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

Rupshi Mitra\textsuperscript{a}, Robert Morris Sapolsky\textsuperscript{b}, Ajai Vyas\textsuperscript{a}

\textsuperscript{a}School of Biological Sciences, Nanyang Technological University, Singapore 637551, Republic of Singapore

\textsuperscript{b}Department of Biology, Stanford University; Departments of Neurology and Neurological Sciences, and of Neurosurgery Stanford University Medical School, Stanford, CA 94305, USA

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Corresponding author

Ajai Vyas
School of Biological Sciences, Nanyang Technological University,
Singapore 637551, Republic of Singapore
PH: +65-6513 7365; Fax: +65-6791 3856; avyas@ntu.edu.sg

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Conflict of Interest

Authors declare no conflict of interest.
Abstract

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 fear of rats for cat odors, a change believed to be parasitic manipulation of host behavior aimed at increasing parasite transmission. It is likely that mechanisms employed by *Toxoplasma 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 *Toxoplasma gondii* and compared with sham-infected animals 8 weeks post-infection. 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 *Toxoplasma gondii* infection causes a dendritic retraction in basolateral amygdala neurons. Such dendritic retraction is accompanied by lower amount 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 fear response provide an animal model relevant to stress hormones interaction with amygdalar plasticity.

Keywords: Anxiety; Behavioral manipulation; Fear; Glucocorticoid; Hormone; Parasite
Introduction

Anxiety disorders are widely prevalent, have huge economic burden and are 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 disappointing 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 clinical search for the treatment of anxiety disorders. Behavioral effects of Toxoplasma gondii present such an opportunity. Toxoplasma gondii removes 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 of cat intestine for parasite sexual reproduction. It is possible that mechanisms employed by Toxoplasma 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, 1999; 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 (Moriceau 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 likely increases corticosterone secretion; and greater corticosterone secretion likely 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 critical role of BLA plasticity and corticosterone, we postulated that *Toxoplasma gondii* alters both of these pivotal nodes in fear response. In this backdrop, we study effects of chronic *Toxoplasma gondii* infection in rats on dendritic arborization of BLA neurons and on circulating levels of corticosterone.
Materials and methods

Animals, parasites and infection

Long-Evans (≈49 days old) were obtained from Charles River laboratories (Willmington, MA). The Stanford University administrative panel for laboratory animal care approved all procedures. A Prugniaud strain of *Toxoplasma gondii* was used. Animals were either infected with tachyzoites (10 X 10^6, i.p.) or mock-infected with sterile phosphate buffered saline. All experiments were conducted between 6 to 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 behavioral effects of *Toxoplasma gondii* are gender-dependant 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 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 cover slip and used for morphological analysis. Custom-designed macros embedded in ‘NIH Image’ software (http://rsbweb.nih.gov/nih-image/) were used for morphometric analysis of digitized images.

Endocrine measurements

Concentration of circulating plasma corticosterone was quantified at baseline 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 heparinised tubes through a tail nick. Blood was drawn between 10 AM to 12 noon (3 to 5 hours after start of the light-phase) when corticosterone is at the trough of its diurnal cycle. Time interval between picking up animal from home-cage and end of blood collection was less than two minutes. This method is known to induce minimal stress during repeated blood collection (Fluttert et al., 2000). The tubes were kept on ice and were subsequently centrifuged to collect the plasma (5415C, Eppendorf; 10000 rpm 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 CORT two standard deviation away from zero on standard curve). This assay method has low cross-reactivity to testosterone (< 0.15%).

**Statistics**

Values are reported as mean ± SEM, and percentage changes are calculated with respect to corresponding control values (N is provided in figure legends). Two way analysis of variance was conducted to discern effects of infection and hemisphere on dendritic length and also when analyzing 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 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.
Results

Toxoplasma gondii infection induced dendritic retraction in basolateral amygdala

We quantified total dendritic length of principal neurons of the BLA eight weeks after infection with either Toxoplasma gondii or a sham 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 main effects of both infection and hemisphere. Neurons from infected animals exhibited reduced dendritic length (Figure 1a, 30% reduction in marginal mean; F(1,152) = 37.6, \( p < 0.000001 \)). Likewise, neurons sampled from right basolateral amygdala exhibited lower dendritic length compared to left (Figure 1b, 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 (Figure 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 75th percentile of infected neurons being lower than 25th percentile of control neurons.

In conjunction to the above-mentioned analysis, we also investigated dendritic retraction through a more conservative approach of using animals rather than neurons as units of analysis. All neurons sampled for an individual animal were averaged to arrive at a single mean value. Infection-induced dendritic retraction was confirmed by this approach (Figure 3;
independent sample t-test; \( t_{(6)} = 2.6, p < 0.05 \); Mann-Whitney U test, \( p < 0.05 \); 4 animals for control and infected group each). The maximum value of dendritic length of infected animals (1733 µm) was lower than minima of control animals (1801 µm).

Neurons in a neighboring brain region, the pyriform cortex, did not undergo dendritic retraction (Figure 4; \( t_{(42)} = -0.5, p > 0.6 \)). This shows that the retraction observed in the BLA does not represent a generic infection-induced atrophy of neurons.

*Toxoplasma gondii* infection reduced circulating levels of corticosterone

Effect of infection on circulating plasma corticosterone was quantified at both baseline and 25 minutes after the presentation of two ml of bobcat urine. A two-way analysis of variance revealed significant effect of infection (\( F_{(1,33)} = 75.5, p < 0.000001 \)), cat odor (\( F_{(1,33)} = 9.5, p < 0.01 \)) and the interaction (\( F_{(1,33)} = 6.8, p < 0.02 \)).

As shown in Figure 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 amount 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 robust increase in corticosterone after cat door exposure (16-fold increase). Amount of corticosterone post-cat odor was reduced in infected animals (47% reduction compared to that in control animal 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. Firstly, plasticity within the BLA and changes in corticosterone levels modulates behavioral change in fear and anxiety (Sajdyk and Shekhar, 1997; Adamec, 1999; Vyas et al., 2002; Vyas et al., 2003; Rainnie et al., 2004; Adamec et al., 2005; Mitra et al., 2005; Mitra and Sapolsky, 2008). Secondly, 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, a; Mitra and Sapolsky, 2010). *Toxoplasma gondii* infection attenuates both of these inter-connected nodes. We suggest that infection with *Toxoplasma gondii* provides a useful model to study molecular mediators of their interaction. It is pertinent to ask if a perturbation model, like *Toxoplasma 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 Schror, 2007; Gomez-Outes et al., 2012) or of the cone snail *Conus magus* eventually leading to powerful and nonaddictive pain-relievers (Klotz, 2006). Similarly, molecular mechanisms employed by this parasite can be hopefully co-opted to reduce secretion of stress hormones and/or to protect against stress-induced dendritic changes in the amygdala.

Anxiety disorders, while heterogeneous, are all characterized by a common theme of excessive fear. Phobia is characterised 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). *Toxoplasma gondii* can be a relevant heuristic tool to understand 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 model 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 endpoint 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 if *Toxoplasma 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., 2007b), 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 *Toxoplasma 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., 2007a) or no tropism at all (Gulinello et al., 2010). Given the importance of the amygdala for fear, subtle tropism or even mere presence of *Toxoplasma 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
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, 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 et al., 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 responsiveness of stress hormones that could underlie behavioral manipulation of fear in rats by *Toxoplasma gondii*. We believe this model is valuable in clinical search for mechanism to reduce stress reactivity and plasticity of the fear system.


**Figure Legends**

**Figure 1.** Two-way analysis of variance revealed significant main effects of infection (A) and hemisphere of sampling (B). Values of estimated marginal means are depicted as mean ± SEM. Ordinate depicts total dendritic length in µm. * p < 0.05, ** p < 0.01. Please note that ordinate does not start at zero.

**Figure 2.** Planned comparisons revealed that infection reduced total dendritic length in both left and right hemisphere. Dot and whiskers depict mean ± SEM. Box plots depict median, 25th percentile and 75th percentile. * p < 0.05, ** p < 0.01, 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 4 control and 4 infected animals (12-32 neurons per animal). Please note that ordinate does not start at zero.

**Figure 3.** Infection reduced animal mean dendritic length, calculated by averaging all neurons from an individual animal. * p < 0.05, student’s t-test. N = 4 animals each for control and infected group.

**Figure 4.** Infection did not affect dendritic length of pyriform cortex, thus precluding a generalized retraction all over the brain. Neurons were sampled from right pyriform cortex, using same brain slices used for quantification of basolateral amygdala neurons. Dot and whiskers depict mean ± SEM. Box plots depict median, 25th percentile and 75th percentile. N= 28 neurons for control and 16 neurons for infected. Neurons were derived from 4 control and 4 infected animals. Please note that ordinate does not start at zero.

**Figure 5.** Infection reduced circulating levels of plasma corticosterone, both at baseline and after exposure to bobcat urine. Dot and whiskers depict mean ± SEM. Box plots depict median, 25th percentile and 75th percentile. * p < 0.05, ** p < 0.01, post-hoc student’s t-test.
N = 8 control and 14 infected animals for baseline, and 8 control and 7 infected animals post exposure to cat odor. Please note that panel left and right panel have dissimilar scales.
Translational Impact Box

1) Clinical Issue

Anxiety disorders are widely prevalent disorders of the fear response. Currently, there is a vast gap in need and availability of treatment options for these disorders. It is a common (and indeed very valid) approach to study fear/anxiety using models of anxiogenesis. This approach takes the form of studying how fear is generated, with the hope of using that knowledge to reduce fear. We have taken a complementary and infrequent approach. We study a naturally occurring form of anxiolysis. We hope that mechanistic knowledge derived from this alternative approach directly guide clinical search in future.

2) Results

We study a perturbation system of fear, whereby the fear experienced by rodents in response to cat odor is abolished due to infection with a protozoan parasite, *Toxoplasma gondii*. This parasite requires transfer from rat to cat for its own sexual reproduction. It generates loss of fear to achieve its transmission. Using this perturbation system, we report that *Toxoplasma gondii* infection results in a selective dendritic retraction of neurons in basolateral amygdala, a brain region important for regulation of emotions. Infection also results in lower secretion of corticosterone stress hormones. Effects of the infection on amygdala anatomy and stress hormones demonstrate a coordinated effect on nodes of the fear response, given that these two substrates interact with each other in a positive feedback.

3) Implications and Future Directions

This report generates mechanistic knowledge that will directly guide clinical search. It is instructive to give a palpable precedent. Outcome of traumatic brain injury in female rats
depends on hormonal state during the injury (Roof et al., 1993), outcome of the injury being less severe in females than males and less severe in pseudo-pregnant females than normally cycling females. A reproductive hormone, progesterone, is the main mediators of the protection in this example. Systematic follow-up studies of progesterone-induced protection have led to a current Phase II clinical trial (Stein, 2011). This example demonstrates evolution of a perturbation model from animal studies to clinical relevance. Similarly, mechanisms employed by this parasite to reduce fear will also be relevant in the clinical search for intervention in fear disorders.
Figure 1

![Graph showing total dendritic length (µm) by infection status and hemisphere.](image)
Figure 2

The diagram shows a box plot comparing the total dendritic length (µm) between control and infected groups for the left and right BLA. The x-axis represents the groups: Control and Infected, with Left BLA and Right BLA on the sub-axes. The y-axis represents the total dendritic length in µm, ranging from 1000 to 3000. Significant differences are indicated by asterisks: * for the right BLA and ** for the left BLA.
Figure 3

The figure shows a comparison of Total Dendritic Length (µm) between Control and Infected individual animals. The graph indicates a significant difference between the two groups, as denoted by the asterisk (*) on the Infected group boxplot.
Figure 5

![Graph showing plasma corticosterone levels](image)