Behavioral changes induced by *Toxoplasma* infection of rodents are highly specific to aversion of cat odors

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The protozoan parasite *Toxoplasma gondii* blocks the innate aversion of rats for cat urine, instead producing an attraction to the pheromone; this may increase the likelihood of a cat predating a rat. This is thought to reflect adaptive, behavioral manipulation by *Toxoplasma* in that the parasite, although capable of infecting rats, reproduces sexually only in the gut of the cat. The "behavioral manipulation" hypothesis postulates that a parasite will specifically manipulate host behaviors essential for enhancing its own transmission. However, the neural circuits implicated in innate fear, anxiety, and learned fear all overlap considerably, raising the possibility that *Toxoplasma* may disrupt all of these nonspecifically. We investigated these conflicting predictions. In mice and rats, latent *Toxoplasma* infection converted the aversion to feline odors into attraction. Such loss of fear is remarkably specific, because infection did not diminish learned fear, anxiety-like behavior, olfaction, or nonaversive learning. These effects are associated with a tendency for parasite cysts to be more abundant in amygdalar structures than those found in other regions of the brain. By closely examining other types of behavioral patterns that were predicted to be altered we show that the behavioral effect of chronic *Toxoplasma* infection is highly specific. Overall, this study provides a strong argument in support of the behavioral manipulation hypothesis. Proximate mechanisms of such behavioral manipulations remain unknown, although a subtle tropism on part of the parasite remains a potent possibility.

behavioral manipulation | fear | parasites | predator

The "behavioral manipulation" hypothesis states that a parasite can alter host behavior specifically to increase its own transmission efficiency (1, 2). After an acute infection, the protozoan parasite *Toxoplasma gondii* latently persists in the brain for the life of an infected host, offering an opportunity to study the behavioral manipulation hypothesis (3). *Toxoplasma* reproduces sexually in a two-species life cycle (4). The sexual phase of its reproduction occurs in the feline intestine, from which highly stable oocysts are excreted in the feces. Grazing animals, including rodents, can then ingest these oocysts. In these hosts, *Toxoplasma* forms cysts and persists in the central nervous system. The life cycle is completed when a cat eats an infected animal. Recent reports indicate that the parasite blunts the innate aversion of rats for the urine of cats, converting this aversion to an attraction (5), although it does not interfere with energetically costly behaviors related to mating success and social status (6). These findings agree with the behavioral manipulation hypothesis, which predicts that parasites will alter only behaviors that are beneficial to their transmission while leaving other behaviors intact.

Several studies have investigated the innate fear of laboratory rodents toward cat odors (7–11). These studies have delineated a neuroanatomical circuit comprising the medial hypothalamic zone and associated forebrain structures. These forebrain inputs correspond to those emanating from the ventral hippocampus and septum on one hand (septohippocampal pathway) and the medial and basolateral amygdala on the other (amygdalar pathway). Interestingly, the medial amygdala, basolateral amygdala, and ventral hippocampus, implicated above in mediating innate fear to predators, are also important for conditioned or learned fear and unconditioned anxiety (12–17). Furthermore, behaviors pertaining to both anxiety and learned fear appear related to those pertaining to innate defensive reactions against predators (such as the aversion to cat urine among rodents). For example, the exploration models widely used to measure anxiety in laboratory rodents are based on the assumption of a conflict between defense (from interspecific and intraspecific threats) and foraging (18, 19). Similarly, fear conditioning measures adaptive memory related to stimuli associated with danger, which is therefore capable of inducing defensive behaviors (15). In view of the shared neural and behavioral substrates, it is possible that *Toxoplasma* not only disrupts aversion to cat urine but also compromises learned fear and anxiety in general. This is in disagreement with the hypothesis that effects of parasitic infection are highly specific.

In this study we sought to resolve these conflicting predictions. We confirmed that *Toxoplasma* infection in rodents blocks the aversion to cat pheromones. Additionally, we demonstrated the specificity of behavioral modifications by *Toxoplasma* by examining a broad range of behaviors concerning anxiety and learned fear. Finally, we attempted to extend behavioral observations with the localization of *Toxoplasma* tissue cysts in the brain.

Results

Health Status of Control and Infected Animals Was Comparable. Body weight gain of control and infected animals was comparable at the end of experiment [supporting information (SI) Fig. 7], indicating the relative benign nature of *Toxoplasma* infection (20). *In vivo* bioluminescent imaging in mice revealed that parasites could no longer be detected in peripheral tissues at 1 month after infection (Fig. 1). Hence, at this point the acute phase had resolved to a chronic infection, further characterized by the presence of parasite cysts in the brain (rats: 194 ± 28 cysts per brain, $n = 6$; mice: 80 ± 13.4 cysts per brain, $n = 8$). None of chronically infected animals exhibited obvious discomfort or "sickness behavior" at 30 days after infection. We started our behavioral experiments at this stage.

Infection Reduced the Normal Aversion to Cat Odor and Converted It into a Mild Attraction. ***Male Long-Evans rats.*** To examine aversion to odors, animals were released into a circular arena, and two opposing quadrants were laced with either bobcat or rabbit urine. Uninfected control animals exhibited robust aversion to bobcat urine, as measured by the low occupancy in the bobcat quadrant (10.4 ± 1.6%) compared with the rabbit quadrant (23.0 ± 5.2%), giving the occupancy ratio of the "bobcat" versus "bobcat plus rabbit" quadrant of 0.345 ± 0.044 (random occupancy would yield

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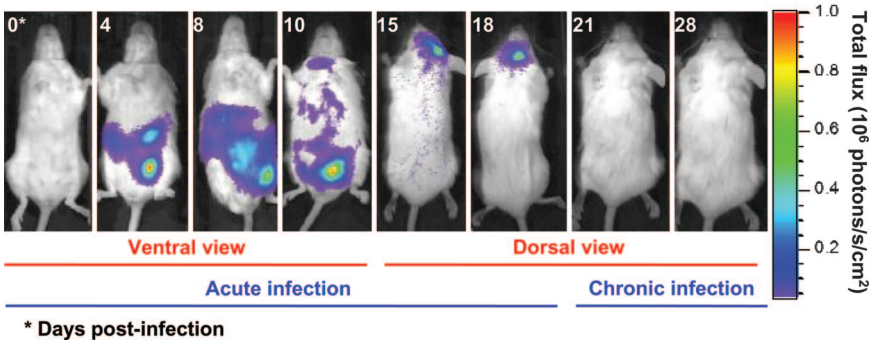

Fig. 1. Infection caused a transient increase in luminescent signals emanating from parasites. The series of images reflects the spread of luminescent signal at successive days after infection. Photon flux is coded by a range of pseudocolors (lowest, blue; highest, red).

a ratio of 0.5) (Fig. 2*A*). Infection significantly abolished such aversion and in fact caused a mild attraction (bobcat: 16.6 ± 3.9%; rabbit: 9.9 ± 2.0%; relative occupancy ratio = 0.611 ± 0.052; n = 10; $P < 0.001$; 77% increase). A clear leftward shift in the cumulative frequency distribution of distance from bobcat urine indicated that infected animals spent relatively more time nearer the bobcat urine than controls (Fig. 2*B*). Representative scatter plots, demonstrating the location of a control and an infected animal relative to odor sources during a trial, are shown in Fig. 2 *C* and *D*, respectively. Locomotor activity during the test was comparable between control and infected animals ($P > 0.5$), in terms of both total distance traveled and number of progression segments.

A towel and a collar permeated with cat odor apparently represented a more aversive stimulus than did bobcat urine, in that both control and infected rats strongly avoided a quadrant containing the towel/collar, as compared with sham towel/collar quadrant (occupancy ratio: control = 0.056 ± 0.023, infected = 0.075 ± 0.030; n = 5; $P > 0.5$).

Female BALB/c mice. Similar to rats, uninfected mice exhibited a marked aversion to bobcat urine quadrant (occupancy ratio: 0.144 ± 0.071; n = 8) (Fig. 3*A*). This aversion was abolished by infection (ratio: 0.435 ± 0.113; n = 13) ($P < 0.05$). Aversion to cat collar was measured by calculating the occupancy ratio in two bisects of a rectangular arena containing either a worn or an unworn cat collar. Whereas control mice showed a moderate aversion to the cat collar (ratio of time spent in cat bisect = 0.408 ± 0.077; n = 5) (Fig. 3*B*), infected mice exhibited an attraction (ratio = 0.760 ± 0.098; n = 5) ($P < 0.05$). Hence, infection significantly blunted avoidance behavior in both rats and mice.

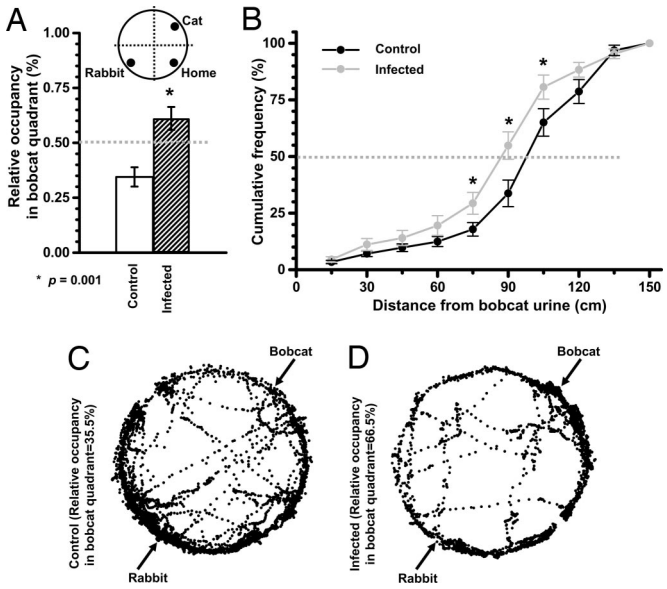

Fig. 2. Infection abolished aversion to bobcat urine in rats, instead producing an attraction. (*A*) In a circular arena, infection increased the ratio of occupancy in the quadrant laced with bobcat urine versus that with rabbit urine. The ordinate depicts relative occupancy in the bobcat quadrant relative to total occupancy in the bobcat plus rabbit quadrant. The dotted line depicts the chance level. Control, n = 10; infected, n = 10. *, $P < 0.01$ relative to control (Student's t test). (*B*) Infected rats spent more of their time nearer to bobcat urine compared with control animals, as illustrated in the leftward shift in the cumulative frequency plot of distance from bobcat urine during the trial. The 50% mark (dotted line) for the total n represents the median values. *, $P < 0.001$ (Student's t test). (*C* and *D*) Representative scatter plots depicting the location of a control rat (*C*) and an infected rat (*D*) during trial, with respect to bobcat and rabbit urine.

Effect of Infection on Aversion to Cat Odors Was Specific, Leaving a Variety of Fear-Related Behaviors Intact. ***Infection did not influence locomotion and anxiety in an open-field arena in rats.*** Total distance traveled (Fig. 4*A*) and median maximal speed during progression (SI Table 2) were not significantly different between infected and uninfected groups ($P > 0.4$). Both groups preferred being away from the center of arena (Fig. 4*B*) and avoided active progression while in the center (SI Table 2). Likewise, both groups undertook a similar number of progression episodes during the trial ($P > 0.5$) (Fig. 4*C*).

Infection did not influence fear conditioning or its extinction in rats. Foot shocks delivered during the training caused a substantial and similar increase in freezing in both control (n = 18) and infected (n = 15) rats ($P > 0.6$) (Fig. 5, Training). Control animals exhibited significant freezing when placed in a conditioned context ($P < 0.0001$)

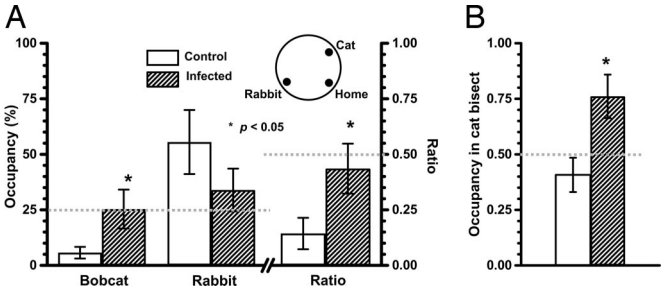

Fig. 3. Infection abolished aversion to bobcat urine and cat collar in mice. (*A*) Infection increased the ratio of occupancy in the bobcat quadrant versus the rabbit quadrant. The dotted line depicts the chance level. Control, n = 8; infected, n = 13. *, $P < 0.05$ relative to control (Student's t test). (*B*) Infection enhanced the occupancy of mice in a bisect of a rectangular arena containing a collar worn by a cat relative to the other half containing a sham collar. Control, n = 5; infected, n = 5.

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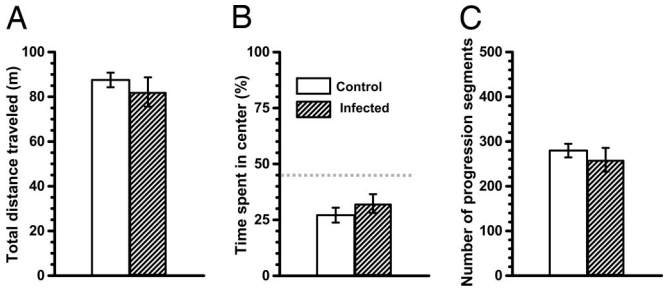

Fig. 4. Infection did not affect anxiety-like behavior of rats in the open-field arena. Control, $n = 15$; infected, $n = 15$. Ordinates depict the total distance traveled during a 30-min trial (*A*), time spent in the center (inner two-thirds of the arena; the dotted line represents the chance level) relative to the total duration of the trial (*B*), and the number of progression segments (*C*).

(Fig. 5, Context). No statistically significant difference was observed between control and experimental groups ($P > 0.3$) (Fig. 5, Context). When tested for cue conditioning, both control and infected rats spent a similar amount of time freezing (Fig. 5, Tone). When presented with thirty successive tones 2 days after testing (Fig. 5, Extinction), time spent in freezing by control and infected animals dropped steadily with no significant difference between the two groups.

Infection did not affect neophobia toward food of novel scent in mice. Control mice consistently avoided food with a novel scent (proportion of scented food relative to total consumption = 28.0 ± 4.3%; $n = 15$). This neophobia was not affected by infection (proportion = 34.0 ± 3.9%, $n = 15$) ($P > 0.3$). Both control and infected mice consumed similar amounts of food during the trial ($P > 0.7$). SI Fig. 8*A* depicts the proportion of novel food consumed by control and infected mice.

Infection did not affect the hippocampal-dependent learning. Infection did not influence spatial learning in rats.

During training, latency to find the hidden platform dropped significantly in both control ($n = 8$) and infected ($n = 6$) animals ($P > 0.001$). ANOVA did not reveal significant differences between experimental groups ($P < 0.5$). Both groups ($P < 0.5$) spent more time in the target quadrant during a probe trial conducted in the absence of a platform immediately after the training (day 0 in Table 1), whereas this preference dropped down to chance level 1 day afterward (Table 1). SI Fig. 9 depicts performance of control and infected animals during the training and at the probe trials.

Infection did not affect social transmission of food preference in mice. Both control ($n = 4$) and infected ($n = 6$) animals preferred cued food smelled previously on the breath of their cagemates (proportion of cued food relative to total consumption: control = 66.3 ± 6.0%, infected = 70.9 ± 10.4%; $P > 0.7$) (SI Fig. 8*B*).

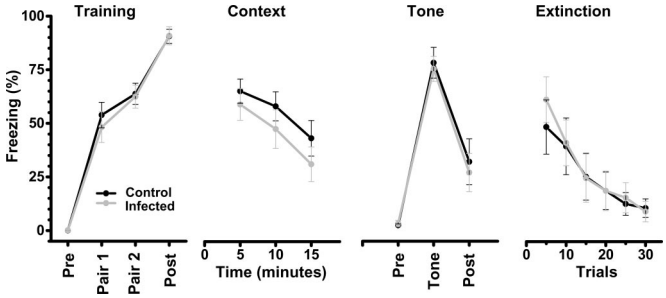

Fig. 5. Infection did not affect freezing during various phases of fear conditioning in rats. The ordinate depicts the percentage of time spent in freezing. Control, $n = 18$; infected, $n = 15$. $P < 0.2$ (Student's t test). Panels (from left to right) represent freezing during training, strength of conditioning to context, strength of cued conditioning, and extinction of cued conditioning.

Table 1. Infection did not affect spatial memory in rats as measured in the Morris water maze

	Occupancy in target quadrant, %	
Mouse	Day 0	Day 1
Control	34.7 ± 6.2	23.8 ± 4.6
Infected	32.6 ± 1.3	20.4 ± 2.7

Data show the preference for the target quadrant during the probe trial conducted immediately after and 1 day after training.

Parasites Could Be Detected in a Variety of Brain Regions, with a Trend Toward Higher Densities in the Amygdalar Region. Bioluminescent signals emanating from* Toxoplasma *could be detected in wide range of acute brain sections. Coronal brain slices (1,000 μm) were obtained across the entire brain (23 slices per brain). Photon flux emitted by luciferase-expressing parasites in infected brains ($n = 7$) was measured. To control for background bioluminescence, slices from rats infected with a Prugniaud strain lacking the luciferase expression ($n = 3$) served as negative control. Control and infected animals did not differ in terms of photon flux emanating from brain slices derived from olfactory bulb and adjoining levels (levels 1–3). But, beginning immediately posterior to that (level 4 and more), photon flux was significantly higher in infected brain tissue than in controls ($P < 0.01$, Student's t test and the Mann–Whitney U test) (Fig. 6). Hence, parasites were present across a wide range of coronal levels of the brain.

Tissue cysts could be detected in several brain regions but were more prevalent in amygdalar structures. Bioluminescence imaging ascertained that parasites could be detected across the entire brain (Fig. 6*A*). We then examined the numerical density of the cysts in different brain regions important for defensive behaviors. Cysts could be observed in all regions to a varying extent (mean numerical density: 0.10 ± 0.01 cyst per cubic millimeter). An ANOVA with brain regions as source of variance did not reach statistical significance ($P > 0.15$) (Fig. 6*B*). As the next step of analysis, we divided the nine brain regions into three groups: amygdalar areas (medial and basolateral amygdala), nonamygdalar areas previously shown to be responsive to cat odor (olfactory bulbs, prefrontal cortex, ventral hippocampus, periaqueductal gray, and hypothalamus) and areas unresponsive to cat odor (caudate putamen and dorsal hippocampus). Interestingly, amygdalar structures showed a higher tissue cyst density (0.16 ± 0.05 cyst per cubic millimeter) (Fig. 6*C*) than in nonresponsive nonamygdalar structures (0.06 ± 0.01 cysts per cubic millimeter) ($P < 0.05$, Tukey's post hoc test) or responsive nonamygdalar structures (0.09 ± 0.01 cysts per cubic millimeter) ($P < 0.05$). No significant differences were found between nonresponsive nonamygdalar structures and responsive nonamygdalar structures. Cyst density did not differ significantly between medial and basolateral amygdala.

Discussion

It has been reported that *Toxoplasma* infection in wild rodents not only reduces an aversion to predator odors, but also, surprisingly, results in the development of an actual attraction (5). We have replicated this finding in laboratory rats and mice, and we have investigated the specificity of these effects in terms of defensive behaviors and also the distribution of parasites in the central nervous system. We report that effects of infection on innate aversion to cat pheromones is remarkably specific, because infection did not diminish learned fear and anxiety-like behavior, although these behaviors are closely related to the innate fear of predator pheromones. Similarly, olfaction and

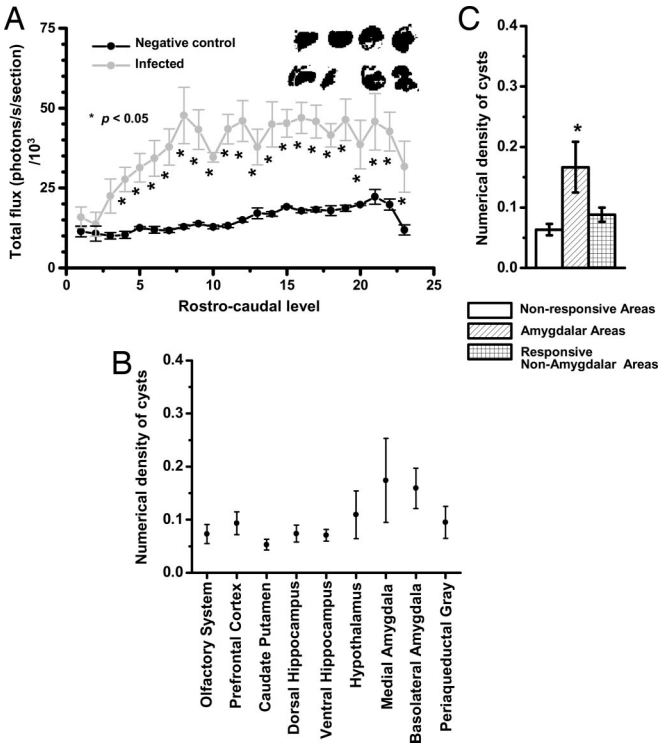

Fig. 6. Localization of tissue cysts in the brain. (*A*) Detection of parasites in acute brain slices using bioluminescence imaging revealed the presence of parasites along a broad range of coronal levels. The ordinate depicts the total photon flux (number of photons per second; section thickness = 1,000 μm). Animals infected with Pru strain parasites lacking the luciferase gene served as controls in this experiment. Negative control, $n = 3$; infected, $n = 7$. (*Inset*) Representative luminescence of eight successive sections (Bregma = 1.70 mm to Bregma = −6.30 mm) derived from an infected animal. A threshold was previously determined by using brain slices of control animals. All pixels registering photon flux more than the threshold are depicted. (*B*) Tissue cysts were observed in a variety of brain regions examined. (*C*) Determination of tissue cyst density using the physical dissector method showed that, among the brain regions analyzed, amygdalar areas (medial and basolateral amygdala) had higher cyst density compared with nonamygdalar areas either responsive to or nonresponsive to cat odor ($n = 6$ animals).

nonaversive learning were not affected. In chronically infected brains, parasite cysts were randomly distributed over the entire brain. However, we observed that cyst density in amygdalar structures was 2-fold higher than nonamygdalar structures.

Specific Behavioral Effects. How specific are these effects? Three alternative explanations could be proposed. First, that these behavioral changes are specific to innate aversion of rodents to the cat pheromones. Alternatively, these effects could reflect a general compromise in defensive behaviors such that infected rodents are less fearful to a range of aversive stimuli. An even broader possibility will be that these effects are merely side effects of generic sickness in the host.

A number of findings argue against these effects being purely a reflection of generic sickness. Previous studies elegantly show that neither social status nor the ability to compete for mates is altered in infected rodents (3, 6), both behaviors that are energetically expensive and require appropriate function of the limbic system. Moreover, in our studies, control and infected rats had comparable growth rates during the course of the experiment. Infected mice were similar in weight to control mice at the time of testing and ate a comparable amount of food during behavioral tasks requiring food consumption. Additionally, nonaversive learning, dependent on the hippocampus and presumably important for survival, was not compromised, as indicated by intact spatial learning in the Morris water maze (rats) and social transmission of food preference (mice). Moreover, we demonstrate that olfaction remains intact after infection, because infected mice retained an aversion to food with a novel odor and were capable of learning from olfactory cues provided by their social group. This is especially important because behavioral tasks measuring innate fear have mainly relied on odors. In addition, both an earlier report (5) and data presented in this report show that the response of infected animals to urine of a mammalian nonpredator, namely rabbit, remains unchanged. Collectively, these observations argue against the possibility that the observed behavioral changes reflect a side effect of generic sickness.

Innate fear of cat pheromones shares considerable neurobiological substrates with learned fear and anxiety. Hence, it could be reasonably argued that these effects reflect a generic reduction in aversive responses of infected animals. We report here that, although *Toxoplasma* infection blocks the aversion toward predator odors, it does not compromise learned fear or anxiety. Hence, the parasite affects only the part of defensive reaction that is important for its transmission, namely innate aversion to cat odors, while leaving other aspects of defensive behaviors intact. Furthermore, it is not merely the case that *Toxoplasma* attenuates the aversion to cat urine; instead, rats and mice develop an actual attraction to the pheromones. This argues against a passive behavioral effect induced by generic malaise or by reduction in a wide spectrum of defensive behaviors. Moreover, infected animals retain aversion to dog urine, a mammalian predator not important for the sexual life cycle of the parasite. Hence, *Toxoplasma* infection specifically abolishes aversion to feline odors.

Prior reports indicate that wild-caught rats with naturally occurring *Toxoplasma* parasites are more active and exhibit smaller latencies to consume novel food compared with rats without an infection (3, 6). In contrast, we did not see differences in locomotion (in rats) or the response to novel food (in mice). The reason for this discrepancy is not clear, but it could readily reflect differences between wild-born and laboratory-bred animals.

Support for Manipulation Hypothesis. The core of parasitism is the ability of an organism to exploit its host. According to the manipulation hypothesis, a parasite may be able to alter the behavior of its host for its own selective benefit (1, 3). Such selective behavioral change is proposed to increase reproductive success of the parasite, usually by enhancing its transmission efficiency. Alternatively, such effects could just be side effects of host sickness or even a fortuitous by-product of the possibility that infection induces hosts to undertake greater risks in pursuit to meet higher energy demands.

Sequential parasitism akin to *Toxoplasma*, where a parasite moves through multiple hosts to complete its life cycle, provides a classic potential of host manipulation. Loss of aversion to cat odors in infected rodents could cause an increase in predation rates and increase the transmission of *Toxoplasma* to cats, a necessary condition for its sexual reproduction. It would be difficult to reconcile specific behavioral effects with the alternative hypothesis that the behavioral effects are by-products of host sickness or adaptation. A direct experimental observation of increased transmission efficiency would require an ethically tenuous comparison of predation rates between control and infected animals. Nevertheless, mathematical models describing a similar prey–predator–parasite system demonstrate that even a small selective increase in susceptibility of an infected prey population would be sufficient to cause a significant increase of parasitic load in predator populations (21).

Proximate Mechanisms of Innate Aversion to Cat Pheromones. Recently, a dedicated circuitry underlying defensive reactions to predator odor has been suggested based on evidence from immediate-early gene activation, lesion studies, and neural connectivity between brain regions responsive to the cat odor (7–11). This

circuitry comprises mainly two forebrain pathways impinging on the medial hypothalamic zone, namely the septohippocampal pathway consisting of the ventral hippocampus and the amygdalar pathway consisting of the medial and basolateral amygdala. Many components of this circuit either overlap or run parallel to those brain regions thought to be important for other domains of emotionality, such as learned fear and/or generalized anxiety (13–17, 22). In light of these shared features, it seems logical to propose that a manipulation that will cause a complete attenuation of innate aversion to the cat odors will also have effects on unconditioned anxiety and conditioned fear. Hence, the specificity of these effects does present a surprise. Interestingly, lack of such cross-stimulus generality has also been demonstrated by specific effects of lesions in brain regions comprising the medial hypothalamic zone defensive system (7). For example, a lesion of dorsal premammillary nucleus is known to affect antipredator defensive reactions without changing response to the foot shock. These findings should further point to a dedicated neurocircuitry mediating antipredator defensive behaviors.

Several studies have investigated the effects of parasitic infections on murine defensive behaviors. For example, mice infected with the nematode *Heligmosomoides polygyrus* exhibit a reduced magnitude of analgesia after exposure to a weasel odor (23). Similarly, mice infected with the protozoa *Eimeria vermiformis* exhibit decreased avoidance of cat odor when compared with uninfected controls (24). Specificity of these behavioral effects has not been investigated in either of the cases, although mice infected with *E. vermiformis* also show reduced spatial performance in nonaversive Morris water-maze task (25). Mice infected with the nematode *Toxocara canis* exhibit a variety of behavioral changes ranging from impaired motor performance and impaired learning to decreased neophobia (26). In this case behavioral changes do not demonstrate a specificity of a degree comparable with that reported in this article. Although this specificity is exciting in its own right, it also raises the possibility that, at some level of analysis, neural substrates of innate aversion to felines can be completely dissociated from substrates of other aspects of fear and defensive reactions. In other words, it points to a set of neural mechanisms or substrates that is dedicated only to the processing of cat odors.

What are the proximate mechanisms? Several potential mechanisms should be systematically weighed and tested. A small inventory of potential mechanisms could include internalization of a few olfactory receptors important for cat odor detection, alteration in emotional valence of cat odor by brain rewiring, preferential localization of *Toxoplasma* to brain areas in septohippocampal or amygdalar regions, preferential localization to areas projecting to septohippocampal and amygdalar systems, uniform distribution but some brain regions being more sensitive to neuronal damage by *Toxoplasma*, and diffusible substances emanating from infected cells or from *Toxoplasma*. These possibilities can be broadly divided in two categories, those requiring that *Toxoplasma* selectively or at least preferentially infect a set of brain areas and those not requiring such tropism. We observe the presence of *Toxoplasma* cyst in a wide variety of brain regions in both mice and rats. Hence, selective localization of *Toxoplasma* in the brain is not indicated. However, the density of cysts in the medial and basolateral amygdala is almost double that in other structures like hippocampus, olfactory bulbs, and prefrontal cortex. This supports the case of a subtle tropism. Interestingly, the amygdala is widely interconnected with a variety of different brain regions (15, 22, 27–29). Hence, a subtle tropism to the amygdala does have the potential to influence innate fear via specific modulation of relevant pathways. Indeed, a recent study reports that development of conditioned aversion in rat pups to an odor depends on corticosterone acting on an intact amygdala (30). Moreover, the presence of the mother during conditioning diminishes both corticosterone secretion and amygdala activation, resulting in development of a net attraction rather than aversion to the odor (30). On the other hand, a more parsimonious account of observations reported here will have to take into account the high variability of the cyst distribution between individuals and the intact amygdala-dependent behaviors like fear conditioning. Nonetheless, subtle tropism remains a potent possibility and should be investigated further. *Toxoplasma* infection also causes neuromodulatory changes (e.g., in the noradrenergic and dopaminergic systems) (31). Hence, alteration of neuromodulation also provides an important avenue for such behavioral manipulation. The enigma with this scenario is how specificity is achieved as a consequence of broad neurobiological alteration.

A recent study investigating *Toxoplasma* infection in the context of schizophrenia is also noteworthy here (32). It has been postulated that *Toxoplasma* has some degree of causal relation to schizophrenia (33). This postulate rests on the positive relationships between the prevalence of *Toxoplasma* antibodies and the development of schizophrenia. A recent article reports that loss of fear to predator odor in infected rats can be reversed by treatment with the antipsychotic drugs haloperidol and valproic acid (32). This study provides one example of the value of integrating behavioral effects of *Toxoplasma* in models of emotional and psychiatric conditions.

In short, data presented in this article provide evidence that the behavioral effects of latent *Toxoplasma* infection in rodents are very specific. This specificity, in turn, provides a strong argument in support of the behavioral manipulation hypothesis. Proximate mechanisms of such behavioral manipulations will require future studies of a very extensive nature, although a subtle tropism for the amygdala on the part of the parasites remains a potent possibility.

Materials and Methods

Animals. Male Long-Evans rats (8 weeks old, three per cage; Charles River Laboratories, Wilmington, MA) and female BALB/c mice (7 weeks old, five per cage; The Jackson Laboratory, Bar Harbor, ME) were used. Animals from these sources tested serologically negative for *Toxoplasma*. The Stanford University Administrative Panel for Laboratory Animal Care reviewed and approved all procedures.

***Toxoplasma* Culture.** We used a Prugniaud strain genetically modified to constitutively express firefly luciferase (under tubulin promoter) and green fluorescent protein (under GRA2 promoter) (S.-K.K. and J.C.B., unpublished data). Parasites were maintained as tachyzoites by passage in human foreskin fibroblast monolayers.

Infection and Experimental Groups. Infected fibroblasts were syringe-lysed by using a 27-gauge needle to release tachyzoites. Animals were either infected with tachyzoites (10^6 for rats and 400 for mice i.p.) or mock-infected with sterile PBS. All behavioral experiments were conducted between 4 and 5 weeks after infection.

***In Vivo* Bioluminescent Imaging in Mice.** Anesthetized mice (2% inhaled isoflurane) were injected with luciferin (150 mg/kg i.p.). After 10 min photon flux from parasite luciferase was measured over 5 min by a cooled CCD camera (IVIS 100; Xenogen, Cranbury, NJ).

Open-Field Exploration in Rats. Exploration in a circular arena (radius = 75 cm) was recorded for 30 min (10 Hz). A ceramic plate, kept in homecage for at least 2 weeks, was placed in one quadrant and served as homebase. NIH Image software was used to generate spatial coordinates. Software for Exploration of Exploration was used to calculate endpoints. Center was defined as the inner two-thirds of the arena.

Aversion to Bobcat Urine in Rats and Mice. The arena described earlier was used, and the home plate was put in one quadrant. Two similar ceramic plates, with 20 undiluted drops of bobcat urine (Leg Up Enterprises, Lovell, ME) or rabbit urine (from a local animal facility), were placed in quadrants adjoining the home quadrant.

Designation of quadrants was randomized across trials. Animal location was tracked during a 60-min trial (10 Hz).

Aversion to a Cat Towel and a Collar in Rats. The design described above was used, except for the nature of predator odor. The experiment was conducted in a circular arena (radius = 75 cm). A cat collar worn for at least 1 month and placed over a cotton towel kept with a cat for 48 h served as a source of predator odor. Occupancy in the cat odor quadrant was calculated over a 60-min trial relative to sham stimuli placed in the opposing quadrant.

Aversion to a Cat Collar in Mice. The cat collar and a sham collar were placed on two opposing sides of a rectangular Plexiglas box (47.5 × 26 × 20 cm). The location of the animal was recorded (0.33 Hz) over a trial of 15 min. A habituation trial without collars preceded the testing. The cat collar was collected during the summer season after at least 1 month of contact with the cat.

Fear Conditioning in Rats. Rats were conditioned in two modified observation chambers (30 × 24 × 40 cm; MedAssociates, St. Albans, VT). A load-cell platform recorded locomotor activity of rats by measuring chamber displacement. Freezing was quantified as the endpoint and was defined as the cessation of all movements except breathing.

Conditioning consisted of two successive presentations of auditory tones (5 kHz, 70 dB, 10 seconds, intertrial duration = 90 seconds) coterminating with foot shock (1 mA, 1 second). The next day, the strength of conditioning to context was measured by placing animals in the same spatial and olfactory context for 15 min. Animals were subsequently tested for cued auditory conditioning by measuring freezing in response to a continuous tone (5 min, 5 kHz, 70 dB) in a different context. The next day, rats were presented with 30 successive auditory tones (5 kHz, 70 dB, 10 seconds, intertrial duration = 50 seconds) to measure the extinction of cued fear conditioning.

Avoidance of Novel Food and Social Transmission of Food Preference in Mice. Animals were habituated to eat powdered chow for 3 days and were subsequently food-deprived for 12 h. To measure neophobia to scented food, mice were presented with two plastic trays containing either scented (2% coriander wt/wt) or unscented chow, and consumption was recorded. To measure social transmission of food preference, two "demonstrator" mice from a cage were allowed to consume scented food for 1 h (2% cocoa or 1% cinnamon, randomly chosen) and returned to the home cage in contact with three other cagemates. After 10 min, the other three mice were separated and presented with two plastic trays containing food with either the cued scent or a novel scent, and consumption was recorded.

Spatial Learning in Rats. Rats were trained in a circular water maze (28°C, radius = 75 cm) to find a submerged platform. Training consisted of six successive blocks of three trials (trial duration = 60 seconds, interblock duration > 10 min). Latency to reach platform was recorded. Retention of spatial memory was tested during probe trials; one immediately after termination of training and another 24 h later. During the probe trial the platform was removed, and occupancy in the target quadrant was recorded over 1 min.

Sequence of Behavioral Experiments. The following sequence was used for rats: open-field exploration, aversion to bobcat urine, and aversion to cat collar and towel. Animals used for fear conditioning and the Morris water maze were naïve to other tests. The following sequence was used for mice: avoidance of novel food, social transmission of food preference, and aversion to bobcat urine. Aversion to cat collar was measured in a separate set of animals.

Bioluminescent Imaging in Acute Rat Brain Slices. Brains were harvested from rats under deep anesthesia. Coronal sections were obtained across rostrocaudal axis by using a precision brain matrix (section thickness = 1,000 μm; Braintree Scientific). Sections were incubated in individual wells of a 24-well plate with 500 μl of PBS containing luciferin (1.5 mg/ml) and ATP (2 mM) for 30 min. Total photon flux was determined over 15 min.

Determination of Cyst Density in Brain Slices. Brains were harvested from animals after transcardial perfusion with 4% paraformaldehyde. Subsequent to cryoprotection in 30% sucrose, 40-μm-thick coronal sections were obtained by using rotary cryotome and stained with hematoxylin and eosin. Cyst density was determined in a stereologically unbiased manner by using a physical dissector approach. Brain regions previously known to be responsive to either cat odor or anxiety-provoking stimuli were individually analyzed. In view of the low number of cysts present, data from these regions were subsequently pooled, and differences in density of cyst in amygdalar and nonamygdalar areas were investigated.

Statistical Analysis. Student's t test was performed to determine statistical significance. Values are reported as mean ± SEM. Wherever appropriate, analysis of variance was conducted, and only the significant differences were further analyzed by the post hoc Student t test. Resultant F values and P values are listed in SI Table 3. Differences in cyst density were analyzed by one-way ANOVA with brain regions as the source of variances. Significant differences were further analyzed by Tukey's post hoc test.

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1. Thomas F, Adamo S, Moore J (2005) *Behav Processes* 68:185–199.
2. Klein SL (2005) *Behav Processes* 68:219–221.
3. Webster JP (2001) *Microbes Infect* 3:1037–1045.
4. Dubey JP (1998) *Int J Parasitol* 28:1019–1024.
5. Berdoy M, Webster JP, Macdonald DW (2000) *Proc Biol Sci* 267:1591–1594.
6. Berdoy M, Webster JP, Macdonald DW (1995) *Parasitology* 111:403–409.
7. Blanchard DC, Canteras NS, Markham CM, Pentkowski NS, Blanchard RJ (2005) *Neurosci Biobehav Rev* 29:1243–1253.
8. Canteras NS, Chiavegatto S, Valle LE, Swanson LW (1997) *Brain Res Bull* 44:297–305.
9. Dielenberg RA, Hunt GE, McGregor IS (2001) *Neuroscience* 104:1085–1097.
10. Markham CM, Blanchard DC, Canteras NS, Cuyno CD, Blanchard RJ (2004) *Neurosci Lett* 372:22–26.
11. McGregor IS, Hargreaves GA, Apfelbach R, Hunt GE (2004) *J Neurosci* 24:4134–4144.
12. Bannerman DM, Rawlins JN, McHugh SB, Deacon RM, Yee BK, Bast T, Zhang WN, Pothuizen HH, Feldon J (2004) *Neurosci Biobehav Rev* 28:273–283.
13. Adamec RE, Blundell J, Burton P (2005) *Neurosci Biobehav Rev* 29:1225–1241.
14. Dayas CV, Buller KM, Day TA (1999) *Eur J Neurosci* 11:2312–2322.
15. LeDoux J (2003) *Cell Mol Neurobiol* 23:727–738.
16. Pawlak R, Magarinos AM, Melchor J, McEwen B, Strickland S (2003) *Nat Neurosci* 6:168–174.
17. Vazdarjanova A, Cahill L, McGaugh JL (2001) *Eur J Neurosci* 14:709–718.
18. Hendrie CA, Weiss SM, Eilam D (1996) *Pharmacol Biochem Behav* 54:13–20.
19. Mechiel Korte S, De Boer SF (2003) *Eur J Pharmacol* 463:163–175.
20. Welch WJ (1990) *J Am Stat Assoc* 85:693–698.
21. Vervaeke M, Davis S, Leirs H, Verhagen R (2006) *Parasitology* 132:893–901.
22. Fanselow MS, Poulos AM (2005) *Annu Rev Psychol* 56:207–234.
23. Kavaliers M, Colwell DD, Perrot-Sinal TS (1997) *Brain Res* 766:11–18.
24. Kavaliers M, Colwell DD (1995) *Parasitology* 111:257–263.
25. Kavaliers M, Colwell DD (1995) *Parasitology* 110:591–597.
26. Holland CV, Cox DM (2001) *J Helminthol* 75:125–135.
27. Pitkanen A, Pikkarainen M, Nurminen N, Ylinen A (2000) *Ann NY Acad Sci* 911:369–391.
28. Usunoff KG, Itzev DE, Rolfs A, Schmitt O, Wree A (2006) *Anat Embryol (Berlin)* 211:721–737.
29. Nathan SV, Griffith QK, McReynolds JR, Hahn EL, Roozendaal B (2004) *Ann NY Acad Sci* 1032:179–182.
30. Moriceau S, Sullivan RM (2006) *Nat Neurosci* 9:1004–1006.
31. Stibbs HH (1985) *Ann Trop Med Parasitol* 79:153–157.
32. Webster JP, Lamberton PH, Donnelly CA, Torrey EF (2006) *Proc Biol Sci* 273:1023–1030.
33. Torrey EF, Yolken RH (2003) *Emerg Infect Dis* 9:1375–1380.