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Toxoplasma gondii infection induces dendritic retraction in basolateral amygdala accompanied by reduced corticosterone secretion

Ruoshi Mitra1, Robert Morris Sapolsky2 and Ajai Vyas1,*

SUMMARY
Pathological anxiety is thought to reflect a maladaptive state characterized by exaggerated fear. Naturally occurring perturbations that reduce fear can be crucial in the search for new treatments. The protozoan parasite Toxoplasma gondii invades rat brain and removes the fear that rats have of cat odors, a change believed to be a parasitic manipulation of host behavior aimed at increasing parasite transmission. It is likely that mechanisms employed by T. gondii can be used as a heuristic tool to understand possible means of fear reduction in clinical settings. Male Long-Evans rats were infected with T. gondii and compared with sham-infected animals 8 weeks after infection. The amount of circulating plasma corticosterone and dendritic arborization of basolateral amygdala principal neurons were quantified. Previous studies have shown that corticosterone, acting within the basolateral amygdala, enhances the fear response to environmental stimuli. Here we show that T. gondii infection causes a dendritic retraction in basolateral amygdala neurons. Such dendritic retraction is accompanied by lower amounts of circulating corticosterone, both at baseline and when induced by an aversive cat odor. The concerted effects of parasitism on two pivotal physiological nodes of the fear response provide an animal model relevant to interactions between stress hormones and amygdalar plasticity.

INTRODUCTION
Anxiety disorders are widely prevalent, have huge economic burden and are a primary condition in co-morbid disorders (Wittchen et al., 2000; Kessler and Greenberg, 2002; Kessler et al., 2005; Baldwin et al., 2010). Current treatments have low success rate and substantial side-effects (Baldwin, 2008; Baldwin et al., 2010; Ravindran and Stein, 2010). Hence, there is a vast gap between need and availability of treatment options. Anxiety disorders are disorders of fear systems, reflecting exaggerated fear mismatched with actual environmental stimuli. Naturally occurring perturbations that reduce fear can be crucial to the clinical search for treatments of anxiety disorders. Behavioral effects of Toxoplasma gondii present such an opportunity. T. gondii removes the instinctual fear of a rat for cat odors, and instead generates an attraction (Berdoy et al., 2000; Webster et al., 2006; Vyas et al., 2007b; Vyas et al., 2007a). This change is believed to be a parasitic manipulation of host behavior, because of the obligatory requirement for cat intestine in parasite sexual reproduction. It is possible that mechanisms employed by T. gondii to reduce fear can provide a heuristic tool for clinical management of excessive fear. Manipulations that enhance dendritic arbors or excitatory drive in the basolateral amygdala (BLA) concomitantly enhance anxiety (Sajdyk and Shekhar, 1997; Adamec et al., 1998; Vyas et al., 2002; Vyas et al., 2003; Rainnie et al., 2004; Adamec et al., 2005; Mitra et al., 2005; Mitra and Sapolsky, 2008), and manipulations that reduce these parameters concomitantly reduce anxiety (Mitra et al., 2009c; Mitra et al., 2009a). Similarly, exogenous application of corticosterone enhances anxiety (Mitra and Sapolsky, 2008) and its antagonism reduces anxiety (Morieau et al., 2004; Mitra et al., 2009b). Interestingly, increases in corticosterone concentrations and in BLA dendritic arbors are inter-linked, a fact that is crucial for regulation of fear and anxiety. The BLA influences corticosterone secretion via facilitatory connections with hypothalamic nuclei initiating stress hormone secretion (Herman et al., 1996; Herman and Cullinan, 1997; Herman et al., 2003). In turn, corticosterone facilitates BLA plasticity via activation of corticosteroid receptors within the BLA (Mitra and Sapolsky, 2008; Mitra et al., 2009b). Thus, greater synaptic connectivity of the BLA neurons probably increases corticosterone secretion, and greater corticosterone secretion probably enhances synaptic connectivity of the BLA. In other words, BLA and corticosterone are connected via a positive feedback loop that creates facilitatory changes in each of them (Mitra and Sapolsky, 2010). The diminution of this positive feedback loop is of immense clinical significance.

In view of crucial role of BLA plasticity and corticosterone, we postulated that T. gondii alters both of these pivotal nodes in the fear response. In this backdrop, we studied the effects of chronic T. gondii infection in rats on dendritic arborization of BLA neurons and on circulating levels of corticosterone.

RESULTS
T. gondii infection induced dendritic retraction in basolateral amygdala
We quantified total dendritic length of principal neurons of the BLA at 8 weeks after infection with either T. gondii or a sham...
TRANSLATIONAL IMPACT

Clinical issue
Anxiety disorders are common but serious disorders that are thought to reflect a maladaptive state characterized by an exaggerated fear response. Current treatments for anxiety disorders have a disappointing success rate and substantial side effects, and there is therefore a large gap between the need for and the availability of treatment options. A common and valid approach to studying anxiety disorders is to use models of aniogenesis. This approach studies how fear is generated, with the hope that this knowledge can be used to develop treatments for anxiety disorders. Studying naturally occurring forms of anxiolysis (reduced anxiety) provides a complementary but rarely used approach to identifying underlying mechanisms and the development of new therapies for anxiety disorders.

Results
In this study, the authors investigate the mechanisms underlying fear reduction in rats infected with the protozoan parasite, Toxoplasma gondii. This parasite, which requires transfer from rat to cat for its own sexual reproduction, abolishes the fear normally experienced by rodents in response to cat odor to ensure its transmission. The authors report that Toxoplasma gondii infection of rats resulted in a selective dendritic retraction of neurons in the basolateral amygdala, a brain region important for regulating emotions. Infection also resulted in reduced secretion of corticosterone stress hormones. Thus, T. gondii infection has a coordinated effect on two pivotal physiological nodes of the fear response that are thought to interact with each other in a positive feedback loop.

Implications and future directions
These findings support the hypothesis that changes in corticosterone secretion and in the basolateral amygdala act in a positive feedback loop to regulate fear. This important mechanistic knowledge about the generation of fear has the potential to guide the search for new anxiolytic therapies. Moreover, these findings identify T. gondii infection as a useful rodent model in which to study molecular interactions between glucocorticoid secretion and the basolateral amygdala, as well as other aspects of the fear response. Further studies on the mechanisms employed by this parasite to reduce fear could lead to testable predictions that will facilitate the search for clinical interventions for fear disorders.

In injection of buffered saline, a time-frame when behavioral effects of the infection are present (Vyas et al., 2007b). We quantified left and right hemispheres separately.

A two-way analysis of variance (ANOVA) revealed significant effects of both infection and hemisphere. Neurons from infected animals exhibited reduced dendritic length (Fig. 1, left; 30% reduction in marginal mean; $F_{(1,152)}=37.6$, $P<0.000001$). Likewise, neurons from right basolateral amygdala exhibited lower dendritic length compared to left (Fig. 1, right; 15% reduction in marginal mean; $F_{(1,152)}=7.6$, $P<0.01$). The effect of interaction between infection status and hemisphere was not statistically significant ($F_{(1,152)}=0.1$, $P=0.75$)

Planned comparisons demonstrated that infection induced dendritic retractions of 30% and 31% in the left and right hemisphere, respectively (Fig. 2; independent sample t-test; $P<0.0001$ for left hemisphere, $P<0.01$ for right hemisphere; P-values after Bonferroni correction for multiple comparisons). The effect of infection was particularly striking in the left hemisphere, with the 75th percentile of infected neurons being lower than the 25th percentile of control neurons.

T. gondii infection reduced circulating levels of corticosterone
The infection on circulating plasma corticosterone was quantified at both baseline and 25 minutes after the presentation of 2 ml of bobcat urine. A two-way analysis of variance revealed significant effect of infection ($F_{(1,132)}=75.5$, $P<0.000001$), cat odor ($F_{(1,132)}=9.5$, $P<0.01$) and the interaction ($F_{(1,132)}=6.8$, $P<0.02$).

**Fig. 1.** Two-way analysis of variance revealed significant effects of infection and hemisphere of sampling. Values of estimated marginal means are depicted as mean ± s.e.m. Ordinate depicts total dendritic length. *$P<0.01$ compared with left hemisphere; **$P<0.000001$ compared with control. Please note that ordinate does not start at zero.

**Fig. 2.** Planned comparisons revealed that infection reduced total dendritic length in both left and right hemisphere. Dot and whiskers depict mean ± s.e.m. Box plots depict median, 25th percentile and 75th percentile. *$P<0.01$, **$P<0.0001$ compared with controls, using Student’s $t$-test after Bonferroni correction; n=18 for control neurons in left hemisphere, 36 for infected left, 48 for control right and 24 for infected right. Neurons were derived from four control and four infected animals (12-32 neurons per animal). Please note that ordinate does not start at zero.
As shown in Fig. 5, exposure to cat odor enhanced plasma corticosterone in both control (independent sample t-test; \(t_{(14)}=-5.2, P<0.001\)) and infected animals \((t_{(13)}=-2.4, P<0.05)\). Infected animals exhibited lower amounts of plasma corticosterone both at the baseline \((t_{(20)}=3.5, P=0.01)\) and after exposure to cat odor \((t_{(19)}=-12.4, P<0.0001)\). Thus, infected animals had lower amount of circulating corticosterone at the baseline (64% reduction). Control animals exhibited a robust increase in corticosterone after cat odor exposure (16-fold increase). The amount of corticosterone after exposure to cat odor was reduced in infected animals (47% reduction compared with that in control animals after cat odor exposure).

DISCUSSION

Our findings support the notion that changes in glucocorticoid secretion and in the BLA play important roles in regulating fear. It is perhaps more fruitful to see these two changes as occurring in two equally important pivots of a positive feedback system regulating fear. This view is based on two related strands of evidence. First, plasticity within the BLA and changes in corticosterone levels modulate a behavioral change in fear and anxiety (Sajdyk and Shekhar, 1997; Adamec et al., 1998; Vyas et al., 2002; Vyas et al., 2003; Rainnie et al., 2004; Adamec et al., 2005; Mitra et al., 2005; Mitra and Sapolsky, 2008). Second, changes in BLA lead to changes in corticosterone secretion, and vice versa (Vyas et al., 2002; Shepard et al., 2003; Vyas et al., 2003; Mitra et al., 2005; Vyas et al., 2006; Mitra and Sapolsky, 2008; Mitra et al., 2009b; Mitra et al., 2009a; Mitra and Sapolsky, 2010). *T. gondii* infection attenuates both of these interconnected nodes. We suggest that infection with *T. gondii* provides a useful model for the study of molecular mediators of their interaction. It is pertinent to ask whether a perturbation model, like *T. gondii*, can lead to testable predictions of clinical utility. Many such models have been successfully used in prior translational research, such as the study of hematophagous leeches leading to anti-clotting factors (Nowak and Schröer, 2007; Gómez-Outes et al., 2012) or of the cone snail *Conus magus* eventually leading to powerful and non-addictive pain-relievers (Klotz, 2006). Similarly, molecular mechanisms employed by this parasite can hopefully be co-opted to reduce secretion of stress hormones and/or to protect against stress-induced dendritic changes in the amygdala.

Anxiety disorders, although heterogeneous, are all characterized by a common theme of excessive fear. Phobia is characterized by abnormal fear responses to stimuli that are related to survival threats in evolutionary history (e.g. blood, enclosed spaces and darkness) (Mineka and Ohman, 2002). Interestingly evolutionarily recent threats do not typically induce phobia (e.g. guns and automobiles). *T. gondii* can be a relevant heuristic tool for understanding phobia in view of the evolutionarily ‘prepared’ nature of fear in both paradigms. Similarly, predator odors have been often used to recreate traumatic stress in animal models of post-traumatic stress disorder (e.g. Roth et al., 2011). This experimental approach can be successfully complemented by the reduced stress reactivity reported in this paper. It should be noted that exposure to cat odor generates anxiety (Zangrossi and File, 1992), an end point that has not been measured in this study.

Generalized anxiety disorder is characterized by excessive worry about a number of things. It is currently unclear whether *T. gondii*...
infection reduces generalized anxiety in animal models. For example, infected rats are reported to show lower anxiety in the elevated plus-maze and social interaction tests (Gonzalez et al., 2007), but not in an open field arena (Vyas et al., 2007b). Infection reduces neophobia to food in wild rats (Webster et al., 1994), but not in laboratory rats (Vyas et al., 2007b). These disparate results probably reflect differences in methodologies. For example, baseline neophobia or anxiety is greatly reduced during domestication of laboratory rodent strains. It can be speculated that a lower baseline renders it less probable to observe a further significant decline of anxiety post-infection.

We have earlier reported that T. gondii exhibits subtle tropism to the amygdala (Vyas et al., 2007b). Other groups have reported a mild tropism of the parasite in other regions (Kittas et al., 1984; Gonzalez et al., 2007) or no tropism at all (Gulinello et al., 2010). Given the importance of the amygdala for fear, subtle tropism or even the mere presence of T. gondii is exciting. We have recently reported that infected rats atypically recruit postero-dorsal parts of the medial amygdala when presented with fearful stimuli, an area normally associated with affiliative behavior (House et al., 2011). It is likely that the infection effects in BLA reported here are part of a larger mechanistic model encompassing both medial and basolateral amygdala.

Our data also shows hemispheric asymmetry in dendritic arborization of BLA neurons, which was present in both control and infected groups. The physiological or behavioral importance of this asymmetry is still unclear. Interestingly, hemispheric asymmetry has been previously reported for potentiation of anxiety after predator exposure (Adamec, 1999; Adamec et al., 2005), pharmacological stressor (Adamec, 2000), kindling (Adamec, 1999) and modulation of memory (Lalumiere and McGaugh, 2005).

In conclusion, we report long-term structural changes in the BLA and in the responsiveness of stress hormones that could underlie the behavioral manipulation of fear in rats by T. gondii. We believe this model is valuable in the clinical search for mechanism to reduce stress reactivity and plasticity of the fear system.

MATERIALS AND METHODS
Animals, parasites and infection
Long-Evans rats (39 days old) were obtained from Charles River Laboratories (Wilmington, MA). The Stanford University administrative panel for laboratory animal care approved all procedures. A Prugniaud strain of T. gondii was used. Animals were either infected with tachyzoites (10^5, i.p.) or mock-infected with sterile phosphate buffered saline. All experiments were conducted between 6 and 8 weeks post-infection, a period known to harbor chronic infection (Vyas et al., 2007b; Vyas et al., 2007a).

Only male rats were used in the present investigation. This limitation is particularly important because a greater proportion of women than men suffer from clinical disorders of anxiety (Kessler et al., 2005) and also because the behavioral effects of T. gondii are gender-dependent in mice (Xiao et al., 2012).

Morphological measurements
Animals were decapitated under deep flurothane anesthesia. Freshly dissected brain tissues containing the amygdala were processed for staining individual neurons using the rapid Golgi method (Vyas et al., 2002; Mitra et al., 2005; Mitra and Sapolsky, 2008; Mitra et al., 2009c). Golgi-stained BLA tissue was sectioned (120 μm thick), mounted with coverslip and used for morphological analysis. Custom-designed macros embedded in NIH ImageJ software (http://rsbweb.nih.gov/nih-image/) were used for morphometric analysis of digitized images.

Endocrine measurements
Concentrations of circulating plasma corticosterone were quantified at baseline (0 minutes) and 25 minutes after exposure to 2 ml of bobcat urine. For blood collection, animals were gently held inside a dark towel and up to 100 μl blood was collected in heparinized tubes through a tail nick. Blood was drawn between 10 am to 12 noon (3-5 hours after start of the light-phase) when corticosterone is at the trough of its diurnal cycle. The time interval between picking up the animal from the home-cage and end of blood collection was less than 2 minutes. This method is known to induce minimal stress during repeated blood collection (Fluttert et al., 2000). The tubes were kept on ice and subsequently centrifuged to collect the plasma (5415C, Eppendorf; 8160 g for 10 minutes). Corticosterone titers in plasma (diluted 11 times) were assessed using a competitive enzyme immunoassay kit (Assay Design, Minneapolis, MN). This assay typically results in a sensitivity value of 27 pg/ml (concentration of corticosterone two standard deviations away from zero on a standard curve). This assay method has low cross-reactivity to testosterone (<0.15%).

Statistics
Values are reported as mean ± s.e.m. Percentage changes were calculated with respect to corresponding control values (n is provided in figure legends). Two way analysis of variance was conducted to discern the effects of infection and hemisphere on dendritic length and also when analyzing the effects of infection and predator odor exposure on corticosterone levels. Independent sample Student’s t-test was employed as post-hoc test. Orthogonal planned comparisons were used to compare the effects of infection separately in left and right hemisphere (Student’s t-test, Bonferroni correction applied). No mean was compared more than once during the planned comparison.

COMPETING INTERESTS
The authors declare no competing or financial interests.

AUTHOR CONTRIBUTIONS
R.M., R.M.S. and A.V. designed the experiments and wrote the paper. A.V. conducted animal infection and tissue harvest. R.M. conducted Golgi staining and morphometric analysis. A.V. and R.M. analyzed the data.

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