Role of testosterone in parasitic host behavioral change in *Rattus norvegicus* : *Toxoplasma gondii* association

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School of Biological Sciences

2016

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change in *Rattus Norvegicus* – *Toxoplasma gondii*
association**

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Abstract

Role of Testosterone in parasitic host behavioral change in *Rattus Norvegicus* – *Toxoplasma gondii* association

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Trophically transmitted parasites are often considered prisoners in the body of their host, relying on incidental predation in order to reach the next stage in their life cycle. In contrast, the ‘behavioral manipulation’ hypothesis posits that parasites exploit the host behavior to facilitate the transmission. *Toxoplasma gondii* is a classic example for study the parasitism and the alteration in host behavior. This project is inspired by the loss of fear in rats post *Toxoplasma gondii* infection. The thesis posits a crucial role for testosterone in mediating parasitic behavioral change in the host. The broad aim is to study the testosterone-amygdalar interactions in behaviorally manipulated *Toxoplasma gondii* infected male rats. I implement *Toxoplasma* induced behavioral changes in male rats as a perturbation model to investigate the manipulated defensive mechanism.

Elevated testicular testosterone during chronic phase of infection has been well documented. Hence as the first specific aim, I studied necessity and sufficiency of testosterone for behavioral change in *Toxoplasma gondii* infected male rats. Results show that alterations in fear and concomitant molecular changes can be eliminated when host rats are castrated post establishment of chronic infection. In addition, results demonstrate that exogenous supply of testosterone within medial amygdala of uninfected castrates recapitulates reduction in innate fear akin to behavioral change attributed to *Toxoplasma gondii*. Overall, these results support a gonadal, rather than brain-dependent, initiation of proximate mechanisms in this association.

Most of testosterone is synthesized in Leydig cells of testes and it is mainly regulated by luteinizing hormone secreted by the anterior lobe of pituitary gland. Steroidogenic process in these cells remains under homeostatic control through reciprocal communication with the brain. In view of the reciprocal effects, it is perhaps more appropriate to view medial amygdala where many of parasite-induced changes are observed in the brain and Leydig cells in testes as two nodes of an interacting network. We demonstrate that molecular changes in the medial amygdala cause increase in luteinizing hormone receptor distantly within the testes. In brief, testicular testosterone and medial amygdala epigenetic changes might constitute a positive feed-forward loop capable of self-sustenance once initiated.

We investigate the interaction of luteinizing hormone and its testicular receptor during infection by *Toxoplasma gondii*. This is important because luteinizing hormone receptor is the starting point of signal cascade regulating testosterone production. My experiments in this regard did not highlight any statistically significant findings although this study had sufficient sample numbers. Future studies about alternative mechanisms are thus indicated.

In summary, I present a model that is built around pre-existing brain-gonad communication in the host species wherein the parasite uses this pre-existing scaffold to manipulate the host behavior.

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1. Introduction

In this thesis, I focus on proximal mechanisms of host behavioral change in *Rattus norvegicus-Toxoplasma gondii* association. During this chapter, I will start with an
5 introduction to this particular host parasite relationship. I will then place the host behavioral change in wider context of host defensive behaviors. Host defense is often traded off with other ecological imperatives like reproduction. Hence I will discuss the conditional strategies the host might use to resolve this trade off. In this backdrop, I will then discuss the possibility that *Toxoplasma gondii* infection represents a shift in
10 these pre-existing host conditional strategies. In light of this intellectual landscape, I will then describe existing gaps in the knowledge and placement of my specific aims with respect to these gaps.

1.1 Rat and Toxoplasma host-parasite relationship

15

The behavioral manipulation hypothesis posits that parasites can change host behavior in such a way that it helps the parasite even at the cost of the host. Specifically, this hypothesis argues that natural selection on the parasite results in an extended phenotype in the host; comprising host behavior change resulting in greater parasite transmission[1]. In the paragraphs below I will discuss this possibility in context of trophically transmitted parasites, although several examples involving non-trophic transmission have also been studied[2]. Trophically transmitted parasites transmit from prey to predators through successive nodes of the food chain. This often involves predation of an intermediate host by the definitive host. A pertinent example is trophic transmission of *Toxoplasma gondii* (henceforth *Toxoplasma*) from intermediate rat host (*Rattus norvegicus*) when rats are predated by cats (Family Felidae)[3]. *Toxoplasma* is a protozoan parasite with a particularly cosmopolitan host range including birds, rodents, livestock and humans. Despite this wide range of intermediate hosts, *Toxoplasma* undergoes gametogenesis and sexual reproduction only in the cat intestines. Moreover, entry into the cat results in eventual shedding of oocysts that are particularly recalcitrant to environment with high potential of infecting more intermediate hosts through fecal-oral route. This route also allows infection of herbivores; an event that will not be possible by transmission of the parasite between intermediate hosts through carnivory. Thus, predation by cats constitutes a bottleneck during the life cycle of *Toxoplasma* and a very plausible selection pressure (Figure 1).

35

Trophic transmission of the parasites is thought to coevolve with high predation rates of the prey[4]. Such robust predation also causes a selection pressure in prey to avoid predators and associated sensory cues. For example, rats under laboratory conditions can often live up to two years before eventually succumbing to age related mortality.

40 Yet in field conditions without protection from predators, life expectancy of rats drop
to around eight months with most of attrition caused by predation[5]. Congruently, rats
exhibit robust and innate aversion to cats and cat odors[6]. Exposure to cat odor results
in a stereotypic fixed action pattern of avoidance even in rats that have not been
previously exposed to cats or cat odors[7-10]. Thus trophic transmission of
45 *Toxoplasma* becomes constrained by innate fear of rats towards predators. The
parasite benefits from greater predation rates, and host benefits from lower predation
rates; representing opposing selection pressures. In this general scenario, *Toxoplasma*
infected rats show reduced aversion to cat odors once the infection has reached its
cystic and chronic phase (see Figure 2A). Such loss of aversion should increase
50 transmission of *Toxoplasma* from rats to cats; although conclusive evidence of greater
predation is currently not available. The narrative then is that natural selection on
Toxoplasma creates an extended phenotypic change in the rat behavior. *Toxoplasma*
manipulates rat behavior by causing a loss of aversion to cats.

Behavioral change in the *Toxoplasma* infected rats is relatively unique in that
55 *Toxoplasma* is also a sexually transmitted parasite in the rats[11]. Sexual selection
generally causes strong aversion to parasitized males in females[12, 13]. Multiple
examples of such mate choice exist across variety of parasite phylogenies[14, 15].
Such discrimination against parasitized males will constrain sexual transmission of the
Toxoplasma to female rats. In this context, infected male rats gain an atypical
60 attractiveness[11]. Uninfected females spend more time near odor of infected males
compared to uninfected male controls; and permit infected males more copulatory
opportunities in a competitive paced mating set up (Figure 2B). This provides another
example of the host extended phenotype that increases asexual transmission of the
parasite through sexual behavior of the hosts.

65 A simultaneous trophic and sexual transmission from the same intermediate host creates an interesting paradox. Sexual transmission is usually associated with lower virulence because dead hosts cannot contribute to sexual transmission. For example, infected male rats will provide trophic transmission but not necessarily terminate any residual chances of sexual transmission. Implications of these disparate demands are
70 not yet well understood. I will further comment on this paradox in the ensuing 'Discussion' chapter (please see paragraph starting from line 930).

In his book Samuel Butler rhetorically asked "why it should be said that the hen lays the egg, and not that the egg lays the hen"[16]. To extend the argument, does natural selection operate on genes or individuals? Is the hen merely "laid" by an egg to make
75 another egg? This differentiation can be difficult to test through experiments. Fortunes of hen and fortunes of egg are too intertwined to tease out if natural selection optimized the genes or the individuals. The behavioral manipulation hypothesis provides an elegant way out of this conundrum. It predicts that parasites can change host behavior, in a way that benefits them but not the host. In other words, genes of a
80 parasite can produce a phenotype "extended" out of parasite's soma. Manipulation of rat behavior by *Toxoplasma* is especially opportune model in this context because both rats and *Toxoplasma* are mainstream model system in neurobiology and parasitology laboratories, respectively. Rats and mice have served as model systems for neurobiology of fear and aversion in multitude of papers, leading to delineation of a
85 well-defined neural circuit [9, 17, 18]. Similarly, *Toxoplasma* has been extensively used for molecular dissection of protozoan pathogenesis [19-21]. Thus, this model is ripe for more definitive investigation of proximate mechanism and synthesis.

90

Figure 1 Life cycle of *Toxoplasma gondii*

95 Sexual reproduction occurs in the intestine of the cat resulting in shedding of (a) diploid oocysts that undergoes sporogony in the environment to yield sporozoites. A-E refers to the stages of development in the enterocytes. Oocysts are extremely environmentally resistant and give rise to infections in a variety of intermediate hosts. During acute infection, rapidly proliferating (b) tachyzoites disseminates within the host. Semi-dormant tissue cysts containing (c) bradizoites give rise to long-term
100 infection and are transmitted asexually following oral ingestion by a variety of host (Adapted from [22]).

Figure 2 Toxoplasma infection causes behavioral change in infected male rats.

Toxoplasma infected rats show loss of aversion to predator odor areas. The rising line
105 above the x-axis represents a relative preference for the cat areas whilst data below the
x-axis indicate avoidance of predator odor by non-infected rats (Panel A)[11].
Uninfected female rats preferred Toxoplasma infected rats to control rats. Preference
ratio depicts time spent in infected bisect divided by control bisect wherein the
females spent more time in arena with infected rat urine (Panel B)[23].

110 1.2 Plausible selection pressures on defensive behaviors

As mentioned earlier, rats experience heavy predation. This ecological mortality could simultaneously result in two selection pressures. The first one is obvious and I discussed it earlier. Since predation negates all residual reproductive opportunities for
115 the prey, a postponement of predation is selected for. This is achieved by evolution of anti-predator defense. Anti-predator defense in rodents involve both static defense like nocturnal life and active defense like avoidance of sensory cues associated with predator presence[6]. These defenses probabilistically reduce the chance of predation and hence experience a positive selection pressure. A logical outcome should then be
120 that all prey animals should be maximally defended constrained only by physical and ecological factors but prey animals are not maximally defended most of the time. The reasons partly relate to various tradeoffs associated with anti-predator defense[24]. Second, the selection pressure that I describe below forms part of that tradeoff.

In simple terms, ecological mortality due to predation can be seen as a constant
125 attrition of the population. In other words, risk of being predated increases monotonically as the age of the individual increases and probabilistic risk of predation accumulates. This is perhaps an over-simplification; for example, older animals might experience lower per-unit risk of predation because of greater accumulation of skills and social learning. Yet in general a temporally accumulating probability of predation
130 usually results in decreasing probability of future survival as age of an individual increases. This will create a selection pressure for early reproduction. As probability of living to a later age declines, slow-and-steady reproductive strategies are disfavored via-a-vis fast and furious reproduction. This is aptly demonstrated by differences in life histories of chimpanzees and humans. It is suggested that relatively lower

135 predation rates in ancestral human populations caused later andrache/menarche and
longer adolescent window for development of hunting skills [25].

Thus heavy predation often results in two simultaneous selection pressures: to increase
anti-predator defense and to increase investment on the current reproduction. Yet these
two imperative are in conflict with each other. Reproductive advertisement in males,
140 for example, require conspicuousness that can be intercepted by predators[26] . And
behaviors required for mate search and approach are in conflict with predator
avoidance.

I will now shift to two possible ways by which conflicting demands of anti-predator
defense and early reproductive investment can be negotiated.

145 **1.3 Conditional strategies in defensive behaviors**

Tradeoff between current reproduction and anti-predator defense is often negotiated through negative frequency dependent selection. A pertinent example is that of crickets (*Teleogryllus oceanicus*) in the Hawaiian Islands[27]. Most males exhibit a stridulatory apparatus consisting of files, teeth and scrapers on hind-wings. This stridulatory apparatus is used to produce acoustic sexual advertisement to attract females. Some of the males contain a flatwing mutation that substantially reduces the size of stridulatory apparatus and precludes production of sexual advertisement. Thus, most of the male crickets stridulate but a minority does not. These ‘sneaker’ males wait on the periphery and attempt to intercept females who are attracted by neighboring callers. The sneaker phenotype is believed to be in negative frequency-dependent equilibrium with calling phenotype.

The sexual advertisement in this case is openly broadcasted and is used by parasitoids to locate cricket prey[28]. Recent introduction of parasitoid insect, *Ormia ochracea*, in Hawaiian Islands has increased mortality of the callers due to preferential parasitism. This parasitoid employs calling advertisement as a beacon to locate males, resulting in a selective disadvantage to the stridulating male crickets. This has, in turn, shifted the equilibrium by increasing reproductive fitness of silent sneaker alleles. While majority of male crickets were callers a few decades ago, now majority of males no longer stridulate[27]. This represents a shift in underlying allele frequency due to natural selection.

Similar phenomena can be seen in gustatory preferences of cockroaches (*Blattella germanica*)[29]. Cockroaches have been extensively ‘hunted’ by humans using poisoned baits for pest control in domestic environments. These baits often use additive like D-glucose that evoke approach behavior in cockroaches. Yet, in response

to baiting, natural selection has recently favored alleles that create an aversive gustatory response to additives that were ancestrally attractive. Thus, sugar in descended populations evoke a defensive response akin to “bitter” taste rather than approach akin to a “sweet” gustatory perception[30]. Similar to the example of crickets above, such shift in behavior represents an underlying shift in allele frequency due to natural selection.

In contrast to static behavior and shift in allele frequencies, many animals exhibit dynamic and inducible anti-predator behaviors without underlying shifts in the genotypes. For example, presence of chemical cues from predator phantom midge larvae induces morphological defenses in the form of neck spines in *Daphnia pulex* water fleas along with shift in life history traits[31]. The same genotype in this case causes an inducible or conditional phenotype in the presence of the predator. Conditional defenses are thought to be selected in ecologies with reliable cues of predators, high cost of defense and unpredictable attacks by the predators[32]; attributes that are common in rodent predation by cats.

Several strands of evidence suggest that rodents resolve defense-reproduction trade-offs using conditional strategies. In other words, relative partitioning to defense and reproduction systematically vary as a function of ecological conditions and physiological milieu. To start with, defensive behaviors in rodents are not inflexible. Individuals demonstrate greater aversion in response to cues like fur and odor from neck glands of the cat because these cues have greater predictive value about bodily presence of the predators. In contrast partial predator cues with less predictive powers generate less fear (*e.g.* urine derived odors generate less fear than cat collars)[33]. Within the same class of predator cues, increasing doses of the cue generate increasing aversion. These observations suggest that aversion to predator is not adaptively

perfected but is calibrated by perceived predation risk. Several studies show similar titration of anti-predator responses in other rodents in response to various ecological factors (succinctly reviewed in [34]).

I will now turn to two independent studies in order to make connections between reproduction and defense more palpable. Male mice demonstrate robust aversion when
200 exposed to odor from a cat or weasel; both natural predators of the mice. Such aversion is accompanied by increased secretion of stress hormones, adrenal glucocorticoids, and reduced secretion of reproductive hormones, testicular testosterone. Yet a brief exposure to sexually receptive female mice results in
205 suppression of both defensive behaviors and endocrine sequelae[35]. This “emboldening” of male mice demonstrates that defenses and reproduction are dynamically adjusted in accordance with incipient environment.

In a similar vein, male mice adjust their investment in sexual advertisement based on perceived predation risk. Mice use competitive scent marking as sexual advertisement.
210 Dominant males leave copious scent marks in the environment which can be identified by females by virtue of polymorphic major urinary proteins present in the urine[36]. These scent marks are open to challenge by competitors who overlay their own scent on preceding competitors. This allows females to register dominance relationships in time and space (“A” marked more and overlaid marks by “B”, but “B” marked less
215 and did not overlay over marks made by “A”). Apropos males who produce copious scent marks also are more likely to produce counter-marks on other males. Being an openly broadcasted advertisement, this olfactory communication is liable for interception by predators. In keeping with risk of predation during scent marking, highly competitive male mice reduce their counter-marking frequency when presented
220 with odors of a ferret[37]. This reduction is specific to predator odor because an

unfamiliar odor for naked mole rat does not influence scent-marking behavior at all. This demonstrates that sexual advertisement in rodents is a conditional behavior that is traded off with risk of predation.

1.4 Recruitment of conditional strategies in host behavioral change

Till now, I have presented two important pillars on which this thesis is built:

- 230 1. Toxoplasma manipulates predator aversion in rats, plausibly to increase its transmission efficiency.
2. Predator aversion in rodents is a conditional behavior that is traded off with reproductive investment.

I now make a case that conditional negotiation of defense-reproduction continuum represents the axis that is manipulated in Rat-Toxoplasma association. In this section I
235 will first summarize mechanisms thought to be involved in behavioral change in this system. I will then extend that to argue for centrality of mechanisms that rely on defense-reproduction trade-off. This represents the third theoretical pillar on which I will subsequently build my specific aims.

As I mentioned earlier, Toxoplasma infection of male rats results in not one but two
240 separate behavioral changes. The first change is reduction in aversion to predator odors. This particular change has been more widely studied in the past. The second change is an increase in sexual advertisement of the infected males. This change has been less studied in comparison[38]. Interestingly both of these changes find parallels in case-controlled human studies. Thus, infected humans demonstrate lower aversion
245 to felid odors[39] and infected male volunteers are also deemed to be more dominant by female volunteers[40]. Are these two behavioral changes brought about by two disjointed proximate mechanisms? Or are these two changes bound by a single proximate mechanism? In the next few paragraphs I argue that a single mechanism brings about both the behavioral changes in the infected male host.

250 During the acute phase of the infection, *Toxoplasma* tachyzoites rapidly divide in peripheral organs. As the infection progresses, the host body mounts an immune response[41, 42]. Subsequent to this immune challenge, *Toxoplasma* migrates to immune-privileged organs in the body and encysts there. These tissue cysts comprise of groups of slowly dividing bradyzoites encased in a cyst wall. Cysts periodically
255 rupture and re-encyst, but otherwise their presence in the invaded tissue remains persistent. The brain represents an important immune-privileged reservoir for the *Toxoplasma*. Blood-brain barrier precludes a free rein to the immune system and forms a refuge for the *Toxoplasma* cysts. Yet the brain is not the only immune-privileged organ in the male body. Testes is likewise secluded from the immune
260 system by a very tight blood testes barrier formed by tight junctions between cells lining the seminiferous tubules. *Toxoplasma* invades rat testes, with cyst burden in testes being comparable to that in brain. Similar invasion of testes is observed in sheep, dogs and rabbits. At least in dogs and rats, *Toxoplasma* makes its way to epididymis and is eventually ejaculated; sexually transmitting to female during the
265 copulation[14, 42]. *Toxoplasma*-infected men have a higher concentration of testosterone when compared to *Toxoplasma*-free controls[43].

Chronic *Toxoplasma* infection in rats enhances testicular steroidogenesis. Thus, infected male rats experience an increase in mRNA of luteinizing hormone receptors. This receptor binds to luteinizing hormone from the anterior pituitary and initiates
270 downstream synthesis of testosterone from its cholesterol precursor[44]. This result in greater testosterone concentration in circulation of the infected males at the baseline; an observation that is also supported by human studies[45]. Work by others in lab show that greater testosterone then enhances the strength of sexual advertisement by the males in form of proteinaceous pheromones[46]. This could, in turn, explain the

275 second behavioral change in the host; *i.e.* increased sexual attractiveness of the infected males.

Importantly, several strands of indirect evidence suggest that testosterone also reduces fear in a variety of animals. For example, male rats treated with exogenous testosterone spend more time in anxiogenic open arms of an elevated plus-maze and
280 spend less time engaged in defensive burying[47]. Similarly, male mice show robust anxiolysis upon exposure to female pheromones or female mice themselves. This anxiolysis is concurrent with reflexive release of testosterone and can be recapitulated by supply of exogenous testosterone in absence of female cues[48]. Within the context of predator aversion, female exposure ‘emboldens’ male mice by reducing subsequent
285 aversion to predators, concomitant with the increase in testosterone[35]. Similarly, deficits in testosterone cause increased freezing and analgesia in rats when exposed to olfactory predator cues[49]. Thus, it can be hypothesized that increase in testosterone after *Toxoplasma* infection should also reduce defensive behaviors. The same biological substrate can lead to both increased sexual advertisement and reduced
290 aversion to predators. In this scheme of proximate mechanism, presence of the parasite in the brain merely becomes incidental. This framework is also attractive because it provides a natural meeting ground for the negotiation of tradeoff between survivorship under predator challenge and reproduction. It should be noted that other proximate mechanisms have also been proposed to underlie the behavioral change in rat-
295 *Toxoplasma* association. I refrain for discussing these here for sake of the brevity. I will return to an integrated analysis of the mechanisms in the discussion section.

300

1.5 Specific Aims

In the preceding sections, I have summarized that *Toxoplasma* infection in rats leads to reduction in predator defense that plausibly increases trophic transmission of the parasite. I have further argued that these behavioral changes are likely linked to flexibility and trade-off between defensive and reproductive behaviors. Given the dual effect of testosterone on fear and sexual advertisement, it is a likely candidate molecule that could mediate conditionality in rodent life history strategies. Prior work show that *Toxoplasma* infection leads to elevated testosterone synthesis in the male rats[44].

In this backdrop specific aims of this thesis are:

A. Necessity and Sufficiency of testosterone for behavioral change

In specific aim 1, I seek to determine necessity and sufficiency of testosterone for reduced predator aversion of *Toxoplasma*-infected male rats. I first ask if testicular testosterone during chronic phase of the infection is required for the host behavioral change. Subsequently, I ask if testosterone acting within a specific brain region can recapitulate behavioral change akin to the infection *sans* *Toxoplasma*. Medial amygdala represents the first site where olfactory information from main and accessory bulbs converges. In view of this convergence, I chose medial amygdala as my target site for chronic infusion of exogenous testosterone during this experiment.

325 **B. Sufficiency of testosterone dependent downstream molecular effectors for host behavioral change**

Having investigated sufficiency of testosterone, I then seek to determine if arginine vasopressin, one of the well-studied downstream effector of the testosterone within the medial amygdala, is sufficient to recapitulate infection-like effects on predator aversion. In this context, it is noteworthy that medial
330 amygdala arginine vasopressin system is believed to be important in titrating reproductive and defensive behaviors.

C. Mechanisms responsible for increase in testosterone synthesis

Prior work shows that Toxoplasma infection results in increased synthesis of testosterone in the infected male rats. I attempt to dissect biochemical
335 mechanisms of increased testosterone synthesis post-infection.

2. Necessity and sufficiency of testosterone for host behavioral change in *Toxoplasma gondii* - *Rattus norvegicus* association.

Association between rats (*Rattus norvegicus*) and their protozoan parasite *Toxoplasma*
340 *gondii* has been widely studied as an exemplar of behavioral manipulation, wherein
parasites manipulate host behavior for their own advantage. Male rats in this
association exhibit reduced fear to cat odor during chronic phase of the infection. This
phenomenon is thought to increase trophic transmission of the parasite from prey to its
definitive felid hosts. Previous studies posit that the rise in testicular testosterone and
345 epigenetic reprogramming within medial amygdala are critical events leading to a
reduction in fear in this model. This narrative then suggests a causal role for peripheral
testosterone acting within medial amygdala, rather than physical presence of the
parasite within the brain itself. This hypothesis has not been tested at present.
Apropos, here we test necessity of testicular testosterone in *Toxoplasma gondii*
350 induced host behavioral change. We demonstrate that alterations in fear and arginine
vasopressin system can be obliterated when host rats are castrated post establishment
of chronic infection. We also demonstrate that exogenous supply of testosterone
within medial amygdala of uninfected castrates recapitulates reduction in innate fear
akin to behavioral change attributed to *Toxoplasma gondii*. These observations argue
355 for a gonadal rather than neuronal initiation of biological changes underlying loss of
fear in the infected rats.

2.1 Introduction

Toxoplasma gondii is a protozoan parasite with a cosmopolitan host range that includes rodents, birds and humans[4, 22, 23]. In rodents, the infection leads to an acute proliferative phase followed by a chronic stage characterized by encystment in immune-privileged organs. Cats serve as definitive hosts for this parasite, allowing gametogenesis, sexual recombination and formation of hardy oocysts. In addition oral-fecal transmission through cats allows subsequent infection of herbivores; an outcome not possible through alternative transmission route of carnivory. Thus *Toxoplasma gondii* might have experienced a selection pressure to increase predation of rodents by cats. In agreement with this suggestion, infected rats reduce their aversion to cat kairomones during chronic phase of the infection in a dose-dependent manner[23, 50, 51].

Several hypotheses have been advanced pertaining to proximate causation of host behavioral change in this model[52]. These include selective invasion of brain regions important for defensive behaviors[41, 53]; or alternatively an epigenetic change in medial amygdala arginine vasopressin (AVP) system secondary to testicular invasion by the parasite and greater synthesis of testosterone[44]. This contrast leads to two unique predictions. Firstly, removal of testes should lead to rescue of host behavioral change if relevant brain changes are secondary to testicular invasion. On the other hand, a targeted testosterone supplementation within medial amygdala should be able to recapitulate behavioral change sans infection. A proximate mechanism dependent on primary invasion of the brain itself will alternatively lead to failure of both rescues by castration and recapitulation by supplementation. In the present report, we experimentally test these predictions. Most importantly, in this study the rats were infected and then castrated while it was the reverse in the previous study.

2.2 Materials and Methods

A. Animals and Infection.

385 Male adult Wistar rats (44–50 days, housed two/cage in NTU vivarium) were procured from 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
390 study. Tachyzoites were maintained in human foreskin fibroblasts. Animals randomly assigned to the infected group were injected with 5×10^6 syringe-lysed tachyzoites (*i.p.*) suspended in 500 μ L buffered saline. Control animals were injected with the saline only. All behavioral experiments started >7 weeks after infection, a time frame consistent with chronic stage of the infection[11, 41, 44].

395 B. Castration

Animals were anaesthetized using a ketamine (90 mg/kg) + xylazine (10 mg/kg) cocktail and maintained using 2-3% isoflurane. Animals were also injected subcutaneously with the analgesic combination of Meloxicam (0.2 mg/kg). A medial incision was made in the scrotum of anaesthetized animal. Testes and vas deferens of
400 each side were sequentially pulled out of the incision. Blood vessels supplying to the testes were sutured. Testes, vas deferens and associated fatty pads were severed just below the point of suture, followed by closure of the scrotum incision.

C. Testosterone supplementation

Stereotactic surgery was performed on castrated males. Cannula was targeted to
405 posterodorsal division of the medial amygdala [AP, 23.2 mm; ML, 63.2 mm; DV, 28.8 mm][54]. Osmotic pumps containing either a testosterone (200 μ L of 25mM in a 3% EtOH solution in ACSF) or vehicular control (3% EtOH solution in ACSF)

were connected to the cannulas. Localized infusion to the medial amygdala was performed for 10 days. On the twelfth day, cat odor avoidance assay was performed.

410 **D. Behavioral testing**

i) Predator odor test

Experiment was conducted in a rectangular arena that had two opposing and identical arms (76X9 cm each) separated by a central hub (9X9X9 cm size). Males were habituated in the maze for three consecutive days for 600 s. Males were tested
415 individually by introduction in the central hub. Each arm contained 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 rat in both arms was quantified over trial duration of 1200 s.

ii) Open-field test

420 The experiment was conducted in a square arena (100cm × 100cm × 30 cm) made of black Plexiglas base with transparent walls. Males were tested for anxiety in an open field test (OFT, customized) for the both the treatments in this study. Males were tested individually by introduction in the center of the arena. Center was defined as the inner two-thirds of the arena. ANY-maze behavioral tracking
425 software was used to calculate the end points.

E. AVP promoter methylation

gDNA extraction was performed by the Trizol method using micro dissected brain tissue. The extracted DNA was used to examine DNA methylation. Methylation of AVP promoter was quantified using methylation sensitive restriction enzyme (MSRE)
430 digestion in combination with qPCR.

435 qPCR reaction[%]
Quantitative PCR reactions were run using an ABI 7500 machine. SYBR Green master mix (AppliedBiosystems, Singapore) was the fluorescence dye used for this reaction. The primers are described in the figures below.

440 All primers were synthesized by AITBIOTECH, Singapore

Primer Name	Primer Sequence 5`-3`
AVP forward [%]	TGCCTGCTACTTCCAGAACTGC
AVP reverse [%]	AGGGGAGACACTGTCTCAGCTC
HPRT forward	GTCATGTCGACCCTCAGTCCCA
HPRT reverse	TCGAGCAAGTCTTTCAGTCCTGT
AVP Promoter 1 forward [%]	GTAGACCGCCACACCTGA
AVP Promoter1 reverse [%]	CCAGACATTGGTGTGTGACC
AVP Promoter 2 forward [%]	GGCCTTTGGCTCTATGTTC
AVP Promoter 2 reverse [%]	TTGAGGGTCACCTGGAAATC

[%]Adapted from [55]

Table 1 List of Primers used for MSRE

445 **F. Statistics**

Normality for all endpoints was examined using Shapiro-Wilk test. Several endpoints exhibited significant departure from the normality. Consequently non-parametric statistics was used for inter-group comparisons (Mann-Whitney U test). Effect size was calculated using Cliff’s delta; a non-parametric statistics [56]. All analyses were
450 performed using GraphPad Prism 6 software. N is depicted in the figures.

2.3 Results

A. Behavioral and molecular effects of *Toxoplasma gondii* were obliterated in male rats castrated six weeks post-infection.

455 *Toxoplasma gondii* infection reduces aversion to cat odors[41]. The infection also creates DNA hypomethylation in promoter region of AVP gene in the MePD[57]. We tested necessity of testicular testosterone for these behavioral and molecular changes, by castrating rats once the infection had reached its chronic stage.

Both control and infected animals exhibited robust aversion to cat urine (Figure 3, 460 occupancy near bobcat urine; Wilcoxon signed rank test against chance = 50%: $p \leq 0.001$). The infected animals demonstrated *greater* avoidance of bobcat urine compared to uninfected controls (Mann-Whitney test: $p = 0.037$). DNA methylation within AVP promoter was quantified using a methylation specific endonuclease treatment and subsequent quantitative PCR. Endonuclease treatment did not produce 465 statistically significant departure from theoretical expectation based on complete methylation (Figure 4; Wilcoxon signed rank test: $p \geq 0.23$). *Toxoplasma gondii* infection did not result in statistically significant change in the methylation status (Mann-Whitney test: $p = 0.739$). Likewise, threshold cycle values (Ct) obtained in presence or absence of the endonuclease did not exhibit significant inter-group 470 differences (Mann-Whitney test: $p \geq 0.7$). Non-parametric estimates revealed a weak effect size of the infection on AVP promoter methylation (Cliff's delta = 0.10)

475

480 **Figure 3 *Toxoplasma gondii* did not reduce aversion to predator odor in male rats castrated six weeks post-infection**

Ordinate depicts percentage time spent in arena bisect containing bobcat urine, relative to trial duration of 1200s. Box plots depict median and inter-quartile range with Tukey whiskers. Mean is depicted with a + sign. Dashed line represents expectation based on chance. *, $p \leq 0.05$; Mann-Whitney test.

485

490

Figure 4 Castration of male host precluded DNA hypomethylation at promoter for arginine vasopressin gene in posterodorsal medial amygdala

Ordinate depicts difference in threshold cycle values (ΔC_t) during quantitative PCR using paired gDNA samples (sans-treatment minus treated with a methylation specific restriction enzyme). Dashed line represents expectation based on complete methylation.

500

B. Chronic exogenous delivery of testosterone in medial amygdala of castrates recapitulated effects of the infection on host behavior.

We next tested sufficiency of testosterone acting within medial amygdala to recapitulate behavioral effects akin to *Toxoplasma gondii* infection. Castrated males
505 were chronically supplied with either placebo or exogenous testosterone within medial amygdala.

Five placebo- and four testosterone-treated animals successfully escaped from the arena before completion of 1200s long trial (Figure 6, escape latency; Wilcoxon signed rank test against total trial duration: $p < 0.01$). Animals treated with
510 testosterone exhibited significantly greater escape latency compared to placebo (Mann-Whitney test: $p = 0.054$), with a robust effect size of -0.73 (Cliff's delta). Testosterone treated animals also conducted more number of sorties to vicinity of the cat odor (Figure 7A; Mann-Whitney test: $p = 0.023$). Likewise testosterone-treated animals spent more time near cat urine (Figure 7B; Mann-Whitney test: $p = 0.016$).
515 Testosterone treatment produced robust effect size of ≥ -0.65 for both number of sorties and time spent near predator odor, with 25th percentile of the testosterone group placed well above the median of placebo-treated animals. Testosterone supplementation did not significantly affect time spent in the anxiogenic center of the open field in an open field arena (Figure 5A; $p = 0.851$). Similarly, inter-group
520 differences for locomotion in open field arena also remained statistically non-significant (Figure 5B; $p = 0.343$).

525 **Figure 5 Testosterone supplementation did not affect exploration in open-field arena.**

Testosterone supplementation did not significantly affect time spent in the anxiogenic center in an open field arena (A) Inter-group differences for locomotion of the open field also remained statistically non-significant (B) $p > 0.05$; Mann-Whitney test.

530 (Sham; n=8 & Testosterone; n=12)

535

Figure 6 Modulation in escape latency induced by exogenous testosterone.

Chronic testosterone supplementation within medial amygdala of castrates increased escape latency from arena containing bobcat urine. *, $p \leq 0.05$; Mann-Whitney test.

540

Figure 7 Chronic testosterone reduced aversion to predator odor

555 Chronic testosterone supplementation within medial amygdala reduced aversion to predator odor as inferred by more sorties to and greater spent time spent near bobcat urine (A and B, respectively). *, $p \leq 0.05$; Mann-Whitney test.

2.4 Discussion

560 Observations reported here demonstrate necessity of intact testes for both host behavioral changes after *Toxoplasma gondii* infection and for epigenetic effects of the infection within medial amygdala. These observations also support sufficiency of the testosterone, acting within medial amygdala, in instituting loss of aversion akin to the behavioral consequences of the infection.

565 *Toxoplasma gondii* invades both brain and testes of the infected male rats[11, 41, 53]. Several studies have focused on localization of the parasite between different brain regions and cell types[58, 59] of the central nervous system. This endeavor is based on a parsimonious assumption that the infection alters behavior through its localized effect within the brain. Despite several studies, preferential localization of the parasite

570 between discrete brain regions remains an equivocal proposition. Some studies have reported mild tropism to amygdala[60] or nucleus accumbens[61] and other have reported a rather random distribution within the brain[53]. These disparate results suggest that the host behavioral change is not dependent on selective invasion of specific brain circuits. Present observations support an alternative narrative where

575 primary mechanism resides outside the nervous system and in male gonads. This suggestion is based on both necessity and sufficiency of testosterone for the observed behavioral effects. Our observations thus support a non-tropic model for the proximate mechanisms. We posit that increased testosterone synthesis after invasion of testes by *Toxoplasma gondii* recruits medial amygdala and its downstream efferents[44, 53]. In

580 agreement with this, selective and chronic infusion of testosterone within the medial amygdala institutes behavioral changes akin to those of the infection. A peripheral origin of the behavioral change in this model is also supported by the observations that

attenuated type 1 *Toxoplasma gondii* strains exhibit the behavioral change in mice without detectable presence of the parasite within brain[51].

585 We have earlier reported that *Toxoplasma gondii* infection in castrated rats does not lead to loss of host aversion to cat odors[44]. Yet those prior observations left the causal links ambivalent. Testosterone is an immune-suppressive steroid[62]. It is thus plausible that castration before the infection altered the potency of immune system and interfered with establishment of the chronic infection. In contrast, the present study
590 employed animals that were gonad-intact during acute phase of the infection. This treatment did not interfere with acute inflammation and establishment of chronic infection *per se*. We further demonstrate recapitulation of the behavior in castrated rats using selective re-supplementation of testosterone within medial amygdala of the brain.

595 In conclusion, our data suggests that behavioral effects of *Toxoplasma* do not require physical presence of the parasite within brain. Behavioral change reflects an interaction between gonads and brain; yet the presence of the parasite inside brain is not necessary for this interaction to manifest. This supports a gonadal, rather than completely brain-dependent, initiation of proximate mechanisms in *Rattus*
600 *norvegicus* – *Toxoplasma gondii* model of parasitic manipulation of host behavior.

3. A putative positive feedback loop between epigenetic changes in the medial amygdala and testicular steroidogenesis.

605 A growing body of evidence suggests that *Toxoplasma gondii* increases testosterone synthesis in the host[44]. Testosterone plays a dual role; firstly it facilitates sexual signals and attractiveness in males of many species. Secondly testosterone can cross the blood brain barrier and abolish the innate fear of cat odor in infected male rats[63]. As a result, the parasite transmission efficiency increases both by sexual and trophic

610 routes. Most testosterone is synthesized in Leydig cells of testes and it is mainly regulated by luteinizing hormone (LH) secreted by the anterior lobe of pituitary gland. Steroidogenic process in these cells remains under homeostatic control through reciprocal communication with the brain. In view of reciprocal effects, it is perhaps more appropriate to view brain and Leydig cells as two nodes of an interacting

615 network. This leads to the investigation carried out in this chapter wherein we study the relationship between testosterone and medial amygdala. Testosterone increases transcription of arginine vasopressin in the posterodorsal medial amygdala through epigenetic means and this formed the base of the hypothesis to test if elevated levels of arginine vasopressin in posterodorsal medial amygdala up regulate testosterone

620 synthesis by itself. We demonstrate that molecular changes in the medial amygdala cause an increase in luteinizing hormone receptor distantly within the testes. This plausibly leads to greater testosterone, in view of a positive influence of the luteinizing hormone receptors on steroidogenesis. In other words, testicular testosterone and medial amygdala epigenetic changes might constitute a positive feed-forward loop

625 capable of self-sustenance once initiated.

3.1 Introduction

As a brief re-cap, protozoan *Toxoplasma gondii* changes behavior of the infected rats by decreasing aversion to predator odors[11, 23, 50]. In the preceding chapter, I
630 demonstrated that testosterone is both necessary and sufficient for the behavioral change. Thus removal of the testes during chronic phase of the infection prevented behavioral effects of the infection. And chronic supplementation of testosterone within medial amygdala of the castrates reinstated loss of aversion to predator odor; a behavioral effect akin to that of the infection.

635 I would like to use those observations as a starting point and expand on the role of the medial amygdala. Olfactory information enters the brain through both the main olfactory bulb and the accessory olfactory bulb[64]. The main olfactory system mainly communicates information about volatile pheromones, while the accessory system conveys information about non-volatile pheromones. Information from both these
640 systems converge on the medial amygdala, which is the first region in the brain with access to both volatile and non-volatile pheromones[64].

Medial amygdala sends downstream projections to the hypothalamus through the medial preoptic nucleus. The efferents form part of the medial hypothalamic zone, important for coupling of incipient olfactory environment with relevant behaviors[65].
645 Thus predator kairomones activate posteroventral medial amygdala (MEApv) and downstream dorsomedial aspect of the ventromedial hypothalamus (VHMdm). Sexual pheromones in contrast activate posterodorsal medial amygdala (MEApd) and downstream ventrolateral aspect of the ventromedial hypothalamus (VMHvl). In summary, defensive and sexual olfactory cues converge in the medial amygdala and
650 then activate the hypothalamus, using parallel circuits[66] (Figure 7).

The hypothalamus is intricately linked with endocrine regulation through hormonal connections to the anterior pituitary and then to adrenals or gonads[67]. Exposure to stressful stimuli, like predator odors, leads to activation of hypothalamus-pituitary-adrenal (HPA) axis. This results in secretion of glucocorticoids that allows the body to
655 initiate a flight-or-fight response. Exposure to reproductive opportunities, on the other hand, initiates activation of hypothalamus-pituitary-gonadal (HPG) axis. This results in secretion of testosterone in male rats (Figure 7).

Thus, two parallel chains can be drawn starting from reproductive and defensive cues to secretion of glucocorticoids and testosterone, respectively. Data presented in the
660 chapter two demonstrates that testosterone in medial amygdala reduces aversion to predator odors. This is congruent with the observation that *Toxoplasma gondii* increases gonadal testosterone in male rats[44]. Testosterone increases transcription of arginine vasopressin in the medial amygdala through epigenetic means[68]. Extra-hypothalamic arginine vasopressin system is involved in sexual behaviors in a variety
665 of species. Thus the hypothesis is that over-expression of AVP by the infection-induced testosterone causes a 'leakage' between parallel circuits processing defensive and reproductive pheromones. This then leads to another possibility. Posterodorsal and posteroventral parts of amygdala are linked to hypothalamus, and eventually to secretion of testosterone and glucocorticoids respectively. Then can a 'leakage'
670 between these two circuits increase testosterone synthesis by itself? Can the greater arginine vasopressin in posterodorsal medial amygdala increase testosterone synthesis by itself? In the present chapter, I test this hypothesis.

Specifically, I determine if blockade of DNA hypomethylation found in the *Toxoplasma gondii* infection rescues effects of the infection on luteinizing hormone
675 receptor in the testes. Luteinizing hormone receptor is an important positive regulator

of the testosterone system and is known to be up-regulated post-infection. Furthermore, I determine if DNA hypomethylation within posterodorsal medial amygdala is sufficient to cause up-regulation of luteinizing hormone receptor in the testes, thus recapitulating effects of the infection.

680

Figure 8 Defensive and Sexual networks interacting with gonads

Predator kairomones activate MEApv and VHMdm. Sexual pheromones in contrast activate MEApd and downstream VMHvl. In brief, defensive and sexual olfactory cues converge in the medial amygdala and then activate hypothalamus; using parallel circuits. Hypothalamus is in turn is linked with endocrine regulation through hormonal connections to the anterior pituitary and then to adrenals or gonads that results in secretion of glucocorticoids or testosterone.

690

3.2 Materials and Methods

A. Animals and Infection.

Male adult Wistar rats (44–50 days, housed two/cage in NTU vivarium) were
695 procured from 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
700 fibroblasts. Animals randomly assigned to the infected group were injected with
 5×10^6 syringe-lysed tachyzoites (*i.p.*) suspended in 500 μ L buffered saline.
Control animals were injected with the saline only. All behavioral experiments
started >7 weeks after infection, a time frame consistent with chronic stage of the
infection[11, 41, 44].

705 B. Methionine treatment

Male Wistar rats (6-8 weeks old) were intraperitoneally injected with 5 million
Toxoplasma tachyzoites, Control animals were injected with saline. Six weeks post
infection; animals were injected with either L – Methionine (Dose 200 mg/kg;
Sigma) or saline solution subcutaneously twice a day for ten days. On the
710 thirteenth day, animals were sacrificed and testes were harvested and flash frozen
in liquid nitrogen.

C. RG-108 treatment

Male Wistar rats (6-8 weeks old) were used. Using osmotic pumps and intracranial
surgery directed at the MEApd, I delivered RG108 (Dose: 1mg/ml, total volume
715 pumped = 200 μ l) for 10 days in brain of uninfected male rats. Cannula
localization was confirmed post-mortem and off target samples were removed

from the analysis. On the eleventh day the behavioral test was conducted and on the thirteenth day animals were sacrificed.

720 **D. Statistics**

Normality for all endpoints was examined using Shapiro-Wilk test. Several endpoints exhibited significant departure from the normality. Consequently non-parametric statistics was used for inter-group comparisons (Mann-Whitney U test). All analyses were performed using GraphPad Prism 6 software. N is depicted in the figures.

725

3.3 Results

A. Effects of *Toxoplasma gondii* on luteinizing hormone receptor abundance were obliterated in male rats treated with methionine.

Host behavioral changes induced by *Toxoplasma gondii* infection are known to be
 730 dependent on epigenetic reprogramming[68]. Specifically, prevention of DNA
 hypomethylation by methionine treatment rescues infection induced behavioral change
 in male rats. In view of these observations, I used systemic administration of
 methionine to determine if rescue of the behavioral change was concomitant with
 rescue of the infection effect on testicular steroidogenesis.

735 For each animal, testicular cDNA was analyzed using quantitative PCR with primers
 for luteinizing hormone receptor, along with GAPDH housekeeping control. Number
 of cycles required to reach a pre-determined product threshold for luteinizing hormone
 receptor was determined, and subtracted with similar value for GAPDH (ΔC_t values in
 Figure 9).

740 Infected animals required smaller number of PCR cycles to reach the threshold
 compared to control animals (Figure 9, ΔC_t for placebo treated animals; Mann
 Whitney test: $p = 0.036$). The effect of the infection was robust with upper range ΔC_t
 values in the infected group being lower than median of control animals. Similarly,
 lower range of controls group was higher than median of the infected animals. Thus,
 745 *Toxoplasma gondii* increased mRNA abundance of luteinizing hormone receptor in the
 testes.

Methionine treatment rescued effects of *Toxoplasma gondii* infection on mRNA
 abundance of luteinizing hormone receptor. Infected animals treated with methionine
 did not significantly differ from control animals with methionine (Figure 9, ΔC_t for
 750 methionine treated animals; Mann Whitney test: $p = 0.63$).

Figure 9 Effects of *Toxoplasma gondii* on luteinizing hormone receptor mRNA in methionine treated male rats

755 *Toxoplasma gondii* infection increased mRNA abundance of Luteinizing hormone receptor (LHR). Ordinate depicts difference in threshold cycle values (ΔC_t) during quantitative PCR using mRNA isolated from testes of animals.

760

765 **B. Administration of RG108 within medial amygdala recapitulated effects of
Toxoplasma gondii infection on luteinizing hormone receptor abundance in testes.**

Methionine in the above experiment was administered systemically through intraperitoneal injection. This was done because specific administration in the brain would have required intra-cranial surgery. Surgical stress often leads to recrudescence
770 of chronic *Toxoplasma* infection, which would have interfered with the infection effects. To circumvent the limitation of systemic treatment, I subsequently compared a centrally infused drug that causes DNA hypomethylation (RG-108) with placebo treated animals. RG-108 was chronically supplied using osmotic pumps specifically directed at the medial amygdala.

775 Animals supplied with hypomethylation agent exhibited significant increase in abundance of the luteinizing hormone receptor in testes (Figure 10; Mann-Whitney: $p = 0.049$). Lower bound of the treated animals was observed to be above the median of placebo treated animals. Likewise, upper bound of control animals was observed to be below median of the infected groups; suggesting a robust effect of the treatment on
780 endpoint.

785

790 **Figure 10 Effects of RG108 on luteinizing hormone receptor abundance**
Treatment with RG108 causes the increase in mRNA abundance of Luteinizing hormone receptor (LHR). Ordinate depicts difference in threshold cycle values (ΔC_t) during quantitative PCR using mRNA isolated from testes of animals.

795

3.4 Discussion

800 It has been previously shown that *Toxoplasma gondii* infection leads to greater
synthesis of testosterone in male rats[44]. The greater steroidogenesis in this case is
believed to arise from an up-regulation of luteinizing hormone receptor. This receptor
is found in Leydig cells of the testes. Upon activation of luteinizing hormone in the
brain, up-regulation of various rate-limiting enzymes involved in the
805 steroidogenesis[69-71].

Data presented in the preceding chapter demonstrates a causal role of testosterone
within medial amygdala for loss of predator aversion in the host post-infection. Data
presented in the current chapter extends that argument by showing that molecular
changes in the medial amygdala, in turn, can cause an increase in luteinizing hormone
810 receptor distantly within the testes. This plausibly leads to greater testosterone, in view
of positive influence of the luteinizing hormone receptors on steroidogenesis. In other
words, testicular testosterone and medial amygdala epigenetic changes might
constitute a positive feed-forward loop capable of self-sustenance once initiated
(Figure 10). One limitation in the interpretation should be noted. we did not conduct
815 infusion in any area extraneous to medial amygdala. This is an important experiment
that should be planned in the future.

820

Figure 11 Positive feedback loop between gonads and medial amygdala

825 Positive-feedback loop wherein testicular testosterone and medial amygdala epigenetic changes promotes up-regulation of AVP in the MEA and the MEA in turn enhances testosterone and also capable of self-sustenance once initiated until a disruptive physiological event terminates this feedback loop.

830 The framework presented above is analogous to challenge the hypothesis proposed to explain the relationship between behavior and testosterone in birds[72]. Testosterone in animals is needed for initiation and maintenance of reproduction. And yet this steroid is costly in terms of metabolic resources and opportunity cost. Hence the testosterone levels are tightly regulated during non-reproductive episodes. A baseline
835 level of testosterone is maintained which is sufficient to exert a negative feedback on its own synthesis by its action on the anterior pituitary. During episodes of reproductive investments, for example during breeding season in seasonal breeders or during male-male conflict over reproductive opportunities, testosterone rapidly rises through concerted collaboration between brain and gonads. The positive feedback
840 circuit posited in Figure 3 represents a mechanism that could underlie such concerted communication.

In conclusion, *Toxoplasma gondii* likely takes advantage of pre-programmed reciprocal communication lines between gonads and brain; resulting in sustained expression of an endocrine and behavioral milieu reminiscent of mating behaviors.

845 **4. Biochemical investigation into nodes of steroidogenesis affected
by the *Toxoplasma gondii* infection.**

Toxoplasma gondii taps on the steroidogenic process in leydig cells to manipulate testosterone synthesis. Therefore, this chapter takes a closer look at steroidogenic
850 metabolism itself. In accordance with the importance of Luteinizing hormone receptor (LHR) shown in the earlier chapter, we investigate the interaction of luteinizing hormone (LH) and LHR in view of infection by *Toxoplasma gondii*. Since LHR is the entry port of signals that regulates testosterone production, further LH-LHR binding initiates a cascade of reactions downstream, which ultimately results in testosterone
855 production. This will lead to identification of actual step(s) of steroidogenesis affected in case of infection. Here we test these downstream molecular events in detail by *in vitro* perturbation to decipher the effect of infection on the steroidogenic pathway. In spite of the fact that my efforts didn't bring about decisive insights, further experiments requiring chronic micro dialysis to measure tone of luteinizing hormone
860 will hopefully elucidate if endocrine regulation from the brain is the locus of the testosterone change.

865

4.1 Introduction

870

The first two chapters take a network approach to study the neural events participating in host behavioral change. To complement this approach, in the third specific aim I sought to take a closer look at the steroidogenic metabolism itself. This approach was designed to dissect the biochemical pathway starting from activation of luteinizing

875 hormone receptor to final synthesis of testosterone itself. This experiment resulted in a null result with no significant inter-group differences. In the present chapter I would like to present my effort in this direction. Although my data did not result in conclusive insights, I believe the experiment itself was well designed and executed.

880

I will first present broad nodes of the steroidogenic pathway in the testes (Figure 12), followed by experimental rationale. In subsequent sections, I will then present results and discuss those.

885

Steroid biosynthesis is a multi-enzymatic process. Steroids like glucocorticoids, mineralocorticoids, progesterone, androgens, and estrogens are produced from common precursor cholesterol[73]. In this process, free cholesterol are transferred to

the inner membrane of mitochondria via the outer membrane. Numerous organs that have the steroidogenesis capacity include the adrenal gland, testis, ovary, brain, placenta, and adipose tissue. Out of which only three endocrine organs (the adrenal gland, testis, and ovary) are specialized in de novo steroid production[74].

890

Leydig cells, present in the interstitial space of testes are the major sites of testosterone production. Physiologically the synthesis of testosterone is regulated by the acute and chronic stimulation by luteinizing hormone (LH) [75]. Luteinizing hormone receptor (LHR) is a critical positive regulator of steroidogenesis [70, 73, 76]. The LHR represents a G-protein coupled receptor (GPCR). When it binds with LH, activation of

Gs protein takes place which in turn stimulates adenylyl cyclase resulting in higher
895 intracellular cAMP level [77].

Furthermore activation of LHR facilitates the transport of cholesterol to inner
mitochondrial membrane with the help of an enzyme called StAR (steroidogenic acute
regulatory protein). This step is considered to be the rate-limiting step in the process of
steroidogenesis. Cholesterol is then converted into the testosterone precursor
900 pregnenolone by the action of an enzyme P450_{scc}. Then pregnenolone moves into the
smooth endoplasmic reticulum where it is converted to progesterone by enzyme 3 β -
hydroxysteroids dehydrogenase (3 β -HSD). Progesterone is in turn converted to 17-
hydroxy progesterone and then to androstenedione by an enzyme 17 α -
hydroxylase/C17-20 lyase (P450_{17 α}). Finally androstenedione is converted to
905 Testosterone by 17 β -hydroxysteroid dehydrogenase (17 β -HSD) [78].

For the purpose of this experiment, I used a sequence of drug treatments to stop the
steroidogenesis at varied stations in this pathway. Accumulated precursors were
quantified. The hope was that systematic analysis of these nodes would illuminate
specific biochemical step influenced by the infection. Specific drug used and
910 endpoints measured are listed in the methods section below.

915

Figure 12 Metabolic steps involved in testosterone biosynthesis in rat testes

Testosterone synthesis is regulated by occupancy of LHR in Leydig cells of testes. Leydig cells produce testosterone using series of modification of cholesterol, each involving distinct enzyme. LH, luteinizing hormone; LHR: luteinizing hormone receptor; cAMP: cyclic AMP; StAR: steroidogenic acute regulatory protein; P450_{scc}: cholesterol side-chain cleavage enzyme; 3b HSD: 3b-hydroxysteroid dehydrogenase; P450_{c17}: C17 hydroxylase/C17, 20 lyase/desmolase; 17b HSD: 17b-hydroxysteroiddehydrogenase). Testosterone often exerts its action after conversion to alternative neuroactive ligands, dihydrotestosterone and oestrogen.

4.2 Materials and Methods

A. Animals and Infection.

Male adult Wistar rats (44–50 days, housed two/cage in NTU vivarium) were procured from 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 5×10^6 syringe-lysed tachyzoites (*i.p.*) suspended in 500 μ L buffered saline. Control animals were injected with the saline only. All behavioral experiments started >7 weeks after infection, a time frame consistent with chronic stage of the infection[11, 41, 44].

B. Leydig cell isolation

24 male Wistar rats were taken (8-10 control and 9 *Toxoplasma gondii* infected). Their testes were removed by surgery and decapsulated under aseptic conditions. The seminiferous tubules were digested in collagenase containing DMEM-F12 (0.25 mg/ml) at 34 °C for 15 min in a water bath. A series of centrifugation was done to finally extract crude testicular interstitial cell suspension.

C. Drug treatment and endpoints

Leydig cells must associate with neighboring cells to produce testosterone. The microenvironment of these cells defines their capacity for producing androgen[79]. Hence, the extracted crude leydig cells were treated with 6 different combinations using hCG, dbcAMP, progesterone, pregnenolone and trilostane.

950 The whole steroidogenic pathway can be divided into **three parts**: first is the production of cAMP (Cyclic Adenosine Monophosphate) by adenylyl cyclase which transmits the signal downstream; secondly movement of cholesterol from cytosol to inner mitochondrial membrane; and, finally conversion of cholesterol to testosterone precursor pregnenolone (Figure 11). Control and Toxoplasma infected leydig cells
955 were treated with the above-mentioned compounds to alter the three key steps pathway. This will lead to identification of actual step(s) of steroidogenesis affected in case of infection. Leydig cells treated with certain increasing concentrations of Human chorionic gonadotropin (hCG) and dibutyryl cAMP (a cAMP agonistic analogue) results in increase testosterone production [80]. Pregnenolone is metabolized to
960 androstenedione, which is an important precursor for testosterone [81]. Progesterone mediates activation of steroidogenic acute regulatory protein (StAR) [82] which is a major precursor of testosterone. Trilostane is an inhibitor of 3 β -hydroxysteroid dehydrogenase (3 β -HSD), which converts pregnenolone to progesterone [83]. The functional activities of these compounds were used to manipulate the steroidogenic
965 pathway (Figure 11). Testosterone and pregnenolone were quantified for these treatments accordingly.

D. Quantification of testosterone and pregnenolone

The testosterone and pregnenolone concentration was estimated by enzyme-linked immunoassay using commercial kit (Enzo Life Sciences, Farmingdale, NY, USA).

970 E. Statistics

Normality for all endpoints was examined using Shapiro-Wilk test. Several endpoints exhibited significant departure from the normality. Consequently non-parametric statistics was used for inter-group comparisons (Mann-Whitney U test). Effect size

was calculated using Cliff’s delta; a non-parametric statistics[56]. All analyses were
975 performed using GraphPad Prism 6 software. N is depicted in the figures

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1020 **4.4 Results**

Table below summarizes central tendency and dispersion measures of control and infected animals under various treatments. None of the inter-group differences were statistically significant.

Treatment	Endpoint	Unit	Group	N	Mean	SD	SeM	p-value	Power*
None	Testosterone	pg/mL	Control	10	358.7	44.2	14.0	0.900	0.052
			Infected	9	355.2	74.1	24.7		
Excess hCG	Testosterone	pg/mL	Control	10	360.8	42.5	13.4	0.550	0.092
			Infected	9	346.8	57.5	19.2		
Excess Pregnenolone	Testosterone	pg/mL	Control	9	1100.4	682.7	230.9	0.940	0.051
			Infected	9	1122.5	537.1	179.0		
Excess trilostane + progesterone	Testosterone	pg/mL	Control	10	11156.3	679.0	214.7	0.360	0.155
			Infected	9	11445.6	668.6	222.9		
Excess trilostane	Pregnenolone	ng/mL	Control	8	8.2	0.9	0.3	0.450	0.115
			Infected	9	8.6	1.3	0.4		
Excess hCG and trilostane	Pregnenolone	ng/mL	Control	8	8.3	0.8	0.3	0.060	0.534
			Infected	9	6.5	2.5	0.8		
Excess trilostane and dbcAMP	Pregnenolone	ng/mL	Control	9	6.5	2.5	0.8	0.005	0.898
			Infected	9	13.0	5.5	1.8		

* Power = 1- β ; where β is probability of type 2 error

Table 2 Central tendency and dispersion measures of control and Toxoplasma infected male rats under various treatments

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Figure 13 Pregnenolone concentration in isolated leydig cells under different treatments

1040 Pregnenolone concentration in isolated leydig cells of control and infected rats under three different treatments namely; “Excess Trilostane”, “Excess Trilostane + hCG” and “Excess Trilostane + dbcAMP”. None of the inter-group differences were statistically significant indicated by Mann-Whitney U test. hCG - human Chorionic Gonadotropin and dbCAMP - dibutyryl cAMP. Leydig cells treated with certain

1045 increasing concentrations of Human chorionic gonadotropin (hCG) and dibutyryl cAMP (a cAMP agonistic analogue) results in increase testosterone production [76]. Trilostane is an inhibitor of 3b-hydroxysteroid dehydrogenase (3b-HSD), which converts pregnenolone to progesterone [79].

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Figure 14 Testosterone concentration in isolated leydig cells under different treatments

Testosterone concentration in isolated leydig cells of control and infected rats under two different treatments namely; “Excess Pregnenolone” and “Excess Trilostane + Progesterone”. None of the inter-group differences were statistically significant indicated by Mann-Whitney U test. Pregnenolone and Progesterone are important precursors for testosterone [81]. Trilostane is an inhibitor of 3b-hydroxysteroid dehydrogenase (3b-HSD), which converts pregnenolone to progesterone [79].

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Figure 15 Testosterone concentration in isolated leydig cells under "excess hCG" treatment

1075 Testosterone concentration in isolated leydig cells of control and infected rats under “no treatment” and under “Excess hCG” treatment. None of the inter-group differences were statistically significant indicated by Mann-Whitney U test. Leydig cells treated with certain increasing concentrations of Human chorionic gonadotropin (hCG) results in increase testosterone production [76].

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4.5 Discussion

Previous reports have shown that *Toxoplasma gondii* infection leads to increase steroidogenesis in the male rats[44]. These studies were primarily based on
1085 measurement of mRNA abundance using quantitative PCR. In the present experiment, I sought to extend these studies in the metabolic domain.

Figures and tables presented in the preceding pages demonstrate that only one of the inter-group differences reached statistically significant levels. In other words, my observations did not have low probability levels to type 1 errors (“probability of false
1090 positive”). Thus it was not possible to confidently conclude that *Toxoplasma* infection altered any of the measure endpoints in testicular steroidogenesis. The p values observed during t-test ranged from 0.005 to 0.94. A power analysis was then conducted to measure the probability of type 2 errors (“probability of false negative”). As indicated in the table 2, none of the inter-group differences demonstrated less than
1095 5% probability of type 2 error (power = 1- β ; where β is probability of type 2 error). Thus I deemed this dataset to be inconclusive.

I believe these experiments were well conceived. Similar design has been used to study steroidogenesis in context of ageing. In several species including rat and human, it has been reported that there is a decrease in serum testosterone concentration with
1100 ageing [84-88]. Enumerating the Leydig cells in the testes of young and old rats shows that loss of Leydig cells does not provide significant explanation to the age related reductions in the serum testosterone level [89-91]. The reduced production of testosterone with aging could be attributed to reduced LHR number, cAMP production and/or PKA activity [92, 93]. Cholesterol transport mechanisms are also reported to be
1105 compromised in aged Leydig cells mostly because of the reductions in the cholesterol transport proteins StAR [94, 95] and in translocator protein (TSPO, also known as

peripheral benzodiazepine receptor, PBR) [96]. The reduced activity of steroidogenic enzymes such as P450_{scc}, 3 β -HSD, P450_{c17}, and 17 β -hydroxysteroid dehydrogenase (17 β -HSD) has also been reported in the aged Leydig cells[94]. Any of the above
1110 stated defects can lead to reduction in production of testosterone in response to LH in aged Leydig cells. Varied studies depicted in this paragraph use the similar experimental design undertaken during my experiments.

Lack of infection effects on enzymatic activity of steroidogenesis pathway is difficult to reconcile with greater testosterone observed after *Toxoplasma* infection. Two
1115 possibilities remain for the reconciliation. The first possibility is that greater testosterone reflects a greater tone of the hypothalamic-pituitary axis, which then results in greater output by steroidogenic enzymes. In other words the increase in testosterone is mediated by central control from the brain rather than peripheral potentiation. Unfortunately our efforts to measure circulating luteinizing hormones did
1120 not succeed. This is not surprising in view of pulsatile nature of pituitary activity. Second possibility is that of paracrine regulation from testicular cells other than Leydig cells. *Toxoplasma gondii* is present within seminiferous tubules of the testes while Leydig cells are present outside the tubule. It can be postulated that Sertoli cells or immune cells resident in the testes are required for the effects of the infection to
1125 manifest. Since I used purified Leydig cells, it is plausible that such paracrine signaling was lost to the experimental methods. Both of these possibilities will explain lack of experimental effects because experiments described here measure maximal and not steady-state rates on enzymatic conversion. Steady-state enzymatic conversions are more difficult to measure in *in vivo* setting.

1130 Future experiments requiring chronic microdialysis to measure tone of luteinizing hormone will hopefully elucidate if endocrine regulation from the brain is locus of the

testosterone change. Likewise, co-culture studies of Sertoli cells and Leydig cells derived from control, and infected animals are required to elucidate possibility of paracrine signaling.

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5. Discussion

In the preceding chapters, I have demonstrated that testosterone is necessary and sufficient for host behavioral change in rat-Toxoplasma system. I have also described experiments that support the existence of a positive feedback loop between epigenetic changes in the medial amygdala and testicular steroidogenesis. Finally I have enumerated some negative evidence for effects of the infection on maximal activity of steroidogenic enzymes.

In this chapter, I will synthesize these observations and place them in wider context of existing knowledge. I will first start with integration of my observations in current narratives of proximate mechanism. I will then place putative interaction between brain and gonads in a wider context of neuroendocrine regulation of behavior. I will then move to the relationship between proximate and ultimate causation of the behavioral manipulation. Subsequently, I will transition to multi-dimensionality of proximate causation in the rat-Toxoplasma model. Finally, I will enumerate some exciting possibilities for future work.

5.1 Mechanistic narratives

Parasites, by virtue of their lifestyle, are prisoners in the body of their hosts. How does
1155 a parasite move from one host to the other? Or how does offspring of a parasite born
inside a host manage to break free? These questions are classical mainstay of the
parasitology research. One frequent route for jumping hosts by parasites involves
trophic transmission, i.e. using natural predation of intermediate host as a vehicle to
complete parasitic life cycle. The main limitation in this otherwise pretty strategy is
1160 that prey typically detest being eaten. Several parasites are hypothesized to get around
this bottleneck by manipulating host behavior and making the infected host more
likely to be eaten. Surprisingly we do not know the biological mechanisms by which
parasites exert such behavioral manipulation (see [97] for sole exception in my
knowledge).

1165 Why is this gap important? Who cares? Phenotypic changes in a host are not always
reflections of adaptation by the parasite. In other words, mere observation of
behavioral change does not tell us much about historical selection pressures. On the
other hand, knowing nuts and bolts of behavioral change can impart more confidence.
If we can demonstrate a biological toolkit with cause-and-effect relationship with
1170 behavioral manipulation, it then argues for an organizing principal generated by
historical selection. If a gene of *Toxoplasma* is needed for behavioral change and if the
gene is conserved in *Toxoplasma* but not in other parasites; it argues for a selection
pressure. So knowing blood-and-sweat biology is important.

1175 The last decade has seen intense interest in the proximate mechanisms of parasitic
behavioral manipulation. A non-exhaustive inventory of host-parasite associations in
this regard includes rat-Toxoplasma, cockroaches stung by emerald wasps, ants
infected with Ophiocordyceps fungi and grasshoppers infected with hairworms. Out of
these models of varied phylogenies, rat-Toxoplasma model has been most widely
1180 studied. The reason this preference is experimental tractability of the system. Rats have
been used as experimental species of choice in neurobiological research for a long time.
There is a rich of work that describes neural event in rat brain mediating innate
aversion to predator odors. Similarly, Toxoplasma has been widely used as model
system for Apicomplexan parasites, in preference to other parasites in this group that
1185 are more difficult to culture and manipulate. The convergence of existing knowledge
had led to several labs successfully bringing together parasitology and neurobiology
and investigating mechanisms of rat behavioral change after Toxoplasma infection.
Focus on the rat-Toxoplasma model has led to three main narratives about proximate
mechanisms. These are three different opinions about the primary event that initiates
1190 host behavioral change.

First of three main hypotheses about mechanisms of behavioral manipulation in
Toxoplasma infected rodents suggests that the parasites invade brain regions important
for host defensive behaviors [41, 98, 99]. This view posits that Toxoplasma has
tropism for specific brain regions; and this tropism then results in modification of
1195 neuronal circuits in the affected brain region. Several studies have tried to enumerate
density of parasites in varied parts of the brain. Some of these studies have suggested
tropism and others have failed to find any conclusive evidence[53]. Either acceptance
or rejection of this hypothesis requires more careful experiments. These tropic
hypotheses also face a curious intellectual problem. As I mentioned earlier,

1200 Toxoplasma cause not one but two distinct behavioral changes: loss of aversion to
predator odors and gain of sexual investment. While loss of aversion can
hypothetically be explained by selective invasion of specific brain region, it remains
unclear how a direct and local change within brain can change reproductive
investment. Moreover, none of the lesion studies conducted during neurobiological
1205 experiments has recreated effects akin to Toxoplasma infection.

Increase in brain dopamine is the second of three main hypotheses about mechanisms
of behavioral manipulation in Toxoplasma infected rodents. Toxoplasma genome
contains two genes with remarkable similarity to mammalian hydroxylase, a rate-
limiting enzyme for synthesis of neurotransmitter dopamine. Dopaminergic neurons in
1210 the brain mediate approach to risk and reward. The hypothesis then posits that
Toxoplasma potentiates approach to risk by increasing dopamine by virtue of
supplying tyrosine hydroxylase. One attraction of this hypothesis is that it does not
require selective tropism. Even if Toxoplasma was found to be randomly distributed
within brain, complementary enzymes needed for dopamine synthesis are only found
1215 within regions with endogenous dopamine production. Thus regional specificity can
be created by an overlap between general parasitic distribution in the brain and
specific presence of dopaminergic enzymes. Yet, it has been very difficult to replicate
increased dopamine in the infected animals, with some studies even showing a
reduction. Moreover, a transgenic Toxoplasma lacking one of the tyrosine hydroxylase
1220 genes does not change dopaminergic tone of the brain. It is thus uncertain if parasite
tyrosine hydroxylases are even expressed or if they have functional role in the host
brain. And at least one study has reported presence of behavioral change in the host
using attenuated Toxoplasma strains that do not cross blood-brain barrier. This
suggests that presence of the parasite within brain is not required at all.

1225 Evidence presented in my thesis supports the third mechanistic model, which is
agnostic to physical presence of the parasite within host brain. This hypothesis
emphasizes role of extra-hypothalamic arginine vasopressin (AVP) containing neurons
within the medial amygdala. In short, these neurons are recruited during perception of
sexual pheromones in infection-naïve rodents. The arginine vasopressin posits that
1230 Toxoplasma – induced up regulation of arginine vasopressin create “leaky” neuronal
activation where sexual neurons are atypically recruited by cat odors post-infection.
This, in turn, creates attraction to cat odor. This thesis posits that testosterone plays a
critical role in this scheme of things. Toxoplasma infection causes more testosterone.
Greater testosterone (or its estrogen metabolites) then causes epigenetic modification
1235 in the promoter for arginine vasopressin within medial amygdala. This results in
greater recruitment of medial amygdala arginine vasopressin system. And finally that
leads to loss of aversion[68]. This sequence is also congruent with the idea that same
set of proximate mechanism causes both host behavioral change. Testosterone can also
cause increase in sexual pheromones, as demonstrated by parallel work conducted by
1240 my colleagues[44]. Additionally this model reconciles the observation that attenuated
strains of Toxoplasma do not cause encystment in brain but do cause loss of predator
aversion.

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5.2 Feedback between testes and brain

In the preceding pages, I have compared three mechanistic models in context of my experimental observations. I posit that physical presence of the parasite in the host
1250 brain is not required. Yet events within the brain form the causal substrate for the proximate mechanisms. Specifically I posit that causal mechanism rests with communication lines between medial amygdala and testes.

Communication between brain and testosterone has been more widely studied in birds due to historical reasons. This body of research has often used ‘challenge hypothesis’
1255 as framework for interpretation. This hypothesis starts with a parsimonious assumption that testosterone is an expensive steroid to produce and maintain. This is supported by both, metabolic and opportunity costs, associated with testosterone. Testosterone is immune-suppressive. Moreover, testosterone enhances sexual advertisement, which then conflicts with other behaviors related to sustenance and
1260 defense. This should lead to a homeostatic mechanism that ensures that testosterone is only produced during episodes when it is needed for reproduction. In keeping with this, testosterone is kept to a minimum baseline during non-reproductive season devoid of sexual opportunities (Figure 16). This level is barely sufficient to engage negative feedback regulation at the level of hypothalamus where occupied androgen
1265 receptors cause reduction in secretion of gonadotropin releasing hormone; thereby shutting down downstream testosterone production. This results in a low and pulsatile availability of testosterone. During breeding season or when reproductive opportunities are present, brain circuits underlying appetitive sexual behaviors are activated. This leads to rapid rise in downstream testosterone to a chronically elevated
1270 tone of the testosterone production at a medium level; which is nonetheless much higher than the baseline. Acute episodes of reproductive pursuit or behavior might

cause transient and facultative rise in testosterone to its physiological maxima. But for most of the time testosterone remains at the medium tone and then goes back to the baseline during sustained absence of reproductive opportunities.

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Figure 16 Homeostatic mechanism of testosterone

Homeostatic mechanism of testosterone ensures that testosterone is only produced during episodes when it is needed for reproduction. Testosterone is kept to a minimum baseline during non-reproductive season devoid of sexual opportunities and elevates when encountered with reproductive opportunities and then during sustained absence

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of sexual cues, it goes back to baseline [72]

This model centers on a communication between brain and gonads to establish tone of testosterone levels. Brain processes information about reproductive opportunities in the environment. Testes then make testosterone that enables sexual behaviors.

1290 Together they generate a conditional program: 'if opportunities are available then make testosterone else desist'. This program leads to rapid rise in testosterone during breeding episodes.

My data suggest that *Toxoplasma* manipulates this pre-existing communication between brain and gonads. More testosterone creates more arginine vasopressin in the

1295 medial amygdala through epigenetic changes. Medial amygdala is involved in processing of sexual pheromones in the environment. Epigenetic changes in medial amygdala thus lead to a positive feedback on gonads, which increase testosterone. That sustains epigenetic change, and so on. This leads to more sexual investment and low fear in hosts; both behavior that are characters of a male who is inclined towards

1300 current reproduction. In support of this perspective, blockade of DNA hypomethylation rescues both aversion to predator odor and abundance of luteinizing hormone receptor in testes. And exogenous establishment of DNA hypomethylation within medial amygdala recapitulates both loss of aversion to predators and greater luteinizing hormone receptor; akin to effects of *Toxoplasma* infection.

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5.3 Relation between proximate and ultimate causation

In his book 'Life and Habit', Samuel Butler rhetorically asked "why it should be said that the hen lays the egg, and not that the egg lays the hen" [16]. This thought carries
 1310 the seed of a gene-centric view of the evolution where individuals bearing the genes are merely considered useful vehicles [100]. Natural selection in this view works at the level of genes (aka selfish gene viewpoint), as opposed to natural selection optimizing individuals or groups of individuals. This gene centric view of natural selection can be difficult to experimentally differentiate from individual-centric view because
 1315 individuals usually are carriers of the genes. In other words, fortunes of egg and fortune of hen are tied together so much that it is experimentally difficult to tease out if natural selection optimized the genes or the individuals.

An elegant way out of this conundrum is offered by behavioral manipulation. This refers to a hypothesis that parasites can change behavior of their host, in a way that
 1320 benefits them but not the host. In other words, genes of a parasite can produce a phenotype in the host. The genes of the parasites have "extended" beyond physical confines on individual parasites body [101, 102]. Prior research has unearthed a multitude of examples of such behavioral manipulations (e.g. commentary by [103-107]). Some fishes infected with parasitic helminthes move to surface of the water; to
 1325 be predated by avian predators that happen to be next host in parasites' life cycle. Grasshoppers infected with hairworm commit suicide by jumping in water so that hairworms can reach an aquatic environment. And rodents infected with *Toxoplasma gondii* develop an atypical attraction to cats, so that the parasite can enter cat intestines where it then undergoes gametogenesis [41, 51, 108].

1330 While a group of scientists, including my team, have studied host behavioral changes with gusto [41, 51, 108, 109]; other group of scientist has expressed reservations about

the inferences [110, 111]. The reservations against extended phenotypes of the parasites emphasizes that it is very difficult to discern historical natural selection through the prism of extant animal behavior. Does an infected grasshopper jump into
1335 water because of selection pressure on hairworm to change specific host behavior? Or is it that an infected grasshopper is an ailing grasshopper whose brain becomes incapable of differentiating safety from danger? Does an infected fish come to the surface of water so those parasites can hitch-hike a ride to avian predators? Or is it because infection increases oxygen demand, necessitating move to more oxygenated
1340 surface water? The only plausible way out of this confusion, short of experimental evolution, is to focus on proximate mechanisms of the behavioral change.

Data presented in this thesis support a model that is built around pre-existing brain-gonad communication in the host species. Ample support exists to buttress the claim that testosterone and extra-hypothalamic parts of the brain do communicate with each
1345 other. What I propose is that the parasite uses this pre-existing scaffold to change host behavior. A specific mechanism (testosterone) aligned to specific behavioral change (loss of fear) argues for organizing principal suggesting historical selection for behavioral manipulation, rather than accidental side-effect [112]. In other words, my data support for behavioral manipulation hypothesis, by demonstrating conformity
1350 between the observed behavioral change and 'a priori design specification that an engineer might use'.

5.4 Future possibilities

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I see at least two lines of future investigations related to research presented here. These represent research directions that I would have taken if I were doing my PhD all over again.

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Toxoplasma gondii exhibits a decided tropism to brain and eyes[113]. These organs are immune-privileged, in the sense that immune cells have limited access to these sites. The parasite then forms bradyzoites-containing tissue cysts that undergo periodic cycles of rupture and encystment (i.e. recrudescence). Interestingly, brain and eyes are not the only immune-privileged sites in the mammalian body. Male testes segregate developing sperms behind a tight barrier that largely excludes entry of immune cells

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[114]. Apropos, presence of *Toxoplasma gondii* beyond blood-testes barrier has been demonstrated in a variety of non-human animals including rats [115], sheep [116], goat [117], dogs [118] and rabbits [119]. This would involve crossing blood-testes barrier, which is one the most impermeable blood-tissue barrier in the mammalian body [114, 120]. Biological mechanisms of such invasion are presently unclear. I

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suggest that study of *Toxoplasma gondii* effects on blood-testes barrier will provide us with a perturbation model to study the physiology of the barrier itself; much as a molecular biologist will use a genetic knock-out to study function of a gene. If we can understand how *Toxoplasma gondii* manipulates permeability of blood-testes barrier, we will also understand what biological factors guide its organization in health and in

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disease.

Secondly, presence of *Toxoplasma* in human ejaculates has not been carefully tested. An early study in 1971 reported the case of three human individuals with *Toxoplasma* in semen; of which two also tested serologically positive [121]. A later study reported post-mortem testicular presence of the parasite in 6 out 10 AIDS patients examined

1380 [122]. Presence of Toxoplasma after AIDS typically reflects reactivation of pre-existing
tissue cysts [123]. Post-mortem presence indicates, but does not prove, that the
parasite was resident in the testes before immune-compromise. Furthermore, both rats
and humans exhibit congruent increase in testosterone, a product of testicular
steroidogenesis, after Toxoplasma infection [43, 124]. In view of these reports,
1385 definitive studies are required to determine if Toxoplasma is a sexually transmitted
infection in the humans. Such sexual transmission is very important from clinical
perspective because a) possibility of vertical transmission; b) sexual intercourse
involving immune-compromised women; and, c) increased odds of developing
Schizophrenia in immune-competent population.

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5.5 Conclusions

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In summary, data presented in this thesis provide evidence that the behavioral manipulation induced by *Toxoplasma* infection pivots around the communication between brain and gonads. This interaction between testosterone and medial amygdala, in turn, provides a strong affirmation in support of the “arginine vasopressin” hypothesis mentioned in preceding sections. The experimental evidences in prior chapters have also demonstrated that testosterone plays a cardinal role in controlling the proximate mechanisms that induces host behavioral change in rat-*Toxoplasma* system.

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On the whole, elevated testosterone levels due to *Toxoplasma* infection induces epigenetic changes in medial amygdala that creates more AVP causing positive feedback loop that sustains the epigenetic changes. I hypothesize that this loop is critical the behavioral manipulation that is seen after *Toxoplasma* infection. Insights into the mechanisms used by *Toxoplasma* to cross blood-testes barrier and manipulate testosterone will require future studies of a very extensive nature. This will open up avenues to understand the biological factors that control its framework in disease.

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6. Annexe

Annexe 1: Abbreviations

AVP	Arginine Vasopressin
BLA	Basolateral Amygdala
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CRH	Corticotrophin-Releasing Hormone
Ct	Cycle Threshold
dbcAMP	dibutyryl cAMP
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
gDNA	genomic DNA
GFP	Green fluorescence protein
hCG	human Chorionic Gonadotropin
HPA	Hypothalamus-Pituitary-Adrenal
HPG	Hypothalamus-Pituitary-Gonad
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
LH	Luteinizing Hormone

LHRH	Luteinizing-hormone- Releasing Hormone
MEA	Medial Amygdala
MEAp	Medial Amgdalar Nuclei, Posterio
MEApd	Medial Amygdalar Nuclei, Posterodorsal
MEApv	Medial Amygdalar Nuclei, Posteroventral
mRNA	Messenger Ribonucleic Acid
MSRE	Methylation Specific Restriction Enzyme
OB	Olfactory Bulb
PBS	Phosphate Buffered Saline
PFA	Paraformaldehye
PVN	Paraventricular Nucleus
qPCR	Quantitative Polymerase Chain Reaction
SEM	Standard error of mean
TH	Tyrosine Hydroxylase
Toxoplasma	<i>Toxoplasma gondii</i>
VHMdm	Ventromedial Hypothalamic Nuclei, Dorsomedial
VMH	Ventromedial Hypothalamic Nuclei
VMHvl	Ventromedial Hypothalamic Nuclei, ventrolateral
VNO	Vomeronasal Organ

Annexe 2: Materials and Methods

1. Animal Work

A. Animals

Adult Wistar rats (44-50 days procured from NUS animal facility) were used. They were housed in groups of two at NTU vivarium. Males and females were housed in same room. Animals were provided with food and water ad libitum and maintained on a 12:12 hour light-dark cycle (lights on at 7 am). All procedures were approved by the NTU institutional animal care committee (APLAAC). Animals were allowed to acclimatize to the animal facility for a minimum of two days before being used for any experiments. This study had 2 groups in each experiment namely, Control and infected group assigned randomly. Number of animals used in each experiment is mentioned as “n” in each figure.

B. Toxoplasma Parasite and Infection

Prugniaud strain of *Toxoplasma gondii* containing GFP and Luciferase inserts was used. The parasites were cultured in human foreskin fibroblast monolayers. Infected fibroblasts were syringe-lysed using 18 gauge (twice) and 25 gauge needles. Male rats were injected intraperitoneally with 5 million parasites suspended in 0.5 ml of PBS. Control males were sham injected with the same volume of sterile PBS. Infected rats were weighed once a week for 4 weeks and checked for any outward symptoms of illness. Behavioral experiments were conducted around 7th week post infection.

C. Confirmation of infection Status

Infection was confirmed by serological detection of anti-Toxoplasma IgG antibodies. Toxoplasma was cultured inside human foreskin fibroblasts in 24-well

plates. 24 hours post infection; the wells were aspirated, washed with PBS and fixed with 4% PFA. They were incubated with 1ml of the serum (1:1000) overnight at 4 degree C. If the animal had been infected with Toxoplasma the serum would contain anti Toxoplasma antibodies, which would bind to Toxoplasma in culture. The bound antibodies were visualized using anti Rat IgG-Cy3 (1:200, Millipore; red fluorescence). The infection paradigm used in this thesis achieved chronic infection in more than 95% of cases.

D. Methionine Treatment

Male Wistar rats (6-8 weeks old) were injected intraperitoneally with 5 million Toxoplasma tachyzoites. Control animals were injected with saline. Six weeks post infection animals were injected with either L – Methionine (Dose 200 mg/kg; Sigma) or saline solution subcutaneously twice a day for ten days. On the eleventh day animals were exposed to cat odor to ascertain fear levels (part of another experiment). On the thirteenth day animals were sacrificed and testes harvested and flash frozen in liquid nitrogen.

Figure 17 Experimental time-line for methionine treated animals

E. RG108 Treatment

Male Wistar rats (6-8 weeks old) were used. Animals were anaesthetized using a ketamine (90 mg/kg) + xylazine (10 mg/kg) cocktail and maintained using 2-3% isoflurane. Additionally animals were injected subcutaneously with the analgesic combination of Meloxicam (0.2 mg/kg) and Lidocaine (1%). Stereotactic surgery was performed and cannulas (Plastic one) were implanted bilaterally into the MEApd (AP = -3.0, L = +/- 3.8, V = -7.0) [162]. MEApd (See Figure below) was chosen as the target region due to its central role in mediating sexual behavior. To artificially create hypomethylation a DNMT inhibitor (RG108, Sigma; Concentration 1 mg/ml, total

volume pumped; 200 μ l) was infused into the MEApd using osmotic pumps (Alzet, model 2002). The solution was pumped for 10 days to provide sufficient time for epigenetic changes to occur. Control animals were infused with the vehicle (6% DMSO in ACSF*). On the eleventh day animals were exposed to cat odor to ascertain fear levels. On the thirteenth day animals were sacrificed.

Figure 18 Experimental time-line for RG108 treated animals

* Preparation of Artificial Cerebrospinal Fluid (ACSF)

Based on recommendation on Alzet webpage
(www.alzet.com)

Solution A		Solution B	
NaCl	8.66 grams	NaH ₂ PO ₄ ·7H ₂ O	0.214 gram
KCl	0.224 gram	NaH ₂ PO ₄ ·H ₂ O	0.027 gram
CaCl ₂ ·2H ₂ O	0.206 gram	Dissolve in 500 ml sterile water	
MgCl ₂ ·6H ₂ O	0.163 gram		

Dissolve in 500 ml sterile water

Mix Solutions A and B in 1:1 ratio. Store at 4°C.

F. Castration

Male Wistar rats (6-8 weeks old) were used. Animals were anaesthetized using a ketamine (90 mg/kg) + xylazine (10 mg/kg) cocktail and maintained using 2-3% isoflurane. Additionally animals were injected subcutaneously with the analgesic combination of Meloxicam (0.2 mg/kg). A medial incision was made in the scrotum of anaesthetized animal. Testes and vas deferens of each side were sequentially pulled out of the incision. Blood vessels supplying the testes were sutured. Testes, vas deferens and associated fatty pads were severed just below the point of suture, followed by closure of the scrotum incision.

G. Testosterone infusion into MEA

Male Wistar rats (6-8 weeks old) were used. Animals were anaesthetized using a ketamine (90 mg/kg) + xylazine (10 mg/kg) cocktail and maintained using 2-3% isoflurane. Additionally animals were injected subcutaneously with the analgesic combination of Meloxicam (0.2 mg/kg and Lidocaine (1%). Stereotactic surgery was performed and cannula (Plastic one) was implanted bilaterally into the MEApd (AP = -3.0, L = +/- 3.8, V = -7.0) [208]. Testosterone (Sigma, Concentration 25mM, total

volume 200 μ L) was infused into the MEApd using osmotic pumps (Alzet, model 2002). Testosterone was dissolved in ethanol first and ACSF added to make the solution a 3% ethanol by volume. The solution was pumped for 10 days to provide sufficient time for epigenetic changes to occur. Control animals were infused with the vehicle (3% EtOH in ACSF). Procedure to make ACSF is been described above. On the eleventh day animals were exposed to cat odor to ascertain fear levels. On the thirteenth day animals were sacrificed.

2. Behavioral Assay

A. Cat Odor Avoidance Assay

The experiment was conducted in a rectangular arena that had two opposing and identical arms (76 X 9 cm each) separated by a central hub (9 X 9 cm size). Males were habituated in the maze for three consecutive days for 600s. Males were tested individually by introduction in the central hub. Each arm had a towel containing either 2 ml of cat or rabbit urine (urine source Rabbit: Biopolis vivarium, Singapore, cat: Predator pee, USA). Urine was topped up every 2 hours. Occupancy of the male rat in both arms was quantified over trial duration of 1200s. The cat and rabbit urine were placed at the two extreme corners of a long straight arm to avoid cross-contamination of smells.

Figure 19 Cat-odor avoidance assay arena

B. Open-field test

The experiment was conducted in a square arena (100cm × 100cm × 30 cm) made of black Plexiglas base with transparent walls. Males were tested for anxiety in an open field test (OFT, customized) 72 hours before exposing the subjects to predator odor test. Males were tested individually by introduction in the center of the arena. Exploration in the arena was recorded for 5 minutes. Center was defined as the inner two-thirds of the arena.

ANY-maze behavioral tracking software was used to calculate the end points. The animals were coded before analysis.

3. Isolation of Leydig cells

Testes were decapsulated under aseptic conditions and Leydig cells were isolated by enzymatic digestion and purified on discontinuous Percoll gradient by the method described by [125]. In brief, testes were decapsulated with fine forceps without breaking the seminiferous tubules and digested in collagenase containing DMEM-F12 (0.25 mg/ml) at 34 °C for 15 min in a thermostated shaking water bath. After this incubation, the tubes were added to DMEM-F12 without collagenase and allowed to stand for 10 min. The supernatant was then aspirated using a Pasteur pipette and

transferred to sterile centrifuge tubes. This procedure was repeated once again to remove additional Leydig cells. The supernatants were combined and centrifuged at $2500 \times g$ for 10 min at 4 °C. After discarding the supernatant, the pellet obtained was re-suspended in 1 ml of DMEM-F12 representing a crude testicular interstitial cell suspension. The reason for choosing crude cell for further treatment is, this interstitial cell preparation includes those cell types that have been seen to secrete paracrine factors *in vivo*. This influences the differentiation of stem leydig cells. These paracrine factors include various growth factors, sertoli cells, stem cell factor, etc., [126].

4. Drug treatment and endpoints

The extracted crude leydig cells were treated with 6 different combinations using hCG (100ng/ μ l), dbcAMP (50 μ M), progesterone (25 μ M), pregnenolone (25 μ M) and trilostane (10 μ M). The treatments are given below:

- i. Excess hCG
- ii. Excess Pregnenolone
- iii. Excess Trilostane + Progesterone
- iv. Excess Trilostane
- v. Excess hCG + Trilostane
- vi. Excess Trilostane +dbcAMP

The above treatments were given to the control and Toxoplasma infected leydig cells. These treatments were used to manipulate different key steps of the steroidogenic pathway to identify the actual step(s) of steroidogenesis affected in case of infection. The amount of testosterone and pregnenolone were quantified accordingly (Figures 12-14).

5. Testosterone and Pregnenolone Quantification

One testes of each animal was homogenized and suspended in diethyl ether (twice the volume of homogenized testes sample). The mixture was shaken and layers were allowed to separate. Top layer containing the solvent was pipetted out in a clean test tube. The procedure was repeated to total of three times and solvent layers were combined. Diethyl ether was then evaporated to dryness. Extracted testosterone was dissolved in one ml of aqueous buffer. Testosterone concentration was estimated by enzyme-linked immunoassay using commercial kit (Enzo Life Sciences, Farmingdale, NY, USA).

6. Microdissection of brain regions

Prior to microdissection all apparatus used were either rinsed in DEPC water (Sigma, USA) and autoclaved or wiped with RNAzap (Sigma, USA). Samples were sectioned at 100 μm in a cryostat at -21°C and mounted onto autoclaved Superfrost glass slides (FischerScientific, USA). The MEApd 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. The reaction composition and run method are described in the figures below.

Figure 20 Region microdissected (adapted from [54])

7. mRNA extraction and cDNA synthesis

Preparation of lysis buffer (components from Sigma USA)

- i. Tris 1M (10X stock) Set pH to 8.0 after autoclaving (working solution 100mM)
- ii. EDTA 0.5M (25X stock) (working solution 20mM)
- iii. Sodium deoxycholate 10% (10X stock) (working solution 1%)
- iv. SDS 10% (10X stock) (working solution 1%)

Note: all solutions were prepared using autoclaved DEPC water.

RNA extraction was performed by the Trizol method. Briefly, tissue was microdissected and, placed in lysis buffer and homogenized in ice cold Trizol (~ double the volume of extraction buffer). Ribolock was added to prevent RNA from

degrading. Choloform (~1/5 volume of Trisol) was added and samples were shaken for 15-30 sec followed by incubation at room temperature for 3min. The samples were centrifuged at 4 degree C and 12000g for 15 min. The aqueous phase was pipette into a new eppendorf 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; 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 μ L DEPC water with 1 μ L 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). 20 μ L reactions were used (described below). cDNA was synthesized at 42°C for 1 hour (Eppendorf Mastercycler personal, Singapore). Samples were stored at -20C for short time or -80C for longer periods.

Materials in one 20 μ L reaction is given below:

Reagent (Thermo Scientific, Singapore)	Volume
5X reaction Buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl ₂ , 50 mM DTT)	4 μ l
Ribolock RNAase inhibitor (20 u/ μ l)	1 μ l
Revertaid M-MuLV Reverse Transcriptase (200u*/ μ g)	1 μ l
Oligo (dT)18 primer (0.5 μ g/ μ l)	1 μ l
DNTP mix 10Mm	2 μ l

8. gDNA extraction

Preparation of lysis buffer

- i.Tris 1M (10X stock) Set pH to 8.0 after autoclaving (working solution 100mM)
- ii. EDTA 0.5M (25X stock) (working solution 20mM)
- iii. Sodium deoxycholate 10% (10X stock) (working solution 1%)
- iv. SDS 10% (10X stock) (working solution 1%)

Note: all solutions were prepared using autoclaved DEPC water.

gDNA extraction was performed by the Trizol method. Briefly, tissues were microdissected, placed in lysis buffer and homogenized in phenol chloroform isoamyl alcohol (~1/2 volume of extraction buffer) and centrifuged at 8000 rpm 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 15min or 4°C for 30 min. Samples were centrifuged at 8000rpm 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.

9. MSRE

Methylation of AVP promoter was quantified using methylation sensitive restriction enzyme (MSRE) digestion in combination with qPCR. MSRE bind to specific CpG site and cut DNA that has not been methylated. Methylation protects the DNA by preventing MSRE from having access to cleavage sites. Primers were designed to bind to regions surrounding the targeted CpG site. Methylated DNA will remain uncleaved by MSRE and there will be template DNA for the subsequent qPCR reaction (this is described in figure below). To examine DNA methylation, 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). HpaII (New England Biolabs, Ipswich, MA) was used to cleave DNA at promoter site 1. For promoter site 1 600 ng of DNA from each animal was diluted in 1 µl of 'NEB buffer 1' in separate tubes. 2 µl of HpaII was added and mixture was incubated for three hours at 37°C. The enzyme was inactivated by treatment at 65 °C for 20 min.

10. qPCR reaction

Quantitative PCR reactions were run using an ABI 7500 machine. The reaction composition and run method are described in the figures below.

Reagent	Volume
Sybr green buffer (AppliedBiosystems, Singapore)	12.5 µL
DEPC	10.5 µL
Forward primer (100mM)	0.5 µL
Reverse primer (100mM)	0.5 µL
DNA (50ng/ µL or 100ng/ µL)	1 µL
TOTAL VOLUME	25 µL

11. Primers Used

All primers were synthesized by AITBIOTECH, Singapore.

Primer Name	Primer Sequence 5'-3'
AVP forward %	TGCCTGCTACTTCCAGAACTGC
HPRT forward	GTCATGTGCGACCCTCAGTCCCA
HPRT reverse	TCGAGCAAGTCTTTCAGTCCTGT
AVP Promoter 1 forward %	GTAGACCGCCACACCTGA

AVP Promoter1 reverse %	CCAGACATTGGTGTGTGACC
AVP Promoter 2 forward %	GGCCTTTGGCTCTATGTTC
AVP Promoter 2 reverse %	TTGAGGGTCACCTGGAAATC

12. Statistical Analysis

Statistical analysis was performed using SPSS software (version 20, IBM). Wilcoxon signed- rank test, Mann-Whitney U test and independent sample t test were used to calculate statistical significance samples, as appropriate. When using non-parametric statistics, exact statistics was used to ensure freedom from normality and distributional assumptions. Two way ANOVA with LSD pot hoc was used for experiments with 2x2 design. Repeated Measure ANOVA was used to compare neuronal counts from the MEApv and MEApd. Box plots in this thesis depict 25th percentile, median and 75th percentile. Central dot and whiskers depict mean and SEM respectively. In bar charts, the bar height depicts mean and whiskers, the SEM. PCR efficiency for each primer set was quantified using a series of sequentially diluted pooled samples that were quantified using qPCR in similar conditions. Primer efficiency was calculated using an online calculator, (<http://www.thermoscientificbio.com/webtools/qpcr/efficiency/>). Relative expression with reference to internal control was quantified using freely available REST software (<http://www.REST.de.com>) [4]. 10000 randomizations were used. This software uses PCR efficiency-calibrated model and randomization tests to obtain relative expression level.

Annexe 3: Reference

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