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# Toxoplasma gondii infection reduces predator aversion in rats through epigenetic modulation in the host medial amygdala

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## Abstract

**Male rats (*Rattus novergicus*) infected with protozoan *Toxoplasma gondii* relinquish their innate aversion to the cat odours. This behavioural change is postulated to increase transmission of the parasite to its definitive felid hosts. Here, we show that the *Toxoplasma gondii* infection institutes an epigenetic change in the DNA methylation of the arginine vasopressin promoter in the medial amygdala of male rats. Infected animals exhibit hypomethylation of arginine vasopressin promoter, leading to greater expression of this nonapeptide. The infection also results in the greater activation of the vasopressinergic neurons after exposure to the cat odour. Furthermore, we show that loss of fear in the infected animals can be rescued by the systemic hypermethylation and recapitulated by directed hypomethylation in the medial amygdala. These results demonstrate an epigenetic proximate mechanism underlying the extended phenotype in the *Rattus novergicus–Toxoplasma gondii* association.**

*Keywords*: arginine vasopressin, behavioural manipulation, DNA methylation, extended phenotype, fear, testosterone

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## Introduction

Many parasites change the host behaviour. Several of these behavioural changes are either part of the host defence or are accidental side effects of the parasitism. Yet, some of the behavioural changes after infection are thought to be the cases of the parasitic manipulation. In these cases, the infection cause specific changes in the host behaviour to increase the transmission of the parasites. Several postulates have been developed to differentiate parasitic behavioural manipulation from the host defensive response and the side effects of the infection (Poulin 1995). These include complexity of the effect, purposiveness of the design, convergence in several lineages, increase in fitness of the parasite due to manipulation and specificity of the effects; all criteria satisfied by the association between *Toxoplasma gondii* and *Rattus novergicus*.

Rats infected with *Toxoplasma gondii* exhibit greater exploration of spaces containing cat odours (Berdoy *et al*. 2000; Vyas *et al*. 2007a,b). This behavioural change seemingly increases parasite transmission because cats are the definitive host of this parasite. It still remains untested if the infection results in actual increase in predation rates (Worth *et al.* 2013), although similar effects have been observed in case of related apicomplexan parasites (Vorisek *et al.* 1998). The loss of fear in the infected rats is a specific phenotype leaving a variety of olfactory and mnemonic behaviours intact (Vyas *et al.* 2007a). While this behavioural change has been typically described as a loss of innate aversion, a subset of infected animals routinely also gains an attraction to the predator odours (Berdoy *et al.* 2000). The gain of attraction suggests that the behavioural change is geared towards greater parasite transmission. In short, behavioural change in the rat host is complex rather than a generalized behavioural drift, is in direction that should typically enhance the parasite transmission, is purposive in terms of gain in attraction rather than mere loss of fear and is specific.

Cat odour activates a well-delineated brain circuit that pivots around posteroventral part of the medial amygdala (MePV) (Dielenberg *et al.* 2001). The MePV

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receives information from the olfactory system and relays it to the hypothalamic nuclei (Scalia & Winans 1975; Swanson 2000; Dong & Swanson 2004). Interestingly, anatomically contiguous and architecturally homologous poster-dorsal medial amygdala (MePD) is robustly activated after exposure to the reproductive pheromones (Goodson & Kabelik 2009). Consistent with this parallel organization, exposure to the cat odour preferentially activates MePV over MePD in uninfected rats. Animals infected with *Toxoplasma gondii* do not exhibit significant difference compared to uninfected controls in terms of the MePV activation after the cat odour exposure (House *et al.* 2011). Yet, infection induces an atypical activation of MePD neurons in addition to the MePV in the rat brain (House *et al.* 2011). In other words, an allegedly sociosexual part of the medial amygdala is hyperactivated in infected animals without much demonstrable difference in defensive part of the same brain region.

The rat MePD constitutes an important node of extra-hypothalamic vasopressinergic system containing profuse number of arginine vasopressin (AVP) neurons. The extra-hypothalamic vasopressinergic system is involved in the male sexual and social behaviours in variety of species. This includes mate recognition in nematode *Caenorhabditis elegans (Garrison et al. 2012)*, flocking and territoriality in birds (Goodson *et al.* 2012), pair bonding in voles (Lim *et al.* 2004) and social recognition in rats (Samuelsen & Meredith 2009; Tobin *et al.* 2010; Blake & Meredith 2011; Hari Dass & Vyas 2014). AVP neurons of extended amygdala are also activated during copulation in the mice (Ho *et al.* 2010). Consistent with its role in sexual behaviours, extra-hypothalamic AVP requires testosterone for its sustained transcription (DeVries *et al.* 1985). In rats, the effect of testosterone on AVP is mediated through promoter hypomethylation, resulting in its enhanced transcription (Auger *et al.* 2011). Related to this, *Toxoplasma gondii* infection also enhances testicular production of testosterone, while the behavioural effects of the infection in male rats require intact testes (Lim *et al.* 2013).

Thus, *Toxoplasma gondii* infection reduces aversion to the cat odours, institutes an attraction to cat odours in a subset of animals, increases testosterone and initiates immediate early gene expression in MePD; a brain region that is part of testosterone-responsive extra-hypothalamic vasopressinergic system with purported role in male sexual behaviour. In this backdrop, we hypothesized that the *Toxoplasma gondii* infection causes changes in the host response to the predator odours by inducing epigenetic changes in the MePD-AVP system, reminiscent of testosterone effects on extra-hypothalamic AVP. The potentiated vasopressinergic system then atypically activates MePD during exposure to cat odour, therefore causing leakiness between defensive and sexual parts of medial amygdala.

## Materials and methods

### *Animals and parasites*

Adult male Wistar rats (44–50 days at the start of the experiment, housed two/cage) were procured from vivarium of National University of Singapore. All experiments were reviewed and approved by Nanyang Technological University institutional animal care and use committee. Animals were maintained on 12:12 h light–dark cycle (lights on at 7 AM) with ad libitum food and water.

Type 2 Prugniaud strain of *Toxoplasma gondii* was used in the study. Tachyzoites were maintained in human foreskin fibroblasts. Animals randomly assigned to the infected group were injected with $5 \times 10^6$ syringe-lysed tachyzoites (*i.p.*) suspended in 500 μL buffered saline. Control animals were injected with the saline only. All behavioural experiments started >7 weeks after infection, a time frame consistent with chronic stage of the infection.

### *Drug treatments*

Starting 6 weeks post-infection, control and infected animals were subcutaneously injected for ten consecutive days with either L-methionine (200 mg/kg body weight, dissolved in saline) or only the vehicle.

Intracerebral cannulas targeting MePD (AP = −3.0, L = ±3.8, V = −7.0) were implanted in uninfected animals for intracerebral administration of RG-108 (inhibitor for the enzyme DNA methyltransferase, Sigma). Standard operating procedures were used for stereotaxic intracranial surgery. During the surgery, animals were anaesthetized using a ketamine (90 mg/kg body weight) and xylazine (10 mg/kg) cocktail with maintenance of anaesthesia using 2–3% isoflurane. RG-108 was bilaterally infused using osmotic pumps through the implanted cannula for ten successive days (Alzet 2002 pump, total volume = 200 μL, 1 mg RG-108 per mL, dissolved in 6% DMSO in artificial cerebrospinal fluid). Control animals were infused with vehicle only.

Behavioural experiments were conducted 24 h after cessation of the drug treatments.

### *Cat odour avoidance assay*

Experiment was conducted in a rectangular arena that had two opposing and identical arms (76 × 9 cm each) separated by a central hub (9 × 9 cm size). Males we habituated in the maze for three consecutive days for

600 s. Males were tested individually by introduction in the central hub. Each arm had a towel containing either 2 mL of bobcat or rabbit urine (urine source Rabbit: Biopolis Research Center vivarium, Singapore; bobcat: Predator Pee, USA). Urine was replenished after every trial. Occupancy of male in control and infected arms was quantified over trial duration of 1200 s.

### *AVP and Fos colabelling using immunohistochemistry*

Animals were exposed to 2 mL bobcat urine in their home cage for 20 min with minimal handling during and after the stimulation and sacrificed 100 min after the termination of the stimulus.

Animals were deeply anaesthetized and transcardially perfused with 4% paraformaldehyde. Free floating brain sections (40 μm thick) were incubated in a cocktail of primary antibodies for 72 h at 4 °C (guinea pig anti-AVP, 1:500, Bachem; and, rabbit anti-Fos, 1:100, Santa Cruz Biotenchology). This was followed by incubation with secondary antibodies at room temperature for 2 h (biotinylated anti-guinea pig; 1: 200 + anti-rabbit DyLight 549; 1:200; obtained from Vector Laboratories). The biotinylated antibody signal was developed using Vectastain elite ABC kit (Vector Laboratories) and tyramide signal amplification system (PerkinElmer). Sections were counter-stained with DAPI for 1 min.

Brain sections between Bregma levels −2.76 mm and −3.24 mm (Interaural 7.28–7.08) were selected for analysis of the medial amygdala. Sections were imaged at 40× magnification and 1.2× digital zoom using a confocal microscope (optically sliced at 4 μm, three set of stacks per animal, Carl Zeiss LSM 710). Neurons positive for DAPI, FOS and AVP were counted. Scores were cumulated per animal and percentages calculated using total DAPI counts. The PVN was analysed using brain section from Bregma −1.72 to −1.92 and Interaural 7.28–7.08 [(Paxinos & Watson 2007), labelled as PaLM].

### *Microdisection of brain regions*

Samples were sectioned at 100 μm in a cryostat at −21 °C and mounted onto autoclaved Superfrost glass slides (Fischer Scientific). The MePD (Bregma −2.76 to −3.24, Interaural 6.24–5.76; (Paxinos & Watson 2007) labelled as MePD) was microdissected using autoclaved glass Pasteur pipettes to obtain micropunches that were transferred to 80 μL of lysis buffer in the eppendorf tubes. An additional 40 μL lysis buffer was pipetted into each of the glass Pasteur pipettes to wash down any remaining tissue into their respective eppendorf tubes. 1 μL of RiboLock (Thermo Scientific, Singapore; RNase inhibitor) was added per tube to ensure that the RNA does not degrade. Paraventricular nucleus (Bregma −1.72 to −1.92 and Interaural 7.28–7.08) was microdissected as a control.

### *Quantitative PCR for AVP*

RNA extraction was performed by the Trizol method. Briefly, tissue was microdissected, placed in lysis buffer and homogenized in ice-cold Trizol (~ double the volume of extraction buffer). Ribolock was added to prevent RNA from degrading. Chloroform (~1/5 volume of Trizol) was added, and samples were shaken for 15–30 s followed by incubation at room temperature for 3 min. The samples were centrifuged at 4 °C and 12 000 *g* for 15 min. The aqueous phase was pipette into a new tube and ice-cold isopropanol (~ same volume as aqueous phase) added. This was incubated at room temperature for 15–25 min and centrifuged again for 15 min. The supernatant was discarded carefully, and the pellet washed three times with 1 mL of 75% ethanol prepared using DEPC water and allowed to air dry at RT for ~15–30 min. It was resuspended in 30 μL DEPC water with 1 μL Ribolock. Samples were immediately converted to cDNA. cDNA synthesis was performed using the RevertAid first strand cDNA synthesis kit from Fermentas (Catalogue number #K1621, #K1622).

Abundance of AVP cDNA was quantified using the standard SYBR green-based real-time quantitative PCR (forward primer TGCCTGCTACTTCCAGAACTGC and reverse primer AGGGGAGACACTGTCTCAGCTC). HPRT was used as internal control (forward primer GTCATGTCGACCCTCAGTCCCA and reverse primer TCGAGCAAGTCTTTCAGTCCTGT). For each sample, threshold cycle numbers required to reach a predetermined fluorescence value were measured. Threshold values of HPRT were deducted from that for AVP to calculate delta Ct values. Quantitative PCRs were run using an ABI 7500 machine.

### *AVP promoter methylation analysis*

Genomic DNA extraction was performed by the phenol–chloroform–isoamylalcohol method. Briefly, tissues were microdissected, placed in lysis buffer, homogenized in phenol–chloroform–isoamylalcohol (~1/2 volume of extraction buffer) and centrifuged at 12 000 *g* for 15 min at 4°C. The aqueous phase was pipetted into a new eppendorf tube and ice-cold isopropanol added (~ same volume as aqueous phase), and incubated at room temperature for 15 min or 4°C for 30 min. Samples were centrifuged at 12 000 *g* for 15 min at 4°C. The supernatant was discarded carefully; pellets washed three times with 1 mL 75% ethanol and allowed to air

dry at RT for ~15–30 min. They were resuspended in 30 μL MilliQ water and stored at −20 °C for short time or −80 °C for longer periods.

Methylation of AVP promoter was quantified using methylation sensitive restriction enzyme (MSRE) digestion in combination with quantitative PCR. Primer, methods and targeted sites were adapted from Auger *et al.* (2011). Briefly, DNA from each rat was pipetted into two tubes in equal amounts: an enzyme-treated and a no-enzyme control tube. These tubes were then processed using the same primers surrounding the targeted HpaII site (unmethylated CCGG) or BstUI (unmethylated CGCG). HpaII (New England Biolabs) was used to cleave DNA at promoter site 1 and BstUI (Fermentas) at promoter site 2 (Auger *et al.* 2011). Hypomethylation in this assay manifests as reduced protection of DNA template from MSRE, and consequently, greater divergence between enzyme-treated and sans-enzyme samples in terms of PCR cycle numbers required to reach a predetermined threshold (Ct).

### *Statistics*

Student's *t*-test test was used to analyse statistical differences between treated and untreated animals and between control and infected animals. Analysis of variance (ANOVA) was used when both infection and drug treatments were administered. Values reported are mean ± SEM. Relative expression with reference to internal control during quantitative PCR was analysed using freely available REST software (Pfaffl *et al.* 2002). This software uses randomization tests to obtain relative expression level with robust statistical testing. 10 000 randomizations were used. All other statistical analyses were carried out using SPSS software (version 17, IBM).

## Results

Arginine vasopressin promoter methylation in MePD genomic DNA was quantified using methylation sensitive restriction enzyme (MSRE) digestion. Two restriction enzymes were used [HpaII and BstUI; promoters and associated gene sequences similar to (Auger *et al.* 2011)].

Treatment with HpaII increased Ct value in infected animals, relative to no-HpaII samples (Fig. 1A; eight of eight animals; $P = 0.008$, exact binomial test, null hypothesis being equal chance of enzyme > no-enzyme and no-enzyme > enzyme). Seven of eight control animals also exhibited increase in Ct value after HpaII application, although the data did not reach statistical significance in this case (Fig. 1A; Delta Ct = 0.662 $P > 0.05$). Taken together, these observations suggest

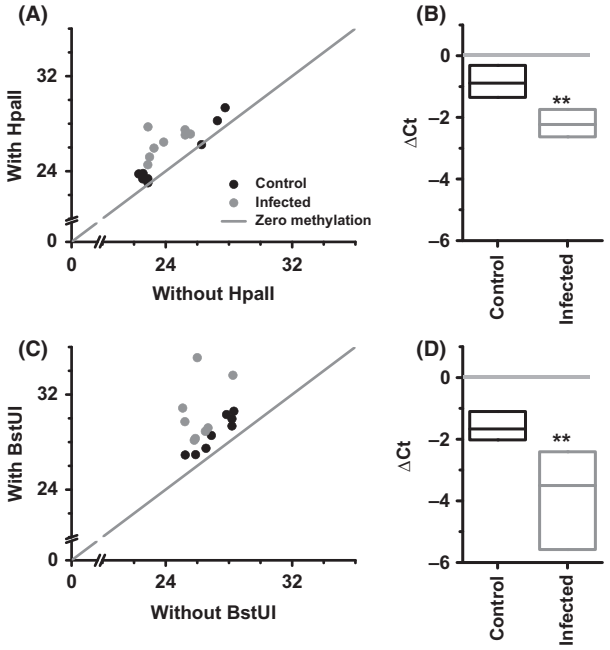


**Fig. 1** *Toxoplasma gondii* infection increased methylation at arginine vasopressin (AVP) promoter in MePD, at CpG islands corresponding to MSRE sites of HpaII (A and B) and BstUI (C and D). Panel A and C depict Ct values for MePD genomic DNA with (ordinate) or without (abscissa) pretreatment with MSRE. Each dot represents one animal. Diagonal grey line depicts expected values in event of complete methylation. Please note break in axes after origin. Panel B and D depict ΔCt values (without MSRE–with MSRE). Horizontal grey line is for complete methylation. Boxes represent median and interquartile range. $N = 8$ animals for each group. $^{**}P < 0.01$.

that HpaII promoter site is not completely methylated in rat MePD. An arbitrary index of departure from chance (x = y; grey line in Fig. 1A) was calculated as $(x - y)^2$ divided by $(x + y)^2$, where x and y are Ct value without HpaII and with HpaII, respectively. Amongst experimental groups, infected animals exhibited greater departure (mean ± SEM: control = 0.0004 ± 0.00013, infected = 0.0028 ± 0.00095; $|t_{14}| = 2.45$, $P = 0.028$, independent sample *t*-test). Consistent with this, infected animals demonstrated greater magnitude of ΔCt values (Ct value for sans-enzyme subtracted from Ct value for corresponding enzyme-treated samples; Fig. 1B; $P = 0.002$, calculated using randomized significance testing in REST). This intergroup difference was striking in that 75th percentile of ΔCt values in infected group was placed below 25th percentile of control animals. These observations suggest that *Toxoplasma gondii* infection creates hypomethylation at the HpaII susceptible promoter site of the AVP gene in the MePD.

Congruent results were observed in case of BstUI susceptible promoter site. All animals in both control and

infected groups exhibited increased Ct values after treatment with the restriction enzyme (Fig. 1C; $P < 0.008$ for both control and infected, exact binomial test). Thus, similar to HpaII promoter site, the BstUI site was also incompletely methylated in gonad-intact male rats. Amongst experimental groups, infection amplified the divergence between observed and chance Ct values (chance being x = y, see preceding paragraph; divergence: control = 0.0009 ± 0.00019, infected = 0.0067 ± 0.00249; $|t_{14}| = 2.39$, $P = 0.039$). Infected animals also demonstrated greater magnitude of ΔCt values compared to controls (Fig. 1D; $P < 0.001$, REST). 75th percentile of ΔCt values in infected group was placed below 25th percentile of control animals, an observation that was similar to that observed for HpaII promoter site (Fig. 1B).

Data presented in preceding two paragraphs demonstrate that *Toxoplasma gondii* infection creates an epigenetic change by reducing DNA methylation of AVP promoter in the MePD. This is likely to enhance AVP transcription. To investigate this, we extracted mRNA from the MePD of control and infected animals followed by cDNA synthesis. Quantitative PCR was used to compare transcript levels of AVP relative to a housekeeping gene, HPRT (Fig. 2A). Difference between AVP and HPRT Ct values was used as index for AVP transcription (ΔCt value). cDNA obtained from infected animals required fewer PCR cycles to reach a predetermined threshold (ΔCt; Fig. 2B, left; $P < 0.05$, REST), suggesting enhanced MePD-AVP transcription. Level of cDNA in PVN was not different between control and infected animals (Fig. 2B, right; $P > 0.05$). This suggest that infection-mediated increase in AVP transcription was specific to MePD, being absent in PVN.

Necessity of hypomethylation in infection-induced behavioural effects was further tested by preventing it using systemic administration of L-methionine. Systemic L-methionine creates global hypermethylation of the DNA. The systematic route of administration was chosen over localized administration to MePD because of the concern that immunological upheaval during intracranial surgery would initiate recrudescence of cystic *Toxoplasma gondii* in the brain. A two-by-two experimental design was employed (control or infected; injected with L-methionine or placebo; 10 animals each group, total 40 animals). L-methionine treatment consisted of subcutaneous injection at 200 mg/kg dose in sterile saline for ten successive days, twice a day starting 6 weeks post-infection. On the twelfth day after start of methionine treatment, we quantified innate fear by measuring aversion to bobcat odour during a 20-min trial (independent measure being occupancy in cat odour bisect divided by sum of occupancy in cat and rabbit odour bisect, %).

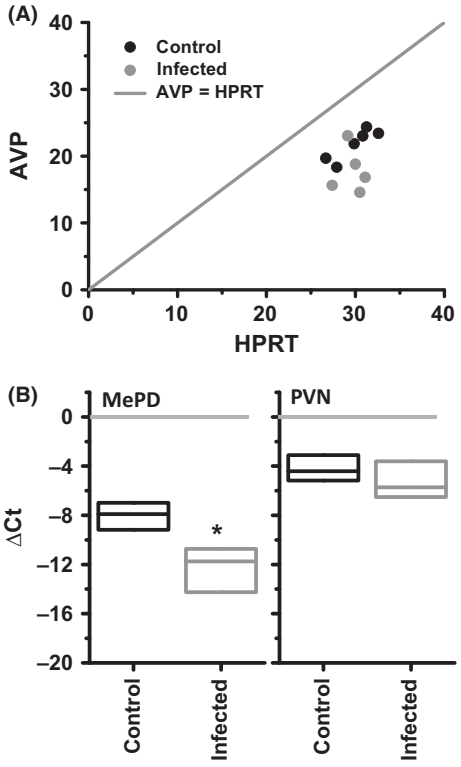


**Fig. 2** *Toxoplasma gondii* infection increased mRNA levels of MePD- arginine vasopressin (AVP). Infected animals required lower number of cycles to reach predetermined signal threshold for AVP primers (A; ordinate), but not for housekeeping control (abscissa). Panel B depicts ΔCt values (Ct for AVP – Ct for HPRT), when using cDNA from MePD (left) and paraventricular nucleus of hypothalamus (PVN, right). $N = 6$ animals for control and five for infected group. *$P < 0.05$.

Analysis of variance revealed significant main effects of the infection status and methionine treatment ($F_{(1,32)} > 15$, $P < 0.001$). Effects of interaction also reached statistical significance ($F_{(1,32)} = 8.2$, $P = 0.007$).

*Toxoplasma gondii* infection resulted in reduction of defensive response in placebo-treated animals (Fig. 3A and 3B; $P = 0.001$, LSD post hoc test). Six of ten infected animals exhibited gain of attraction to cat odour (Fig. 3A, chance = 50%). Methionine treatment did not induce significant changes in the behaviour of control animals ($P = 0.124$). Furthermore, methionine treatment completely abolished gain of attraction to cat odour in infected animals and renormalized defensive behaviour to the level observed in control animals (Fig. 3C and 3D; $P < 0.001$ for comparison between infected placebo and infected methionine; $P = 0.348$ for comparison between control placebo and infected methionine). All animals treated with methionine spent more time near rabbit odour compared to cat, regardless of infection status. These observations demonstrate that a hypomethylation event is necessary to

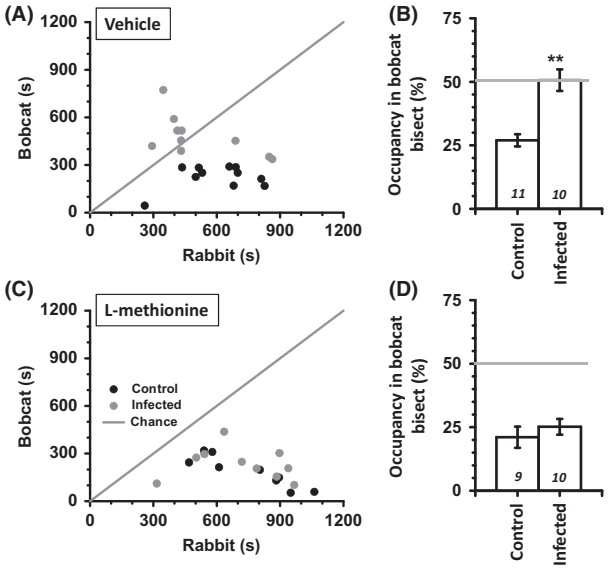


**Fig. 3** Systemic administration of hypermethylating agent, L-methionine, prevented behavioural effects of *Toxoplasma gondii* on aversion to cat odour. Panel A and C depict time spent in bisects containing either rabbit urine (abscissa) or bobcat urine (ordinate), without (panel A) or with L-methionine treatment (panel C). Diagonal grey line depicts chance (rabbit = bobcat). Panel B and D depict time spent in bobcat bisect relative to sum of time spent in bobcat and rabbit bisect (chance = 50%), without (panel B) or with (panel D) L-methionine treatment. N is appended in bar of panel C and D (in *italics*). $^{**}P < 0.01$.

induce behavioural effects of the infection on defensive behaviours.

We further investigated whether a localized hypomethylation within MePD was sufficient to recapitulate behavioural effects of the infection. Using osmotic pumps and intracranial surgery directed at MePD, we delivered RG108 (an inhibitor of DNA methyltransferase enzyme; total volume: 200 μL, concentration: 1 mg/mL) for 10 days in brain of uninfected male rats. Behaviour testing was performed on the twelfth day after the start of the infusion. Cannula localization was confirmed post-mortem, and off-target samples were removed from the analysis. RG108 infusion significantly reduced innate fear to cat odour, compared to placebo (Fig. 4A and 4B; $|t_{17}| = 2.52$, $P = 0.022$, independent sample *t*-test). These observations suggest that hypomethylation in MePD is sufficient to reduce defensive behaviour, recapitulating behavioural effects of the infection sans parasite.

Hypomethylation of MePD-AVP promoter (Fig. 1) results in enhanced AVP levels (Fig. 2). Such an up-regulation might increase the probability of vasopressinergic recruitment more typical of male sexual behaviour and might create leakiness between parallel but interconnected circuits of the medial amygdala. Hence, we further compared recruitment of MePD-AVP

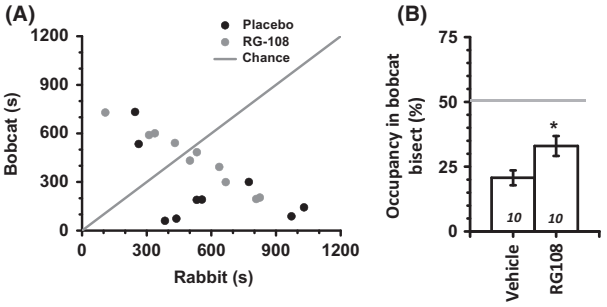


**Fig. 4** DNA hypomethylation in MePD was sufficient to reduce aversion to cat odour in rats devoid of *Toxoplasma gondii* infection. Panel A depicts time spent in bisects containing rabbit and bobcat odour. Panel B depicts parentage time spent in bobcat bisect. N is appended in bar of panel B (in *italics*). $^{*}P < 0.05$.

neurons during cat odour exposure in control and infected animals. Expression of Fos was used as a proxy for neuronal activation. Neurons expressing both AVP and Fos were quantified in MePD and MePV (Fig. 5A), downstream brain region serving as internal negative control.

ANOVA revealed significant interaction between infection status and brain region ($F_{(1,10)} = 8.21$, $P = 0.017$). In case of MePD, exposure to bobcat urine robustly increased number of colabelled neurons in infected animals compared to controls (Fig. 5B, left; 160% increase; $|t_{10}| = 2.56$, $P = 0.028$, post hoc independent sample *t*-test). No statistical difference was evident between MePV colabelled neurons with or without infection (Figure 5B, right; $|t_{10}| = 0.31$, $P = 0.765$, statistical power = 0.13). The infection did not induce significant changes in AVP and Fos colabelling in bed nucleus of stria terminalis and ventromedial hypothalamus (Table 1). Finally, infection status did not affect number of Fos-positive neurons in either MePD or MePV at baseline without exposure to the cat odour ($P > 0.9$).

## Discussion

Rats infected with *Toxoplasma gondii* relinquish innate aversion to cat odours and instead develop an attraction (Berdoy *et al.* 2000; Vyas *et al.* 2007a,b). Based on present observations, we propose that enhanced testosterone production in the testes of infected animals (Lim *et al.* 2013) reduces methylation of MePD-AVP promoter. This results in up-regulation of the AVP. Such an up-regulation increases probability of vasopressinergic activation more typical of male sexual behaviour and creates leakiness between parallel but interconnected circuits of the medial amygdala. This is supported by the present observations that greater number of AVP neurons are activated upon cat odour exposure in the

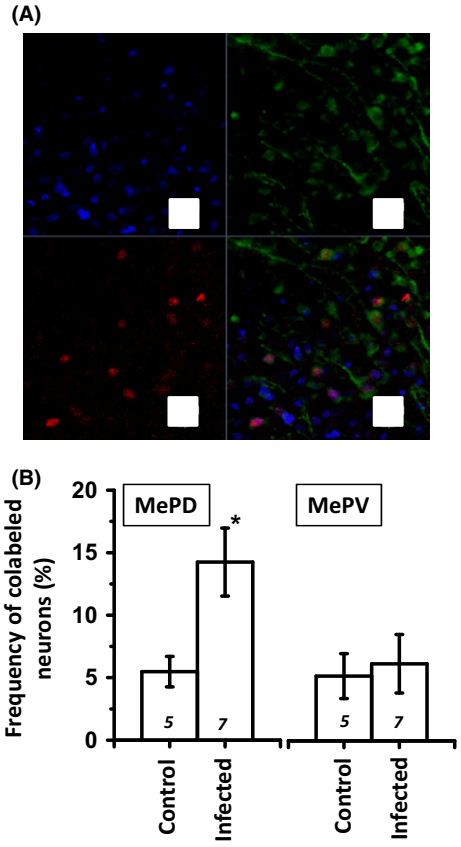


**Fig. 5** *Toxoplasma gondii* infection increased colabelling of arginine vasopressin (AVP) and Fos in MePD neurons after exposure to bobcat odour. Panel A depicts a representative image depicting colabelling. Fos is stained in red colour (DyLight 549, emission = 568 nm), and AVP is in green (Fluorescein, emission = 517 nm). DAPI is in blue. Scale bar = 30 μm length/width of white square. Panel B depicts frequency of colabelled neurons (%) in MePD (left) and MePV (right). N is appended in bar of panel B (in *italics*). *$P < 0.05$.

**Table 1** Comparison of arginine vasopressin (AVP)–Fos colabelled neurons in bed nucleus of stria terminalis and ventromedial hypothalamus after exposure to cat odour

| | Control | Infected | *P* | Power |
|---|---|---|---|---|
| Bed nucleus of stria terminalis, medial | 9.4 ± 2.1 | 6.9 ± 1.9 | 0.41 | 0.14 |
| Ventromedial hypothalamus, dosromedial | 7.2 ± 1.2 | 8.3 ± 2.5 | 0.74 | 0.07 |
| Ventromedial hypothalamus, ventrolateral | 9.1 ± 3.7 | 12.1 ± 2.8 | 0.51 | 0.10 |

*N* = 5 control and 7 infected animals.

infected animals. The role of testosterone-dependent epigenetic change is also supported by the observations that the effects of the infection can be blocked by castration (Lim *et al.* 2013) or pharmacological hypermethylation in intact animals and recapitulated by sans-infection hypermethylation (present report). In congruence, human subjects infected with *Toxoplasma gondii* exhibit greater testosterone (Flegr *et al.* 2008) and lesser aversion to cat odours (Flegr *et al.* 2011). Alternatively, it is also plausible that AVP promoter hypomethylation and testosterone increase represent two independent consequences of the infection, without a causal link between these two.

Prior studies show that interference of dopaminergic transmission rescues effects of the *Toxoplasma gondii* infection on the rat behaviour (Webster *et al.* 2006). The infection also enhances dopamine release in mouse brain slices (Prandovszky *et al.* 2011). Additionally, *Toxoplasma gondii* genome contains two genes with remarkable homology to the mammalian tyrosine hydroxylase (Gaskell *et al.* 2009; Prandovszky *et al.* 2011), a rate-limiting enzyme in the dopamine synthesis pathway. These observations suggest that the brain dopamine system might be involved in mediating behavioural change post-infection. Interestingly, AVP and dopamine system can be viewed as an integrated system that negotiates challenges and opportunities in an ambivalent umwelt, titrating behavioural responses to the dynamic trade-offs (O'Connell & Hofmann 2011a, b). In agreement with the possible interactions between dopamine and vasopressinergic MePD, lesions of the medial amygdala block dopamine release in medial preoptic nucleus with concomitant reduction in sexual behaviour (Dominguez *et al.* 2001), and chemical stimulation of the medial amygdala enhances dopamine release in this nucleus (Dominguez & Hull 2001).

Several studies have demonstrated that DNA methylation changes in the brain can mediate a change in the behaviour [listed in (Lester *et al.* 2011)]. For example, maternal grooming of rat pups modulates methylation status of glucocorticoid receptor promoter in the hippocampus, resulting in changes in stress sensitivity (Weaver *et al.* 2004). Outside the realm of the brain, proteinaceous urinary pheromones in male mice provide another example of epigenetic influence on the behaviour. Promiscuity-mediated increases in major urinary proteins in this species is epigenetically regulated through promoter hypomethylation (Nelson *et al.* 2013). These studies suggest that changes in the DNA methylation are integral to the behavioural plasticity in accordance with the changing environment. We suggest that *Toxoplasma gondii* utilizes this pre-existing phenotypic plasticity in its host by changing DNA methylation in the MePD-AVP. Extra-hypothalamic AVP is indeed known to be regulated by testosterone through epigenetic means (Auger *et al.* 2011), reminiscent to hypomethylation caused by the infection.

Several prior studies have postulated the role of epigenetic processes in the metabolic programming of the host cells to the advantage of a pathogen or parasite (Silmon de Monerri & Kim 2014). For example, *Mycobacterium leprae* promotes de-differentiation of the infected Schwann cells to a more stem cell like progenitor state, thus creating greater opportunity for the bacterium to spread (Masaki *et al*. 2013). Moloney murine leukaemia virus is another pertinent example. The proviral insertion of this retrovirus causes DNA hypermethylation in the flanking sequences of the host genome (Jahner & Jaenisch 1985). The resultant transcriptional silencing contributes to the latent maintenance of the provirus in the host genome. Thus, parasites can influence host metabolism through DNA methylation, and host themselves have pre-existing epigenetic mechanisms to change their behaviour. In this report, we extend this argument by proposing that parasite-induced changes in host DNA methylation can manipulate the host behaviour.

Parasitic manipulation of host behaviour is often invoked as rhetorical tool to argue that natural selection acts on genes and not necessarily individuals. In this framework, change in behaviour of infected rats can be viewed as a phenotype of parasite's genes. This assertion assumes that *Toxoplasma gondii* infected rats are consumed more readily by cats; an outcome that has been suggested but not yet proved (Vorisek *et al*. 1998; Worth *et al*. 2013). Within limits of this assumption, current observations present the possibility of an epigenetic mechanism for *Toxoplasma gondii* induced extended phenotype.

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## Data accessibility

Raw data used for analysis are available at the Dryad data repository (doi:10.5061/dryad.pb282).