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Loss of predator aversion in female rats after *Toxoplasma gondii* infection is not dependent on ovarian steroids

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ABSTRACT

Toxoplasma gondii infection reduces aversion to cat odors in male rats. Relevant proximate mechanisms include interaction of gonadal testosterone and brain nonapeptide arginine-vasopressin. Both of these substrates are sexually dimorphic with preferential expression in males; suggesting either absence of behavioral change in females or mediation by analogous neuroendocrine substrates. Here we demonstrate that *Toxoplasma gondii* infection reduces aversion to cat odor in female rats. This change is not accompanied by altered steroid hormones; cannot be rescued by gonadal removal; and, does not depend on arginine-vasopressin. Thus behavioral change in males and female occur through non-analogous mechanisms that remain hitherto unknown.

1. Introduction

Male rats infected with protozoan parasite *Toxoplasma gondii* (henceforth *Toxoplasma*) exhibit reduced aversion to cat odor (Berdoy et al., 2000; Vyas et al., 2007). This behavioral change is presumed to reflect a parasitic manipulation because cats are definitive host for the parasite. It remains undetermined if the infection indeed leads to increased predation by cats under naturalistic circumstances (Worth et al., 2014). Meanwhile neuroendocrine mechanisms of the host behavioral change have been extensively studied in male laboratory rats (Hari Dass and Vyas, 2014b; House et al., 2011; Lim et al., 2013; Vyas, 2013). Several hypotheses have been proposed to account for loss of aversion to predator odor in the infected males (Gaskell et al., 2009; McConkey et al., 2013; Prandovszky et al., 2011; Vyas, 2015b). The most parsimonious of the three posits that toxoplasma infection in male rats results in an increase in testicular testosterone synthesis (Lim et al., 2013; Vyas, 2015a). The testosterone then crosses the blood-brain barrier and causes hypomethylation of the arginine vasopressin gene within the posterodorsal medial amygdala (Auger et al., 2011). This subsequently leads to increased arginine vasopressin production which then drives the behavioral change (Hari Dass and Vyas, 2014a,b). This narrative has a significant limitation in that the proposed mechanism includes nodes that are sexually dimorphic. Testosterone is a predominantly male reproductive hormone that is produced in relatively smaller quantities in females (Goymann and Wingfield, 2014). Similarly medial amygdala arginine vasopressin is testosterone dependent for its transcription (Bluthe et al., 1990; Dantzer and Bluthe, 1991; Dantzer et al., 1988). This suggests that either the behavioral change is absent in females; or that it is mediated by analogous mechanisms involving steroids synthesized by ovaries. The later assumption is plausible because much of testosterone in males gets locally aromatized to estrogen after entry into the brain. We investigated these possibilities.

2. Materials and methods

2.1. Animals and infection

Female rats of Wistar Hans strain were used, procured from InVivos Singapore. Animals were 7–8 weeks at start of experiments. A type 2 *Toxoplasma* strain, Prugniaud, was used for the infection at dose of 5 million lab-grown tachyzoites per animals (*i.p.*). Corresponding control animals were injected with sterile buffered saline. Routine management of animals and parasites was similar to earlier studies (Hari Dass and Vyas, 2014b). All experi-

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mental procedures were reviewed and approved by local institutional animal use and care committee.

2.2. Removal of ovaries

Surgery was performed using aseptic techniques under isoflurane anesthesia (2.5% gaseous isoflurane with pure O2). After placing animals in ventral recumbency, ovaries were approached bilaterally using incisions caudal to ribcage. Ovaries were excised after suturing fallopian tubes. The wound was subsequently sutured. Animals assigned to sham surgery group were operated in similar manner, except that fallopian tubes were not tied and ovaries were not excised.

2.3. Experimental groups and statistics

Animals were randomly divided into experimental groups. In experiments with two experimental groups, control and infected, inter-group differences were analyzed using independent sample student's *t*-test. Effect size was calculated using Cohen's *d*. In experiments analyzing effects of ovarian steroids, control and infected animals were further divided into three surgical treatments: sham surgery with implantation of an empty silastic capsule, removal of ovaries and implantation of empty capsule, removal of ovaries and implantation of capsule filled with estradiol. Silastic capsules were 15 mm long and resulted in constant delivery of estrogen in non-placebo configuration, as confirmed by invariant presence of cornified cells in vaginal lavage for duration of the experiment. Infection, or mock infection, proceeded after visual confirmation of wound healing post-surgery. Analysis of variance was conducted to estimate main effects of infection and surgical treatment; and interaction between infection and surgical treatment. Fisher's LSD test was used for two non-orthogonal planned comparisons between control and infected animals after sham surgery and after removal of ovaries without accompanying estradiol supplementation.

2.4. Aversion to bobcat odor

Aversion to cat odor was quantified after establishment of chronic infection. Animals were first habituated to the testing arena (two rectangular arms of 76 × 9 cm each, connected by a central junction of 9 × 9 cm); for two consecutive days in absence of odor and one additional day in presence of a novel odor (vanilla extract). Subsequently females were placed in the arena pre-seeded with bobcat urine during estrus phase of their ovulatory cycle (2 ml, Maine Outdoor Solutions, placed at one corner, trial duration = 20 min).

2.5. Quantification of steroid hormones

Plasma steroids were measured in triplicates from trunk blood obtained during the sacrifice. Commercial enzyme-linked immunoassay kits with minimal cross-reactivity were used (Enzo Life Sciences, USA). Median coefficient of variation between three technical replicates was ≤15%. Estrogen, progesterone and testosterone were separately quantified

2.6. Detection of Toxoplasma in ovaries

We used PCR amplification for B1 gene of *Toxoplasma* to detect presence of the parasite in genomic DNA from excised ovaries of six control and six infected females (primers similar to (Dass et al., 2011)).

2.7. Quantification of arginine vasopressin

Posterodorsal medial amygdala was microdissected from harvested brains, mRNA was isolated and revers-transcribed to cDNA. Standard SYBR green bases assay was used to quantify cDNA for arginine vasopressin (primers similar to (Hari Dass and Vyas, 2014b)) and three housekeeping genes (HPRT, β-actin and 18sRNA). PCR cycles needed to reach a pre-determined fluorescence threshold at early linear phase of the amplification were determined (Ct, threshold cycle number). Three replicates were used for each determination (median coefficient of variation between replicates ≤15%). Ct values for arginine vasopressin were normalized by subtracting geometric mean of Ct values for three housekeeping genes.

3. Results and discussion

We randomly divided 24 adult female rats in two experimental groups. Each group received either 5×10^6 *Toxoplasma* tachyzoites (Prugniaud strain) suspended in 500 µl phosphate buffered saline or buffered saline alone (*i.p.*). Infection did not result in sickness behavior or decrease in weight gain during the experimentation. Aversion to cat odor was quantified after establishment of chronic infection (>7 weeks, confirmed by presence of anti-*Toxoplasma* IgG in blood). *Toxoplasma*-infected female rats exhibited statistically significant loss of aversion to bobcat urine in this assay. This was reflected as increase in occupancy of the arm containing bobcat urine (Fig. 1A; independent sample student's *t*-test, $|t|_{22} = 3.86$; $p = 0.001$; effect size: Cohen's $d = 1.58$) and also greater number of entries made into this arm (Fig. 1B; $|t|_{22} = 5.17$; $p < 0.001$; Cohen's $d = 2.11$). Effect of infection on cat odor aversion was not

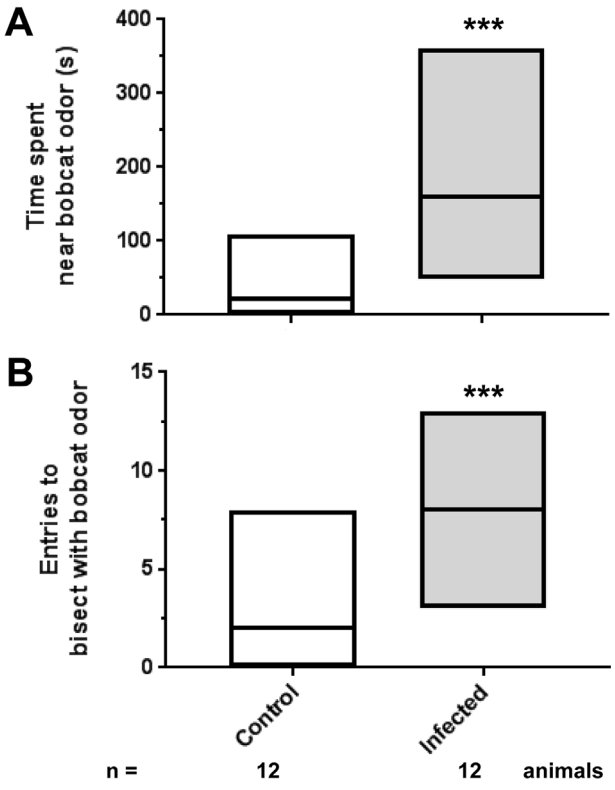

Fig. 1. Female rats chronically infected with *Toxoplasma gondii* showed reduced aversion to bobcat odor. Ordinate depicts time spent near bobcat odor (A) or entries made into bisect containing bobcat odor (B). Bars depict median and inter-quartile range for control (non-shaded) and infected (shaded) females. ***$p < 0.001$, independent sample *t*-test.

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