavioral changes induced by *Toxoplasma* are brain and parasite independent and whether variance in CNS infection
relates to variance in behavioral outcome, it will in future studies be crucial to systematically understand each variable of infection rather than amplify heterogeneity in an already heterogeneous parasite-host interaction.

**Neurobiology of Toxoplasma infection**

*Tropism*. The brain circuitry underlying innate fear in rodents exposed to predator odors involves rapid and stereotyped limbic and cortical activity (Dielenberg et al., 2001; Canteras, 2002; Choi et al., 2005). Specifically, during exposure to cat odors, accessory olfactory nuclei which respond highly to cat odors synapse directly onto amygdalar sub-nuclei which, via efferent connections to the hypothalamus, elicit rapid and stereotyped behavioral aversion responses downstream via the thalamus, basal ganglia and cortex. A plausible hypothesis is that *Toxoplasma* infection in the brain exhibits tropism toward regions involved in the innate fear response, based on the idea that *Toxoplasma* is likely to inspire manipulations in its proximal cellular environment. Indeed, early studies found that *Toxoplasma* density was significantly higher in amygdalar regions necessary for generation of innate fear responses (Vyas et al., 2007). However, subsequent studies in rats and mice observed no specific tropism (Berenreiterová et al., 2011; Gatkowska et al., 2012; Evans et al., 2014).

*Mechanism.* *Toxoplasma* can chronically encyst inside of neurons in the host CNS, placing it in a privileged position to alter individual, chronically-infected neurons or the surrounding cellular environment. Studies of infection in cell lines *in vitro* have observed
electrophysiological membrane disruptions during invasion (Suss-Toby et al., 1996), an increase in dopamine release in challenged dopaminergic neurons (Prandovszky et al., 2011) and tachyzoite infections in dendrites, soma and axons of cultured neurons, leading to both hypo- and hyper-responsive signaling in these neurons (Haroon et al., 2012a). Recent observations with a Cre-secreting Toxoplasma parasite both in vitro and in vivo raised the intriguing possibility that Toxoplasma secretes effector proteins into neurons it does not fully invade, based on observations that many of these uninfected but injected cells show increases in phosphorylated STAT6 (Koshy et al., 2012), a cytokine-responsive, host transcription factor (Wurster et al., 2000). This builds on previous findings that Toxoplasma can affect other host transcription factors and block host cell defenses (Saeij et al., 2007; Steinfeldt et al., 2010), arguing that Toxoplasma has the potential to change individual cells via either invasion or injection.

None of the above parasite-dependent changes in the proximal cellular environment preclude the possibility that Toxoplasma is influencing the host via parasite-independent mechanisms, for example, chronic immune activation (Cekanaviciute et al., 2014; Evans et al., 2014). Growing evidence for the role of immune activation includes the observations that loss-of-aversion behavior remains even after parasite clearance (Ingram et al., 2013) and that the protective effect of infection on stroke and Alzheimer’s mice can be explained as a redirection of immune resources by Toxoplasma away from the respective insult sites.

However, an alternate interpretation for the persistence of loss-of-aversion phenotype after Toxoplasma has cleared is that Toxoplasma has altered neurons in a way that persists long after the parasite is gone, a hypothesis supported by the above
observations that *Toxoplasma* can alter host cell transcription factors. This hypothesis is especially intriguing in light of evidence that *Toxoplasma* cyst location does not seem to directly relate to any behavioral changes, nor does there appear to be tropism to any specific brain region of behavioral interest. Though no specific changes have been reported in neurons injected but not invaded by *Toxoplasma*, such a hypothesis would predict that locations and numbers of these injected but uninvaded neurons might correlate with changes in behavior. Such a mechanism would also exponentially expand the potential range of *Toxoplasma* impact in the brain. For example, if *Toxoplasma* could alter nuclear transcription factors in a single neuron from an injection anywhere along its processes, a single parasite in the cortex could inject into a striatal axon afferent, altering that neuron’s signal transmission to elsewhere in the brain even though the parasite and neuronal cell body are many millimeters apart. If this were the case, even a small amount of parasites could have widespread, multiplicative impact throughout the host brain.

**Behavioral effects in humans**

*Neuropsychiatric disease*. The anticipated genetic determinants of neuropsychiatric diseases like depression, schizophrenia and obsessive compulsive disorder (OCD) have mostly failed to materialize, increasing scrutiny toward environmental, infectious and social factors for their potential etiological significance. As an infectious agent, *Toxoplasma* is uncommon for a non-viral pathogen in its ability to physically persist in the central nervous system (CNS) for the lifetime of the host, often invading or encysting inside of neurons (Dubey et al., 1998). From a privileged location in the host CNS, multiple known consequences of *Toxoplasma* infection are plausibly relevant to
psychiatric disease, including persistent immune activation (Evans et al., 2014; Yarovinsky, 2014), changes to neurotransmitter levels (Stibbs, 1985; Prandovszky et al., 2011), hijacking of metabolic pathways and loss of function in host neurons (Haroon et al., 2012a). Nor are these consequences of infection mutually exclusive, leaving open the possibility that *Toxoplasma* infection is a multifactorial insult on the host CNS that, even if failing to induce Toxoplasmosis, is nonetheless capable of altering the cellular and neurological landscape of the human brain. In support of this hypothesis, recent studies in humans have found positive associations between *Toxoplasma* and depression (Groer et al., 2011; Hsu et al., 2014), suicide (Arling et al., 2009; Okusaga et al., 2011; Okusaga and Postolache, 2012) and schizophrenia (Yolken et al., 2009; Nimgaonkar and Yolken, 2012; Park et al., 2012; Fabiani et al., 2013). An association with schizophrenia and affective psychosis is observed even with maternal seropositivity at birth (Brown et al., 2005; Mortensen et al., 2007; Xiao et al., 2009), arguing that congenital Toxoplasmosis may play a role in later development of mental illness.

A meta-analysis of all 38 studies published between 1956 and 2011 on the association of *Toxoplasma* and schizophrenia, with a total sample size of 6058 patients and 8715 controls, calculated a combined odds ratio (OR) of 2.71 (95% CI 2.21–3.38). At the time, this ranked *Toxoplasma* as one of the top non-genetic risk factors for schizophrenia (Torrey et al., 2012). Since 2011, four additional studies have reported on an association between seropositivity for *Toxoplasma* and schizophrenia, two of which found an association (Alipour et al., 2011; Nascimento et al., 2012) and two of which failed to (Emelia et al., 2012; Khademvatan et al., 2014). A limitation of many of these studies is an inability to know the timing of first *Toxoplasma* exposure, and therefore the
duration of infection relative to onset of schizophrenia. Methods for detecting Toxoplasma antibodies cannot differentiate timing of initial Toxoplasma infection past a few months, precluding longitudinal analysis and seriously limiting the power of the studies to claim causality.

A single study using repeated, longitudinal serum samples from individuals discharged with schizophrenia from the U.S. military found an association between Toxoplasma acquired within six months of diagnosis (Niebuhr et al., 2008). However, a variable amount of serum samples were collected for each patient prior to being pooled, potentially weighting those individuals infected for a longer time and with more serum samples. Using OR criteria from the Torrey et al., 2011 meta-analysis, which ignores repeated sampling, and raw seropositivity data from Niebuhr et al., 2008 reveals that overall association between Toxoplasma and schizophrenia in Niebuhr et al., 2008 was actually low (OR 1.22; 95% CI 0.65 – 2.27) and not significant (p = 0.53). Nonetheless, this study was the first to attempt a longitudinal association between Toxoplasma and a neuropsychiatric illness and is a model for the type of study needed in the future. A follow-up study, also using serum from the U.S. military, did not involve prospective risk and found only a slight hazard ratio of Toxoplasma for schizophrenia (Li et al., 2013).

A wide range of other positive psychiatric associations have been found in single studies, which report links between Toxoplasma and OCD (n=42 patients, OR 3.88) (Miman et al., 2010b), Parkinson’s disease (n=52 patients, OR 2.53) (Miman et al., 2010a), and Alzheimer’s disease (n=34 patients, OR 2.46) (Kusbeci et al., 2011). However, it should be emphasized that these are single studies with small sample sizes and that there is not enough work to generate a combined odds ratio. By analogy, ORs
from individual studies showing positive associations between *Toxoplasma* and schizophrenia ranged from 1.22 to over 10.0 with equivalent sample sizes.

**Integrating behavioral and neurobiological findings**

Linking individual variation in behavior to individual variation in infection burden is difficult partly because of the heterogeneity of infection and partly because of the difficulty of detecting *Toxoplasma* in the brain. Much of the early work on *Toxoplasma* and behavior failed to address individual variation, leaving open the possibility that loss-of-aversion or other behavioral effect sizes could actually be stronger, masked within group-wide effects of pooled subjects. However, recent genetic advances in detecting *Toxoplasma* in the host have resulted in the ability to track not only the parasite via fluorescence, bioluminescence or both (Saeij et al., 2005; Vyas et al., 2007) but also, by means of a Cre-secreting parasite and reporter plasmids, the specific cells it invades (Koshy et al., 2010; Koshy et al., 2012). Thus the ability to track the parasite down to single-neuron resolution creates the potential for comparisons of individual behaviors to the exact and complete anatomical burden of infection in the brain.

Yet another approach to link behavior to neurobiology involves a specific pharmacological rescue of *Toxoplasma*-induced behavior. For example, if all dopamine receptor antagonists restored aversion behavior in infected mice and all serotonergic, glutamatergic and GABAergic receptor antagonists failed to, these observations could motivate a closer look at dopaminergic neurons and circuits as they function in the infected host brain. Indeed, a previous attempt to rescue loss-of-aversion in *Toxoplasma*-
infected rats used three candidate drugs administered a week after infection: dapsone with pyrimethamine, which inhibits tachyzoite proliferation in the acute stages of infection; valproic acid, a mood stabilizer, which has been shown to inhibit *Toxoplasma* growth *in vitro*; and haloperidol, a dopamine D2 receptor antagonist also shown to inhibit *Toxoplasma* growth *in vitro* (Webster et al., 2006b). Remarkably, all three treatments prevented the loss-of-aversion phenotype from developing in infected rats. However, because individual animals were not tested prior to drug treatment, and because each drug was administered during the acute stage before the parasite likely made it to the brain, not much can be concluded about *Toxoplasma* interaction with the drug in the brain *per se*, as there was no proper rescue of the phenotype, but instead a prevention.

**Conclusions and Experiments**

In summary, decades of studies on the effect of *Toxoplasma* on rodent behavior have established chronic *Toxoplasma* infection as a compelling and reliable rodent model for fear and anxiety. Although the specific manner and mechanism by which *Toxoplasma* alters its host are still to be determined, recent efforts to develop technology to track the parasite, to unpack individual variation and to pharmacologically rescue the phenotype indicate that the field is moving from phenomenological investigations of establishing a *Toxoplasma* phenotype to mechanistic investigations of the causes underneath.

In the chapters that follow we report a two-fold investigation. First, we looked in *Toxoplasma*-infected rats at limbic brain regions responsive to both cat odors and appetitive, sexual stimuli. The purpose of this study was to determine whether or not the ensemble of neural activity in these regions could explain loss-of-aversion in infected rats as either a loss-of-aversion or perhaps a gain-of-attraction to the cat odor. We report that
infected rats had significantly increased neural activity in amygdala regions responsive to sexual stimuli, arguing that the loss-of-aversion behavior might be, at least in part, driven by an increased attraction to the cat odor stimuli (House et al., 2011).

Next, we investigated the relationship of *Toxoplasma*-induced behavioral changes to cat urine and the location of injected (“*Toxoplasma*-interacted”) neurons, using a Cre-secreting strain of *Toxoplasma* and a reporter mouse. The purpose of this study was to investigate the potential role of effector secretion by *Toxoplasma* and to characterize total numbers and location of all neurons that interact with *Toxoplasma*, either through invasion or injection. In addition, we investigated, in the same mice, the effect of *Toxoplasma* infection on administration of the dopamine receptor D2 antagonist haloperidol, with the aim of studying the relationship between *Toxoplasma* and dopamine challenge in the brain. We report that total numbers and location of *Toxoplasma*-interacted neurons did not correlate with any measured behavioral changes, but that the number of *Toxoplasma*-interacted neurons in the corpus striatum correlated with protection against haloperidol-induced behavioral changes.
Table 1. Summary of Behavioral Effects of *Toxoplasma* in Mice and Rats

<table>
<thead>
<tr>
<th>Name et al</th>
<th>Yr</th>
<th>Mouse</th>
<th>Toxo</th>
<th>Sex</th>
<th>Route</th>
<th>Week p.i.</th>
<th>Test</th>
<th>Finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingram</td>
<td>13</td>
<td>BALB/c</td>
<td>RHΔmp5 / CEP</td>
<td>Male</td>
<td>IP (500 tachys)</td>
<td>16 w</td>
<td>Forced choice</td>
<td>loss-of-aversion</td>
</tr>
<tr>
<td>Wang</td>
<td>13</td>
<td>BALB/c</td>
<td>PRU</td>
<td>Both</td>
<td>Maternal</td>
<td>10 w</td>
<td>Memory, etc</td>
<td>learning &amp; memory deficits</td>
</tr>
<tr>
<td>Gatkowska</td>
<td>13</td>
<td>C57HsdM</td>
<td>ME49</td>
<td>Both</td>
<td>IP (20 cysts)</td>
<td>3 &amp; 6w</td>
<td>n.a.</td>
<td>increased motoneurons</td>
</tr>
<tr>
<td>Costa</td>
<td>12</td>
<td>B6</td>
<td>ME49</td>
<td>Female</td>
<td>IP (10^-4 tachys)</td>
<td>9w</td>
<td>Open field, etc</td>
<td>increased exploration</td>
</tr>
<tr>
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<td>12</td>
<td>CD-1</td>
<td>VSG</td>
<td>Both</td>
<td>Maternal</td>
<td>4 &amp; 8 w</td>
<td>Barnes maze</td>
<td>short-term memory deficit</td>
</tr>
<tr>
<td>Jong</td>
<td>12</td>
<td>Tg2567</td>
<td>Me49</td>
<td>n.a.</td>
<td>IP (15 cysts)</td>
<td>24 w</td>
<td>Y, Water, etc</td>
<td>reverses memory deficits in AZ</td>
</tr>
<tr>
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<td>12</td>
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<td>DX</td>
<td>F</td>
<td>Oral (4 cysts)</td>
<td>4 &amp; 8 w</td>
<td>Open field</td>
<td>loss-of-aversion</td>
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<td>BALB/c</td>
<td>PRU</td>
<td>F</td>
<td>IP (400)</td>
<td>8-16w</td>
<td>3 chamber</td>
<td>loss-of-aversion</td>
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<tr>
<td>Gatkowska</td>
<td>12</td>
<td>B6</td>
<td>Me49</td>
<td>M</td>
<td>IP (20 cysts)</td>
<td>3 &amp; 6w</td>
<td>Open field, etc</td>
<td>reduced exploration</td>
</tr>
<tr>
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<td>10</td>
<td>B6</td>
<td>Me49</td>
<td>M</td>
<td>IP (1000)</td>
<td>7w</td>
<td>Open field, etc</td>
<td>motor coordination / sensory</td>
</tr>
<tr>
<td>Hermasz</td>
<td>08</td>
<td>Swiss</td>
<td>Me49</td>
<td>F</td>
<td>IP (1000 cysts)</td>
<td>48w</td>
<td>Open field, etc</td>
<td>reduced exploration, motor</td>
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<td>Hoekstra</td>
<td>07</td>
<td>B*</td>
<td>HIF</td>
<td>n.a.</td>
<td>Oral (15)</td>
<td>10w</td>
<td>8 arm, etc</td>
<td>memory / learning deficit</td>
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<td>07</td>
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<td>PRU</td>
<td>F</td>
<td>IP (400)</td>
<td>4-5w</td>
<td>Forced choice</td>
<td>loss-of-aversion</td>
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<td>07</td>
<td>BALB/c</td>
<td>Me49</td>
<td>F</td>
<td>IP (40 cysts)</td>
<td>3.5w</td>
<td>Morris Water</td>
<td>increased TNF</td>
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<td>07</td>
<td>Strain A</td>
<td>Beverly</td>
<td>M</td>
<td>Maternal</td>
<td>13w</td>
<td>Locomotion</td>
<td>increased locomotion</td>
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<td>04</td>
<td>Rat</td>
<td>Strain A</td>
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<td>Maternal</td>
<td>15w</td>
<td>Y-maze</td>
<td>decreased nephobia</td>
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results in sensorimotor deficits but normal cognitive behavior despite widespread brain pathology. Microbes Infect 12:528-537.


Webster JP, Lamberton PH, Donnelly CA, Torrey EF (2006) Parasites as causative agents of human affective disorders? The impact of anti-psychotic, mood-


Chapter II. *Toxoplasma gondii* In the Rat: Predator Cat Odors Activate Pathways of Sexual Arousal in *Toxoplasma gondii*-Infected Rats

This work has been published as:


Author contributions:

Conceived and designed the experiments: PKH, AV, RS.

Performed the experiments: PKH

Analyzed the data: PKH, AV

Wrote the paper: PKH
i. INTRODUCTION

Cat odors induce rapid, innate and stereotyped defensive behaviors in rats at first exposure, a presumed response to the evolutionary pressures of predation. Bizarrely, rats infected with the brain parasite *Toxoplasma gondii* approach the cat odors they typically avoid. Since the protozoan *Toxoplasma* requires the cat to sexually reproduce, this change in host behavior is thought to be a remarkable example of a parasite manipulating a mammalian host for its own benefit. *Toxoplasma* does not influence host response to non-feline predator odor nor does it alter behavior on olfactory, social, fear or anxiety tests, arguing for specific manipulation in the processing of cat odor. A fascinating phenomenon in behavioral biology is the ability of parasites to manipulate host behavior for their own benefit. A handful of examples are noted for insect (Biron et al., 2005; Kuris et al., 2007; Libersat et al., 2009) hosts, but rarely so in mammals. The extraordinary effectiveness of the mammalian blood brain barrier denies most pathogens access to the privileged central nervous system, the seat of will. *Toxoplasma* is an obligate, single-celled protozoan parasite capable of crossing into the central nervous system of any warm-blooded vertebrate. *Toxoplasma* requires the cat intestine to reproduce sexually, is shed in cat feces, and must make its way from the ground to another cat host (Dubey et al., 1998).

*Toxoplasma* manages this in part by infecting ground-dwelling rodents who, remarkably, begin selectively preferring areas with cat urine (Berdoy et al., 2000b; Webster et al., 2006a; Vyas et al., 2007). Infected rats retain normal defensive behavior to non-feline predator odor and normal performance on memory, anxiety, fear and social tasks (Vyas et
This specific preference for cat odor is likely an adaptive manipulation by *Toxoplasma*, increasing infected rat predation rates and facilitating *Toxoplasma* transmission to the cat. Little is known about how *Toxoplasma* inspires this manipulation. By two weeks post infection, *Toxoplasma* has settled throughout the rodent host brain in spherical cysts approximately 50-70 μm in diameter (Dubey et al., 1998). We investigated the effect of *Toxoplasma* on the neural activity in limbic system regions involved in both ‘defensive’ and ‘reproductive’ innate behavior. Neural activity was quantified using the immediate early gene c-Fos, a proxy for neural activity.

**ii. RESULTS**

**Neural activity in limbic brain regions**

We first confirmed limbic activity during exposure to either cat urine or an inaccessible estrous female. As expected (Dielenberg et al., 2001; Canteras, 2002; Choi et al., 2005) in uninfected rats, cat urine increased neural activity in the ‘defensive’ ventromedial hypothalamus, dorsomedial part (VMHdm) (Figure 1B and Table S1). Exposure to an estrous female rat increased activity in the ‘reproductive’ posterodorsal medial amygdala (MEApd) (Figure 1B and Table S1) (Choi et al., 2005). *Toxoplasma* infection made rats spend more time exploring cat urine (Figure 1A) and increased neural activity in the MEApd, VMHdm and basolateral amygdala (BLA) (Figure 1C and 1D) during this exploration. During exposure to an estrous female, *Toxoplasma* increased activity in the VMHdm and BLA, but did not significantly alter MEApd activity (Figure 1E) relative to
uninfected controls. *Toxoplasma* infected rats had reduced volumes of both the MEApd and the posteroventral medial amygdala (MEApv).
Figure 1. Toxoplasma Infection Alters the Limbic Response in Rats Exposed to Cat Odor. (A) Toxoplasma infected rats spent more time exploring cat urine than uninfected rats. (B) In uninfected animals, female odor induces relatively greater ‘reproductive’ MEApd activity and relatively diminished ‘defensive’ VMHdm activity, as expected. (C) In infected animals, activity levels in the MEApd are the same, regardless of whether it is a ‘reproductive’ or ‘defensive’ odor (D) Neural activity in infected male rats, normalized to uninfected controls, after exposure to cat urine. Infection increased neural activity in the BLA, MEApd and VMHdm. (E) Neural activity in infected male rats, normalized to
uninfected controls, after exposure to an estrous female. Infection again increased neural activity in the BLA and VMHdm, but did not change MEApd levels. (F) Infection elevated ‘reproductive’ MEApd levels during exposure to cat odor to levels similar to uninfected animals exposed to female. Abbreviations: MEApd (posterodorsal) medial amygdala; VMHdm (ventromedial hypothalamus, dorsomedial part); BLA (basolateral amygdala). P values listed where appropriate.
Figure 2. Models of Limbic Activity and the Effect of Toxoplasma Infection on the Processing of Cat Odor.

(A) Schematic model of limbic activity in a male rat. Exposure to an inaccessible estrous female activates the ‘reproductive’ pathway, producing robust MEApd activity and eliciting approach behavior. (B) Exposure to cat urine activates the ‘defensive’ pathway, producing robust activation of excitatory VMHdm neurons and defensive aversion behavior. Excitatory VMHvl neurons, countering inhibitory projections from the MEApd and suppressing any approach behavior. (C) Proposed model for Toxoplasma rats during exposure to cat urine. Robust MEApd activity biases toward approach behavior. Aversion behavior remains, but is lessened. Raw density of c-Fos (large print) ±SEM (small print) activity is given for each region during relevant odor exposure. AOB, accessory olfactory bulb; MEA, medial amygdala; VMH, ventromedial hypothalamus. Models adapted from Choi 2005.
iii. DISCUSSION

Rats have separate ‘defensive’ and ‘reproductive’ pathways gating innate behavioral response to, respectively, predator or sexual stimuli (Choi et al., 2005). Given the precipitous pressures of both reproduction and predation, these pathways run as direct projections from the olfactory bulb to the limbic system and generate rapid and stereotyped behavioral output (see Figure 2A and 2B). Thus although functionally distinct, the limbic ‘defensive’ and ‘reproductive’ pathways run in parallel through the medial amygdala and hypothalamus in close anatomical proximity. Previous findings of increased *Toxoplasma* cyst density in these areas compel the possibility that *Toxoplasma* is somehow perturbing its surrounding neural environment and thereby manipulating the host response to cat urine. We find, indeed, that *Toxoplasma* infection perturbs the ‘defensive’ pathway in the infected rat during exposure to cat urine, shifting neural activity to the nearby ‘reproductive’ pathway, specifically the MEApd (see Figure 2C). The MEApd projects robustly to hypothalamic nuclei involved in sexual arousal and the generation of approach behavior. The MEApd is responsive to a variety of social odorants (Goodson and Kabelik, 2009), but responds strongest to opposite-sex mating stimuli. Lesioning the MEApd reduces, specifically, attraction to opposite-sex odors (Maras and Petrulis, 2006). Interestingly, *Toxoplasma* brought MEApd activity during cat urine exposure to levels of uninfected rats during female exposure (Figure 1F). This suggests that the specifically increased magnitude of MEApd activity in male *Toxoplasma* infected rats is biasing the processing of the cat urine toward the sexual, ‘reproductive’ pathway (Figure 2D). Plausibly, this shift is altering the salience of the cat
urine stimuli and mitigating the defensive response by creating, in its stead, a competing attraction to the cat urine.

Little is known about how, if at all, *Toxoplasma* cysts exert themselves in the host brain. Much work remains to be done, based on striking findings that *Toxoplasma* raises whole brain dopamine levels in rats by up to 15% (Stibbs, 1985) and that dopamine receptor antagonists block rodent host attraction to cat urine (Webster et al., 2006a). These data suggest a link between dopamine, a primary neurotransmitter in decision-making and reward, and the altered behavior. Intriguingly, the *Toxoplasma* genome contains a homolog of tyrosine hydroxylase (Gaskell EA, 2009) the rate-limiting enzyme in the vertebrate synthesis of dopamine, raising the possibility that *Toxoplasma* is altering dopamine levels by synthesizing its own tyrosine hydroxylase.

From the ground, *Toxoplasma* finds its way into other hosts besides rats, including cows, sheep, pigs and many grazing livestock. Ingestion of undercooked meat from infected livestock and the profligacy of private cat ownership are responsible for a strikingly high number of human chronic *Toxoplasma* infections. Approximately one-third of humans are seropositive for *Toxoplasma* across the world (Lafferty, 2006) and several recent studies find infection increases risk for schizophrenia (Leweke et al., 2004; Cetinkaya et al., 2007; Torrey and Yolken, 2007) and obsessive compulsive disorder (Miman et al., 2010c), diseases noted for elevated dopamine levels and disturbed amygdala function (Benes, 2010). Our results are therefore of wide interest, as the ability of *Toxoplasma* to
dramatically alter host behavior and proper amygdala functioning may extend beyond the rat into ancillary *Toxoplasma* hosts, including humans.

iv. MATERIALS AND METHODS

*Experimental Design*

Animals were split into four groups: cat-urine uninfected (n = 9), female-odor uninfected (n = 9), cat-urine infected (n=9) or female-odor infected (n = 9). On the day of sacrifice, animals were exposed to either cat odor or an inaccessible estrous female. Brains were collected and regions of interest were analyzed for c-Fos activation. For stereological analysis, twelve animals were split into two groups: stereology-control (n = 6) and stereology-infected (n = 6).

*Animals*

Male Long Evans rats were housed in groups of three, kept on a 12 hr light/dark cycle and given food and water ad libitum. Behavioral testing occurred during the light cycle.

*Toxoplasma injection* The *Toxoplasma* infected groups (n = 18 for cat-urine infected groups, n = 6 for stereology-infected group) were injected i.p. with approximately $10^7$ *Toxoplasma* tachyzoites. We employed a Prugnaud strain of *Toxoplasma*, maintained as tachyzoites by passage in human foreskin fibroblast monolayers. Infected fibroblasts were syringe lysed using a 27-gauge needle and injected into animals. Animals were either infected i.p. with *Toxoplasma* tachyzoites or mock-infected with sterile PBS. Behavior experiments and c-Fos quantification was performed six weeks post-infection.

*Odor Exposure and Behavior*
For cat-urine groups, a towel with 1 ml bobcat urine was clipped to a rack above the home cage for 20 min. For female-odor groups, an inaccessible estrous female was placed in the home cage for 20 min, separated from the male rats by a plastic divider with holes in it. Male rats could not touch the female. Video recordings were scored by A.V. Briefly, in the videos post hoc ‘incentive zones’ were created around the feline urine or the towel and the number of nose pokes into this area were scored across the twenty minute period.

**Tissue Fixation**

Animals were sacrificed 90 min after the end of the 20 minute exposure to either cat odor or a female rat. Animals were deeply anaesthetized and transcardially perfused with 4% paraformaldehyde (PFA) made in 0.1 M phosphate buffer (PB). The brains were removed from the skull and postfixed in 4% PFA overnight. Blocks containing the amygdala and hypothalamus were cut on a cryostat and subsequently sectioned into 40 μm thick sections.

**Immunohistochemistry**

Sections were incubated in 1% H202 for 15 min and then incubated for 90 hrs at 4°C with a c-Fos primary antibody (1:2000, sc52 Rabbit Polyclonal, Santa Cruz Biotechnology) diluted in PBS (0.1 M PBS with 0.2% Triton-X and 0.1% BSA). Sections were then incubated for 1 hr in a secondary antibody solution (1:400, biotinylated anti-rabbit IgG, Vector Laboratories), followed by incubation for 1hr in Vectastain Elite ABC Reagent (1:25, Vectastain Elite ABC Kit, PK6101 Rabbit, Vector Laboratories). Next, sections were incubated for 6 min in a DAB solution (DAB Substrate Kit SK-4100, Vector Laboratories). Sections were mounted and cover slipped. Sections were washed in
0.1 M PBS for 30 min between each of these steps and all steps were done under agitation.

*b-Fos Counting*

For technical reasons, not all brains could be counted. Two animals were lost in the cat-urine uninfected group (leaving n = 7), one in the female-odor uninfected group (leaving n = 8) and one in the cat-urine Toxoplasma group (leaving n = 8). Regions of interest were traced in Stereo Investigator software and scored. Only darkly-labeled oval shaped nuclei were counted as b-Fos positive. The area of the region of interest was scored using the Cavalieri Estimator tool in Stereo Investigator software. The number of positive nuclei was divided by the area of the region to arrive at b-Fos density per region of interest.

*Stereology*

A systematic and randomly sampled series of sections through regions of interest was used to estimate volumes. Specifically, 40 μm coronal sections throughout the entire region of interest were cut and cresyl-violet stained. The area of the ROI in every fourth section was estimated using the Cavalieri Estimator tool in Stereo Investigator software. The first section in the series was randomly selected from among the first four sections. The distance between the upper surfaces of the sections was 160 μm (4 x 40 μm). Areas were recorded for each ROI as described above and total volumes were calculated using the Stereo Investigator software.

*Statistical Analysis*

Behavior was analyzed using one-way analysis of variance (ANOVA) to compare between uninfected and Toxoplasma infected groups exposed to cat urine. Values are
reported as mean ± SEM throughout. For c-Fos counts and volume data, independent-samples T test was conducted. A P value of < 0.05 indicates statistical significance throughout.

REFERENCES


onset schizophrenia. European Archives of Psychiatry and Clinical Neurosciences 254:4-8.


III. CHAPTER 3. *Toxoplasma gondii* in the Mouse: Individual Variation to Acute Haloperidol in *Toxoplasma gondii*-infected Mice Correlates with Infection Presence in the Corpus Striatum

This work has not been published. It will be submitted soon as, likely:


Author contributions:
Conceived and designed the experiments: PKH, SB, RS.
Performed the experiments: PKH, SB
Analyzed the data: PKH
Wrote the paper: PKH
Materials & Reagents: AK
i. INTRODUCTION

Recent studies have observed that *Toxoplasma*-induced loss-of-aversion remains in infected mice after parasite clearance from the brain (Ingram et al., 2013), raising the possibility that in addition to *Toxoplasma* altering the local cellular environment it might also be inspiring changes in the host brain that persist long after it has cleared. *A priori*, multiple mechanisms could explain these findings, including a long-lasting, active inflammatory immune state, induction of apoptotic or necrotic neuron cell death or the secretion of effector molecules into neurons to induce long-lasting changes. The later hypothesis is supported by *in vitro* and *in vivo* work using a Cre-secreting *Toxoplasma* strain and observations that a vast majority of neurons *Toxoplasma* is interacting with by injecting effector molecules do not actually end up invaded (Koshy et al., 2010; Koshy et al., 2012). *Toxoplasma*-interacted neurons are therefore candidates for neurons that are the targets of effector molecules which might underlie the behavioral changes seen in infected rodents.

The purpose of these studies was to investigate the relationship between location of *Toxoplasma*-interacted neurons in the mouse brain and changes in aversion behavior induced by *Toxoplasma*. To do this, we used a Cre-secreting Type II *Toxoplasma* strain to infect a Cre-dependent reporter mouse line, as described previously (Koshy et al., 2010; Madisen et al., 2010). This allows us to resolve every individual neuron and cell in the brain that *Toxoplasma* has interacted with, either via infection or injection.

Another goal of the present studies was to investigate a potential relationship between dopamine and *Toxoplasma*-induced behaviors in the brains of infected mice. *Toxoplasma* has at least two genes for tyrosine hydroxylase, the rate-limiting enzyme in
the biosynthesis of dopamine (Gaskell EA, 2009), increases whole brain dopamine levels 14% (Stibbs, 1985), and increases dopamine secretion after infection in dopamine PC-12 neuronal cell lines (Prandovszky et al., 2011). Intriguingly, two weeks of haloperidol, a dopamine D2 receptor antagonist, has been shown to prevent loss-of-aversion in *Toxoplasma*-infected rats (Webster et al., 2006b). The consistent observation that *Toxoplasma* increases locomotor activity (Hay et al., 1983a; Webster et al., 1994; Afonso et al., 2012; Gatkowska et al., 2012) is also intriguing given that similar amphetamine-induced increases in locomotor activity, which are thought to be the result of postsynaptic dopamine receptor activation (Bardo et al., 1990), are also blocked by haloperidol (Bardo et al., 1990). In the present experiments, infected and uninfected mice were repeatedly tested for aversion behavior in both drug and no drug conditions. Infected mice were challenged with haloperidol during the chronic stage of infection and thus the effect of haloperidol on chronic infection was measured in the context of aversion.

**ii. RESULTS**

**Aversion behavior**

Mice were exposed to the arena for habituation on each of two days prior to the first behavioral assessment. **Figure 1** illustrates the effect of *Toxoplasma* on mouse behavior during exposure to low (.5 mL) and high (2 mL) doses of cat urine. For distance to cat in percentage of arena (**Fig. 1a**), two-way ANOVA with dose as repeated measure revealed no effect of infection ($F_{(1,47)} = 1.040; $ NS), a significant effect of dose ($F_{(1,47)} = 12.46; p = .0009$), and a significant interaction effect between infection and dose ($F_{(1,47)} = 14.25; p = 0.0004$). *Post-hoc* Bonferroni analysis revealed that high dose cat urine increased
distance to cat in uninfected mice but not in *Toxoplasma* mice. In addition, *Toxoplasma* had no effect at low dose relative to uninfected mice, but decreased distance to cat in the high dose condition. For total distance moved (**Fig. 1b**), two-way ANOVA with dose as repeated measure revealed no effect of infection ($F_{(1,47)} = 2.971$; NS), dose ($F_{(1,47)} = 0.01$; NS), or interaction effect between infection and dose ($F_{(1,47)} = 3.82$; NS). *Post-hoc* Bonferroni revealed that *Toxoplasma* had no effect at low dose, but increased total distance moved in the high dose condition. For percentage of total time spent freezing (**Fig. 1c**), two-way ANOVA with dose as repeated measure revealed a significant effect of infection ($F_{(1,47)} = 4.430$; $p = 0.04$), no effect of dose ($F_{(1,47)} = 1.609$; NS), and a significant interaction effect between infection and dose ($F_{(1,47)} = 10.87$; $p = 0.001$). *Post-hoc* Bonferroni analysis revealed that high dose cat urine increased freezing in uninfected mice but not in *Toxoplasma* mice. In addition, *Toxoplasma* had no effect at low dose but reduced freezing in the high dose condition relative to uninfected mice.

**Figure 1**, D E and F, illustrate the effect of *Toxoplasma* on behavioral outcomes by rank during exposure to high dose. Mann-Whitney U analysis revealed a significant effect of *Toxoplasma* by rank at the high dose in distance to cat (**Fig. 1d**, $p = 0.02$), total movement (**Fig. 1e**, $p = 0.01$), and freezing (**Fig. 1f**, $p = 0.007$).

**Figure 2** is a movement heat map of all mice during high-dose (2 mL) cat urine exposure, sorted from left to right by time spent in the cat area, defined as the tenth of the arena closest to the cat urine. *Toxoplasma* increased cat area occupancy (data not shown), defined as the time spent in cat area divided by total time spent in cat area and rabbit area (uninfected mean $= 0.28 \pm 0.03$; *Toxoplasma* mean $= 0.40 \pm 0.02$; $p = 0.008$; unpaired t test). *Toxoplasma* also increased the percentage of sorties starting from the rabbit area.
which ended in the cat area (data not shown, uninfected mean = 0.53 ± 0.03; *Toxoplasma* mean = 0.64 ± 0.02; *p* = .01, unpaired t test).

**Number and distribution of *Toxoplasma*-interacted neurons**

Table 1 shows the distribution by brain region of *Toxoplasma*-interacted neurons. All infected mice had some fluorescent neurons and no uninfected mice had any fluorescent neurons in the brain. *Toxoplasma* mice (n=35) had a mean of 1281 ± 198.8 *Toxoplasma*-interacted neurons and the number of these neurons varied widely from mouse to mouse, ranging from a high of 3948 neurons to a low of 73 neurons. The location also varied widely, as no single brain region had *Toxoplasma*-interacted neurons in all mice and no single region had significantly different density by one-sample t test relative to mean density across all regions (see Table 1). Whole-brain totals of *Toxoplasma*-interacted neurons did not correlate with any behavioral outcomes during the high dose cat urine exposure. A regression analysis revealed no significant relationship between total number of *Toxoplasma*-interacted neurons in the brain and distance to cat in percentage of arena (r²<0.001, NS), cat area occupancy (r² = 0.003, NS), total distance moved (r² = 0.14, NS) or percentage freezing (r² = 0.08, NS). An additional exploratory regression analysis of all brain regions from Table 1 against all behavioral outcomes revealed no significant relationship between number of *Toxoplasma*-interacted neurons in any individual brain region and any tested behavior.
Effect of single-dose, acute haloperidol on aversion behavior

Acute haloperidol administered i.p. 30 minutes before behavior at a dose of 0.25mg/kg had no effect on average distance to cat or cat area occupancy (data not shown). For total distance moved, a two-way ANOVA revealed a significant effect of drug ($F_{(1,45)} = 70.48; p < 0.0001$) and no effect of infection ($F_{(1,45)} = 3.00; p = 0.09$) or interaction effect between drug and infection ($F_{(1,45)} = 0.01; NS$). Post-hoc analysis revealed haloperidol reduced distance moved in both uninfected and *Toxoplasma* mice. Figure 3, A and B, provide evidence that *Toxoplasma* is interacting with haloperidol-induced changes in freezing. For percentage freezing in the acute haloperidol condition (Fig. 3a), a two-way ANOVA revealed a significant effect of drug ($F_{(1,45)} = 173.1; p < 0.0001$) and a significant effect of infection ($F_{(1,45)} = 11.03; p = 0.001$), and no interaction effect between drug and infection ($F_{(1,45)} = 0.77; NS$). Post-hoc Fisher’s LSD revealed that haloperidol increased freezing in both uninfected and *Toxoplasma* mice and that *Toxoplasma* reduced freezing after administration of haloperidol compared to uninfected mice. Because all mice underwent repeated behavioral testing, the difference in amount of freezing from the no drug condition to the acute haloperidol condition (“delta freezing”) was analyzed to account for individual variation in baseline freezing rates. For delta freezing from no drug to acute haloperidol condition, (Fig. 3b), a two-way ANOVA showed a significant effect of drug ($F_{(1,45)} = 286.5; p < 0.0001$) and no effect of infection ($F_{(1,45)} = 1.913; NS$) or interaction effect between drug and infection ($F_{(1,45)} = 3.589; NS$). Post-hoc Fisher’s LSD revealed that *Toxoplasma* mice given haloperidol had less delta freezing from no drug levels than uninfected mice given acute haloperidol.
**Correlation of Toxoplasma-interacted neurons to behavior**

A regression analysis revealed no relationship between total number of *Toxoplasma*-interacted neurons and freezing in the haloperidol group ($r^2 = 0.21$, NS) nor a relationship between total *Toxoplasma*-neurons and delta freezing from no odor to acute haloperidol ($r^2 = 0.003$, NS) (data not shown). **Figure 3**, C and D, illustrate the relationship between *Toxoplasma*-interacted neurons in the corpus striatum and freezing. Regression analysis revealed that the number of *Toxoplasma*-infected neurons in the corpus striatum and freezing were significantly related (**Fig. 3c**) for infected mice given haloperidol ($r^2 = 0.38$, $p = 0.008$) but not for infected mice given saline ($r^2 = 0.07$, NS). Additionally, number of *Toxoplasma*-infected neurons in the corpus striatum and delta freezing were significantly related (**Fig. 3d**) for infected mice given haloperidol ($r^2 = 0.32$, $p = 0.01$) but not for infected mice given saline ($r^2 = 0.003$, NS).
Figure 1. *Toxoplasma* Induces Hyperactivity and Loss-of-aversion in Mice Exposed to Cat Urine. *Toxoplasma* reduced average distance to the cat urine (A), increased activity (B), and decreased freezing (C) in the presence of high dose (2 ml) cat urine but not in the presence of low dose (.5 mL) cat urine. Ranked data from high dose cat urine exposure revealed that *Toxoplasma* decreased average distance to cat urine (D), increased locomotion (E), and reduced freezing (F). Bars in (A - C) represent ± SEM. Whisker boxes in (D - F) represent 25th to 75th percentile with whiskers from min and max.
Figure 2. Individual Variation in Aversion Behavior. Heat maps of time spent in rectangular area (cartoon, left) with inaccessible cat urine on top and inaccessible rabbit urine on bottom, ordered from left to right by time spent in area closest to cat urine. Each column represents an individual mouse. Colors of heat map are averaged times across all mice, not per individual. *Toxoplasma* mice are noted by a red bar underneath; uninfected mice by a grey bar.
Table 1. Distribution of Toxoplasma-interacted neurons in the brains of mice

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<td>superior colliculus</td>
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<td>4.23 ± 2.64</td>
<td>0.92 ± 0.57</td>
</tr>
<tr>
<td>PAG</td>
<td>2.83</td>
<td>0.43 ± 0.25</td>
<td>0.43 ± 0.25</td>
</tr>
<tr>
<td>VTA</td>
<td>0.72</td>
<td>0.11 ± 0.07</td>
<td>0.11 ± 0.07</td>
</tr>
<tr>
<td>corpus collosum</td>
<td>10.29</td>
<td>0.11 ± 0.07</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>corpus striatum</td>
<td>24.88</td>
<td>11.34 ± 2.05</td>
<td>0.46 ± 0.08</td>
</tr>
</tbody>
</table>

\(a\)Data are mean ± SEM
Figure 3. Toxoplasma Protects Against Haloperidol-induced Catalepsy. Haloperidol injection 30 min prior to aversion behavior reduced locomotion in both uninfected and Toxoplasma mice. Toxoplasma decreased freezing compared to uninfected mice when given haloperidol (A). Toxoplasma reduced the delta freezing from no drug condition to acute HAL in infected mice given haloperidol (B). The number of Toxoplasma-infected neurons in the corpus striatum correlated with freezing in infected mice given haloperidol but not in infected mice given saline ($p = .008, r^2 = .38$) (C). The number of Toxoplasma-infected neurons in the corpus striatum correlated with delta freezing from no drug condition to acute HAL in infected mice given haloperidol but not in infected mice given saline ($p = .01, r^2 = .32$) (D). Whisker boxes in (A) represent 25th to 75th percentile with
whiskers from min and max. Bars in (B) represent mean delta freezing ± SEM. * $p < 0.05$; ** $p < .01$; *** $p < .001$.

iii. DISCUSSION

Loss-of-aversion to cat urine

This study demonstrates that *Toxoplasma* infection alters the behavioral aversion response of male mice to cat urine and disrupts haloperidol-induced catalepsy. Uninfected mice exposed to a high dose of cat urine exhibited classic aversion behavior (Dielenberg et al., 2001; Vyas et al., 2007), noted by a decrease in time spent near the aversive stimulus, a decrease in locomotor activity and an increase in freezing. When exposed to an aversive level of cat urine, *Toxoplasma*-infected mice were more active, spent time on average closer to the cat urine, froze less, and made more exploratory bouts into the part of the arena closest to the cat urine. A low dose of cat urine did not elicit aversion behavior in uninfected mice and no effect of *Toxoplasma* infection was observed at this dose, providing evidence that *Toxoplasma* is not generally altering locomotor or freezing activity but is doing so specifically at a stimulus dose strong enough to elicit aversion. A high variance was observed in the *Toxoplasma* group on all aversion behaviors, arguing that *Toxoplasma* is a heterogeneous infection, with behavior alterations in some but not all infected mice. In support of this, the extent of infection also varied widely in the brains of mice, as measured by total numbers of *Toxoplasma*-interacted neurons. High variance in the behavioral response to haloperidol in infected mice was also observed and explained by extent of *Toxoplasma* interaction in the striatum, linking individual variation in behavior to individual variation at the neural
level in the host brain. These results will be discussed for their potential neurobiological significance, their implications for aversion behavior and their implications for the impact of *Toxoplasma* on the host central nervous system.

**Variance in loss-of-aversion**

A plausible hypothesis is that the variance in amount of *Toxoplasma*-interacted neurons in specific brain regions or in the entire brain explains the variance in loss-of-aversion behaviors in infected mice. For example, some mice had only a few dozen individually scattered *Toxoplasma*-interacted neurons in the entire brain and others had many thousands clustered in groups of hundreds in specific brain regions. If *Toxoplasma* is altering signaling in neurons it infects, one might expect that the location or extent of *Toxoplasma* infection in the brain could directly result in the observed loss-of-aversion phenotype. However, neither the amount of *Toxoplasma*-interacted neurons in the entire brain nor the amount in any specific brain region correlated with any measured behavioral aversion outcomes. One possible explanation is that the changes to behavior are mediated by, for example, a parasite-induced inflammatory response that is independent of the precise location of the parasite. In support of this, a recent study found that the loss-of-aversion to cat urine in infected mice persists long after the parasite has cleared from the brain (Ingram et al., 2013).

Another possible explanation lies in the multiple interpretations of the observed ‘interaction’ between *Toxoplasma* and individual neurons. The measured interaction between *Toxoplasma* and individual neurons in this study was based on observed fluorescence due to Cre-mediated recombination. The Cre-secreting *Toxoplasma* strain used has Cre fused to Toxofilin, a unique *Toxoplasma* protein secreted during the parasite
cellular invasion process (Koshy et al., 2010). However, early work with this strain indicates that *Toxoplasma* does not always invade cells even after initiation of invasion, and these studies note that a majority of Cre recombination events do not colocalize with active *Toxoplasma* infections (Koshy et al., 2012), but were instead a likely consequence of aborted or failed invasions. Because the Cre-recombination event is constitutive and permanent even if the parasite clears and because it is not evident *ex post facto* whether or when a neuron was successfully invaded, it is difficult to know precisely the manner in which an individual neuron interacted with *Toxoplasma*. Cre-recombination could have resulted from multiple different interaction histories between *Toxoplasma* and an individual neuron, for example: (1) a successful, active *Toxoplasma* invasion, (2) a successful but subsequently cleared *Toxoplasma* invasion, (3) a failed invasion, (4) an aborted invasion, (5) post-mitotic cell division, or (6) a previously unobserved mechanism, such as uptake of extracellular Cre after parasite-induced lysis. It is unlikely that spontaneous Cre-recombination events occur, as no fluorescent signal was detected in any uninfected mice. The detection of Cre-mediated recombination therefore has single-neuron spatial resolution but very poor temporal resolution, as it is impossible to distinguish an interaction that occurred in the early stages of infection from one that occurred immediately before sacrifice. For these reasons, it is unclear whether amount of *Toxoplasma*-interacted neurons scales in a meaningful way with the amount of *Toxoplasma* in the brain or the impact on the host. Nonetheless, the observed fluorescence is a good approximation for where *Toxoplasma* has been, if not a direct measure of where it was at sacrifice or at behavior. It is unlikely that *Toxoplasma* invaded a region of the brain and left no trace, though it remains possible that *Toxoplasma* kills
some host neurons, an outcome which would not be detected by these methods. In summary, though the total amount of *Toxoplasma* did not correlate with any behavioral measures, this does not rule out the possibility that *Toxoplasma* invasion in a subset of *Toxoplasma*-interacted neurons is responsible for the observed variance in loss-of-aversion phenotypes in infected mice.

**Acute haloperidol**

This study provides behavioral and neuroanatomical evidence that *Toxoplasma* is interacting with the dopamine D2 receptor (D2R) antagonist haloperidol. Haloperidol will reliably induce a decrease in locomotor activity in mice, a presumed consequence of D2R antagonism releasing dopamine-mediated tonic inhibition of striatal GABA interneurons (Patel et al., 1998). The striatum, especially the dorsolateral part, has an important role in the regulation of voluntary movements, and acute haloperidol is thought to deregulate the coordinated activity of the striatum, leading to catalepsy in mice and extrapyramidal side effects in humans. As evidence for this, a single, acute dose of haloperidol induces an increase in striatal cFos positive neurons and multiple studies find that this increase in striatal cFos is predictive of catalepsy in mice (Merchant and Dorsa, 1993; Robertson et al., 1994; Patel et al., 1998). In the present study, acute haloperidol administered 30 minutes prior to behavior elicited the expected locomotor deficit in uninfected mice. *Toxoplasma*-infected mice, however, had less of an increase in freezing in response to acute haloperidol, an effect which remained even when accounting for the baseline levels of freezing from pre-drug exposures to cat urine. Again, a large variance was observed in the infected group, some of which responded strongly to haloperidol and others weakly. Interestingly, both the amount of freezing and the change in freezing from
the pre-drug cat urine exposure correlated with amount of *Toxoplasma*-interacted neurons in the striatum. These results are intriguing in light of evidence that *Toxoplasma* has a putative tyrosine hydroxylase gene (Gaskell EA, 2009) and that *Toxoplasma* infection is correlated with increased dopamine levels in whole brain and culture (Stibbs, 1985; Prandovszky et al., 2011). If *Toxoplasma*, for example, is chronically increasing striatal dopamine in those mice with striatal infections, these mice could effectively be buffered against the D2R antagonism of haloperidol, leading to the observed effects.

This study provides behavioral and neuroanatomical evidence for the heterogeneity of *Toxoplasma* infection in male mice and behavioral and neuroanatomical evidence for an interaction between *Toxoplasma* and haloperidol. This is the first study, to our knowledge, to link *Toxoplasma* infection in a single brain region with a drug-based interaction in infected mice specifically targeting the dopamine system. These results are therefore relevant to the growing evidence linking *Toxoplasma* and psychiatric disorders like schizophrenia and obsessive compulsive disorder (Leweke et al., 2004; Cetinkaya et al., 2007; Torrey and Yolken, 2007; Miman et al., 2010c), both of which are thought to involve disregulation of dopamine signaling. Haloperidol is commonly prescribed for both disorders, and our findings suggest that seropositivity for *Toxoplasma* might be an important variable in the pharmacological treatment of these diseases.

iv. MATERIALS AND METHODS

*Experimental animals.* Male Cre-reporter mice (background C57/B6) originally purchased from Jackson Laboratories (#007906) were bred in-house. In these mice, Cre-
dependent recombination releases a LoxP-flanked ZsGreen-fluorescent cassette at the Rosa26 locus in all cells. All mice were between 5-7 months old at infection. 

Infection with Toxoplasma. A Cre-secreting PRU Type II strain of Toxoplasma gondii was used for all infections. In the Toxoplasma group, mice (n=63) were infected with 400 tachyzoites and in the uninfected group, mice (n=14) were mock-infected with saline. Twenty-seven of the Toxoplasma mice (43%) died by 8 weeks post-infection and the remaining mice (n=36) were used for subsequent behavior.

Experimental Design. Aversion behavior was done on days 1, 7, 10, and 25 subsequent to two days of 5 minute habituation to the arena and 30 minute habituation to the behavior room. For the first aversion test, a low dose of cat urine was chosen for previously established sub-aversion thresholds in uninfected mice (data not shown). For the second behavioral test, the dose of cat urine was increased to a dose previously shown to induce aversion in uninfected mice (data not shown). After the second test, half of mice from each group were randomly selected to receive either haloperidol (HAL) drug treatment or saline. (Toxoplasma-HAL, n=18; Toxoplasma-saline, n=18; Uninfected-HAL, n=6; Uninfected-saline, n=6). Haloperidol was administered i.p. every day for 2 weeks. The day after cessation of haloperidol, the fourth behavioral test was administered. Mice were sacrificed 7 days later.

Behavioral Apparatus. A custom, plastic (183cm x 9cm x 16cm) arena was used for all behavior experiments. The middle chamber of the rectangular arena was separated from the ends by two vertical dividers with small holes in them to allow odor to pass through. Mice could freely move about the center chamber but could not physically interact with the urine.
**Predator Aversion Behavior.** Animals were habituated to the behavioral room for 25 minutes and the arena for 5 min twice, once on each of the two days prior to the first behavioral test. For the first behavior test, .5mL of cat urine (PredatorPee.com, Hermon, Maine) and 2 mL of rabbit urine (Foggy Mountain, Lexington Outdoors, Inc., Lincoln, Maine) were placed on opposite sides of the arena. Mice explored the arena for 15 minutes. A ceiling-mounted infrared camera video recorded all mice. For all subsequent behavior tests, 2mL of cat urine was placed opposite 2mL rabbit urine. All four behavioral tests were conducted similarly, though, habituation was only done once in the two days preceding the first test.

**Drugs.** Haloperidol (Sigma, St. Louis MO) was dissolved in glacial acetic acid and titrated with sodium hydroxide to a pH of 6.8. It was then diluted with PBS in order to administer a dosage of 0.25mg/kg via i.p injection at 10mL/kg. 30 minutes before the third behavior, a randomly selected half of both the *Toxoplasma* and uninfected mice.

**Histology and Neuronal Counting.** Animals were sacrificed 7 days after final behavioral experiment via decapitation. Animals were deeply anaesthetized and transcardially perfused with 4% paraformaldehyde (PFA) made in 0.1 M phosphate buffer (PB). The brains were removed from the skull and postfixed in 4% PFA overnight. Brains were cut on a cryostat into 40 μm thick sections. Fluorescence was detected and counted in commercial software (Stereo Investigator; MBF Biosciences). Coronal mouse brain atlases from Allen Brain Institute were used for neuroanatomical counting.

**Behavior and Data Analysis.** Videos from all behavior experiments were analyzed in EthoVision 8 (Noldus; Leesburg, VA). All statistical analysis was done in Prism 6 (Graphpad; La Jolla, CA).
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