# Role of arginine vasopressin in modulating plasticity in defensive behavior

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2014

Shantala Arundathi Hari Dass. (2014). Role of arginine vasopressin in modulating plasticity in defensive behavior. Doctoral thesis, Nanyang Technological University, Singapore.

https://hdl.handle.net/10356/61632

https://doi.org/10.32657/10356/61632

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**May 2014**

# Contents

# Acknowledgments

This study was carried out with the financial aid of the School of Biological Science, Nanyang Technological University and the Ministry of Education under the supervision of Dr. Ajai Vyas. This thesis is truly a testament of Ajai's mentorship. Throughout my four year stint at his lab he guided me without ever micromanaging my experiments. This helped me evolve as a researcher. He created a lab environment where no idea or doubt was too trivial. His advocation for an ecological grounding of behavioral studies made the scope of this thesis more far reaching. Research is rarely a solitary endeavor. Some of these results are the fruits of teamwork. Anthony undertook (and succeeded) at the daunting task of teaching me stereotactic surgery. He always generously loaned my any equipment that I need. Anand bore the brunt of my lack of knowledge all things computer related. His mini tutorials started with ctrlc/ctrlv (which ofcourse was not used during the writing of this thesis!) and extended till the formatting of my thesis. Linda jokes that she is the reason I joined the lab. While this isn't the case she, literally, made my journey here so much sweeter. Donna's painstaking effort in setting up our behavior facility made my experiment work less tedious. Dhiraj, Deborah and Vineet helped me at various stages of my experiments. Samira patiently and meticulously read (and reread) this thesis for errors. The newest members in our extended lab, Yamini, Akshaya, Archana and Dr. Koe brought an infectious sense of fun with them.

Mahati, despite being miles and a few time zones away, was always there to help me troubleshoot my experiments. My father, to me, epitomises a scientific spirit. He approaches a good pizza and quantum physics with the same enthusiasm. He has often shown me a different way to approach my results, especially when it comes to statistics. He and my mother have been my strongest champions. It is evident that this list runs long; I am thankful to everyone who helped my intellectually or morally.

# Abstract

## Role of arginine vasopressin in modulating plasticity in defensive behavior

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Social behavior can be viewed as a varying output of an approach-avoidance conflict. For example a potential mate elicits a strong 'approach' response and a predator induces the very opposite. Social behavior must be plastic to best adapt to a continuously changing (biotic and abiotic) environment. Different external states are mirrored by changes in levels of circulating sex hormones; presence of a mating opportunity elevates testosterone levels while presence of a stressor depresses it. I hypothesize that the plasticity in social behavior is a function of changes in circulating sex hormones; i.e. testosterone in males. Arginine vasopressin (AVP) is a neuropeptide widely implicated in social behavior. In the extrahypothalamic regions of the brain it is highly sensitive to testosterone. Castration causes a drop in AVP projections in the extrahypothalmic zone, medial amygdala (MEA); a change that can be attributed to an androgen dependent hypermethylation of the AVP promotor. For these reasons extrahypothalmic AVP was selected as a candidate target. I focused my studies on the MEA, a part of the social behavior network, which has a central role in social behaviors such as reproductive and defensive behavior. The infection of rats by *Toxoplasma gondii* is a well suited system to study social behavior. Infected male rats display an attraction to their feline predators. This is a shift of the approach-avoidance conflict from avoidance to approach. I show that this change can be generalized to sexual aspects of the social behavior, coupled with a corresponding increase in

testosterone levels. The changes in behavior were apparent in a long-time frame rather than being transient, implying a long lasting robust proximate mechanism. This suggested epigenetic regulation as an ideal candidate. AVP promoter contains two androgen sensitive methylation sites. Infection decreased methylation at both these sites in a sub-nucleus of the MEA. Global hypomethylation (methionine injection; s.c.) in infected males was sufficient to reverse *Toxoplasma gondii* infection induced behavior changes. Post methionine treatment infected males were no longer attracted to cat odor. Furthermore, hypomethylation by treatment with a DNA methyl transferase inhibitor (RG108) in MEA decreased fear response towards cat odor. I demonstrate that testosterone is necessary to drive the infection induced changes in behavior; localized supplementation of testosterone in the MEA of castrates reduces their fear response. Further an overexpression of AVP in the MEA is sufficient to cause a reduction in the fear response. Based on these findings I propose a model for plasticity of social behavior wherein changes in external environment are mirrored by changes in testosterone levels in males. This in turn changes levels of AVP in the MEA via androgen dependent methylation sites. Decreased methylation of the AVP promoter regions corresponds to increased AVP expression and an 'approach' bias in social behavior and vice versa. Taken together these posit a central role for epigenetic modulation of AVP in the MEA as a mediator of plasticity in social behavior.

In culmination I propose that social behavior is collectively governed by a trinodal 'extended social behavior network'. This consists of reciprocally connected nodes; the environment, gonadal milieu and neural circuits.

# List of Figures and Tables; Results

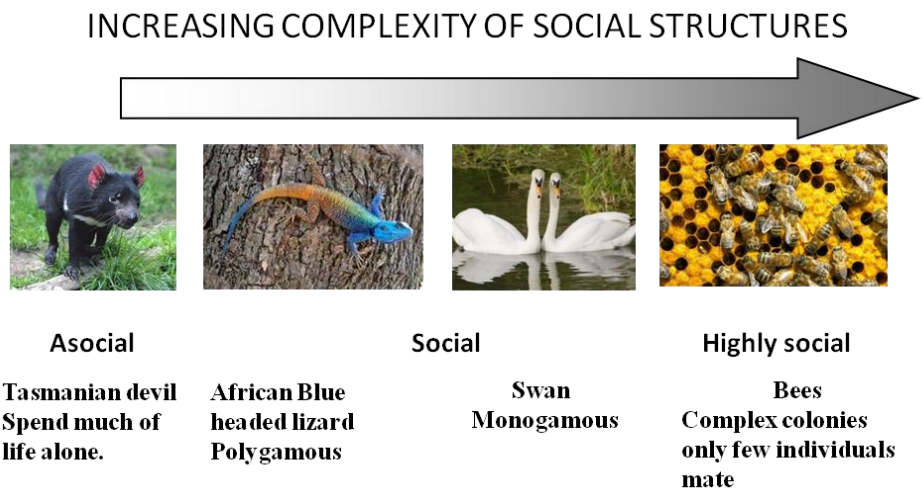
# List of Figures and Tables; Miscellaneous

# 1. Introduction

## 1. Social behavior in evolutionary context

Sociality classically refers to the formation of groups or aggregates, in contrast to solitary living [1]. In such a scenario animals interact constantly impacting each other's behavioral outcomes. These bidirectional interactions form the basis of complex social networks. One of the most daunting and, yet, intriguing aspects to social behavior is the sheer extent of diversity. These behavior show both convergent and divergent patterns of evolution (Box 1 Page 11; Evolution of social structures). Lekking is a pertinent example. In several phylogenetically unrelated species, males form groups, assembling at a common place to exhibit and attract females. In some species, like long-tailed manakin birds, males form cooperative groups in order to woo females [2]. Another relevant example of convergent evolution is mobbing behavior where beach-dwelling species of birds cooperatively mob predators [3, 4]. Interestingly, closely related species that dwell on steep cliffs do not exhibit mobbing behaviors, showing that the social mobbing behavior is driven by natural selection acting on survival benefit of mobbing (divergent evolution), rather than purely a reflection of phylogeny. In a similar vein, Prairie voles and Montane voles adopt two very different strategies to monogamy despite being phylogenetically related [5, 6]. Prairie voles form monogamous pair-bonds [7], presumably in response to sparser food supplies. Males with polygamous attributes fare poorly in this environment because progenies tend to die in the absence of both parents pooling resources for care. Montane voles, on the other hand, are polygamous because an abundant food supply means males with greater partners have greater reproductive success.

**Box 1: Evolution of Social structures**




Social behavior strategies have evolved to best suit the ecological and life history traits of the species and provide maximum advantage to organisms. Their primal function is to boost reproductive opportunities and survival chances. Hence sociality will only evolve if aggregation is favored over a solitary life style, i.e. the benefits imparted by sociality outweigh the costs imposed on an individual by it.

| | Cost | Benefits |
|---|---|---|
| Foraging | Increased competition for limited food resources | Division of labor increases foraging efficiency especially when resources are scarce |
| Predation | Increased groups size causes increases conspicuousness towards predators | Safety is provided by the dilution effect wherein the chance of an individual getting predated in a group is reduced. |
| Disease Transmission | Social interactions provide a route for disease spread | |

Pair bonding has also arisen repeatedly in mammals as a response to unstable social environments requiring mate guarding [8, 9]. Existence of both convergent and divergent evolution suggests that social behaviors are adaptive in some ecological conditions, rather than being accidental results of phylogeny or drift.

The fitness values of social behavior (as opposed to individual behavior traits) are under studied. At this juncture the notion of social competence comes in handy. It is the ability to optimize the expression of social behavior as a function of the available social information [10]. Social competence demands that social behavior cannot be intransigent. It is driven by the premise that animals live in a constantly changing social environment. Social behavior needs to be plastic or context dependant to provide maximum fitness value. Information about the external environment and the internal status (reproductive, health etc) need to be factored into the social behavior machinery. Seasonal breeders are a good illustration. Changes in day length trigger an elevation in testosterone levels, enhancing display of territorial and sexual behaviors. Sexual signals are energetically expensive. These displays of sexual wealth such as peacock feathers, antlers and major urinary proteins (MUP) in mice are all testosterone dependant [11, 12]. Energy that would have otherwise been involved in aspects of survival such as immunity gets diverted to the production of these sexual ornamentations. Mistimed display of sexual signals and behavior would be futile and energetically reckless. Animals need to change their behavior strategies with the onset of breeding season. Timely display of sexual behavior is especially important in species, such as Garter Snakes, that have only a narrow window when reproduction is favored. Understanding plasticity of social behavior could shed light onto the adaptive value of social behavior.

Chapter 1

There is an extreme diversity in social behavior. A glimpse at the natural world shows varied mating strategies such as polygamy in Montane voles, monogamy in Prairie voles, satellite mating strategies as those seen in Pupfish. There are extreme strategies such as those seen in wasp and ants; colonies consist of a single reproducing female supported by sterile individuals.

This immense diversity has caused studies to be constrained to specific behaviors in a specific/group of species. However the most important selection imperatives for all animal are the need to survive and reproduce; or in other words maximizing genetic contribution to offspring and minimizing death during reproductive years (or days or hours) [13]. This commonality implies that there is a unified theme to social behavior. Hoffmann and co-authors present an overarching theoretical construct in which to study social behavior of a variety of taxa. They propose that despite variations in ecology and sensory modalities, social life of animals can be viewed as simultaneous series of challenges (competitors, predators, diseases) and opportunities (mates, food, offspring) [14]. Because of simultaneous presence and often ambivalent predictive cues, approach to the opportunities and avoidance of challenges must be negotiated vis-à-vis each other. In their formulation, the task of social brain is then the negotiation of this trade-off which is conserved across variety of taxa. This model posits that interactions of neuropeptides with dopamine system provides one way of resolving trade-off between approach and avoidance. This model provides a unified framework for studying social behavior. The social behavior is very diverse and highly species specific. Such a perspective reduces them to fundamental approach avoidance dilemmas. This enables researchers to extrapolate findings across species and promotes a more coherent understanding. This framework can also be used study plasticity in social behavior seen within life-span of an individual animal. Social behavior systems must intrinsically be plastic to be context appropriate.

Plasticity in social behavior can be viewed as shifting the output point in this approach-avoidance scale.

At the opposing sides of this approach- avoidance continuum lie behaviors such as reproduction and maternal care (strongly approach) and antipredatory responses (strongly avoidance). I will discuss in detail the specifics of these extremes (i.e defensive behaviors and sexual behavior), with special emphasis on rodents to highlight the possibility of a unified machinery driving social behavior.

**2. Defensive behavior**

While developing anti-predatory strategies an animal is faced with conflicting tasks of minimizing exploratory activity (to reduce risk of predation) and sustaining exploratory activity (required for foraging and finding mates) [13]. Behavior at either extreme of this approach-avoidance conflict is likely to be evolutionary disadvantageous, leading to stabilizing selection at the middle of this conflict depending on prevailing ecological conditions.

Defensive behaviors have been well characterized in mammals. Both rats and mice exhibit robust avoidance in response to predators [15] and olfactory cues of predators [16, 17]. This avoidance is a hard-wired fixed action behavior, not requiring learning through prior exposure to predators or predator odor. Exposure to predators or their odor is aversive in its emotional salience, as demonstrated by the ability to generate analgesia [18], conditioning and long-term anxiety [19].

In rodents, predatory kairomonal cues are detected by both main and vomeronasal olfactory system, consisting of projections from nasal epithelium to main and accessory olfactory bulb, respectively [20, 21]. Both main and accessory olfactory bulb sends direct synaptic projections to medial amygdala (MEA) [22, 23]. Lesions of MEA significantly reduce the display of innate fear

upon exposure to either cat odor or a live cat [24, 25]. Amidst sub-regions of MEA, postero-ventral part of the medial amygdala (henceforth MEApv) is robustly recruited during exposure to predator odors, as demonstrated by extensive expression of Fos protein in this subnucleus after exposure to cat odor [17]. Fos is an immediate early gene that has often been employed as proxy for neuronal activity [26].

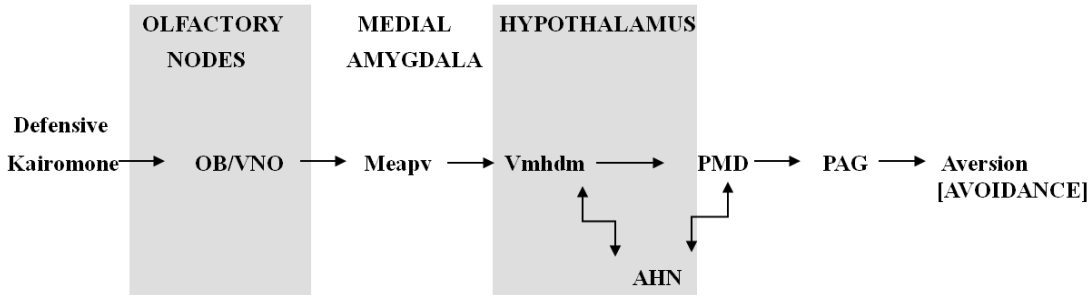



**Figure A: Defensive neural network**

Descending projections from the amygdala to the hypothalamus are especially important in innate defensive behaviors. Hypothalamus can be longitudinally divided into three zones; periventricular, medial and lateral [27]. Initially, electrolytic lesions identified the ventromedial and dorsomedial sub-nuclei as the key regions involved in innate fear [28]. The functional localization was further generalized to the broader medial zone as electrical stimulation here mimicked behavior elicited by natural threats [29-31]. Subsequent tract-tracing experiments have segregated the medial hypothalamic zone (MHZ) into two circuits. The first circuit comprises of anterior hypothalamic nucleus (AHN), dorsomedial ventromedial hypothalamus (VMHdm) and dorsal premammillary nucleus (PMD) [32, 33] (reviewed in [27]) . Likewise, the second circuit consists of medial preoptic nucleus (MPN), ventrolateral ventromedial hypothalamus (VMHvl),

and ventral premammillary nucleus (PMV) [33, 34] (reviewed in [27]). It is noteworthy from the current perspective that exposure to cat odor increases Fos expression in rat AHN, VMHdm and PMD [17, 35]. Congruently, chemical lesions of PMD profoundly diminishes freezing and escape in response to cat odor [36-38]. This part of the medial hypothalamus is also sensitive to contextual information via projections from lateral septum to the AHN [39], suggesting its role on conditioning of spatial context to predatory exposure.

The major target of the MHZ is periaqueductal gray (PAG) [37, 40], a set of brain regions situated around the central aqueduct in the mid-brain. For example, dense projections can be demonstrated from the VMHdm and PMD to the PAG. This is supported by disruption of defensive behaviors in rats post-lesion of the PAG [41].

The MHZ also has projections to the amygdala and bed nucleus of stria terminalis (BNST, a part of extended amygdala system) [38]. This could create a feedback loop for the modulation of innate behavior. Cat odor exposure also increases Fos expression in the locus coeruleus [17]. Interestingly, this brain region has projections to the olfactory bulbs. Activation of the locus coeruleus increases neuronal activity of mitral cells in the olfactory bulb. This could be important to improve signal to noise ratio in response to an otherwise feeble olfactory trace of predators.

The paraventricular nucleus (PVN) also exhibit Fos expression post cat exposure [17]. Interestingly, the PVN has a central role in regulation of stress response [42]. Related to this predatory exposure results in a robust increase in corticosterone [43]; a steroid stress hormone secreted by the rat adrenal glands. In fact some scientists have posited that predator stress can serve as an ethologically appropriate stress paradigm [44]. Activation of stress endocrine response, starting from the PVN and resulting in corticosterone secretion, might be important for

the long lasting effects of cat odor exposure. In support of this, disruption of corticosterone synthesis ameliorates increase in anxiety normally induced by predator exposure.

**3. Sexual behavior**

Females incur a disproportionately greater cost of parental care (compared to males) in most of rodents. This results in greater number of males, than females, being reproductively available at any given time, leading to a skewed operational sex ratio [45-47]. A typical consequence of this skewedness is existence of mate choice in limiting gender (females in this case) and intra-sexual competition to procure reproduction in opposite gender (males in this case) [48, 49]. Male rats and mice express their sexual worth by olfactory cues excreted with their urine, including volatile pheromones and urinary proteins [50-53]. Consonant with secondary sexual characteristics of many other species, these olfactory cues are testosterone dependent in rats and mice [52-55]. This dependency is central to their role as sexual signals, a relationship that is based on energetic requirements of the testosterone production [56, 57]. Resources that would have otherwise been used to boost survival [e.g. bolstering immunity and combating stress] get diverted to the production of testosterone [58]. In addition, testosterone has an immune-suppressive function and increases production of reactive oxygen species, further increasing its opportunity cost. Hence only fit males will be able to engage in the production of increased testosterone. This imparts an 'honesty' to these sexual signals, akin to conspicuous consumption by the resourceful. Consistent with this idea, female rats prefer gonad-intact male rats over castrated and higher-testosterone males over lower-testosterone males [59].

Thus, testosterone concentrations seem to pivotal to sexual behavior. I now turn my attention to neural substrates in male rodent brain involved in sexual behaviors. Many of these brain regions, not surprisingly, are responsive to testosterone and/or its locally aromatized form estradiol.

Brain regions involved in regulating social behavior are collectively encompassed in the Social Behavior network (SBN). These were initially described in mammals, but subsequent work has shown their presence in birds, reptiles, amphibians and fish [60-62]. The similarity extends to both structural (e.g. hodological relationships) and functional domain (e.g. distribution of sex steroid receptors). All the regions of the SBN are connected via bidirectional projections [39, 63, 64] and are rich in steroid receptors [65-69]. Steroid receptors are vital to the plasticity of the SBN because information regarding the external and internal milieu is conveyed to the brain via steroids such as testosterone and corticosterone.

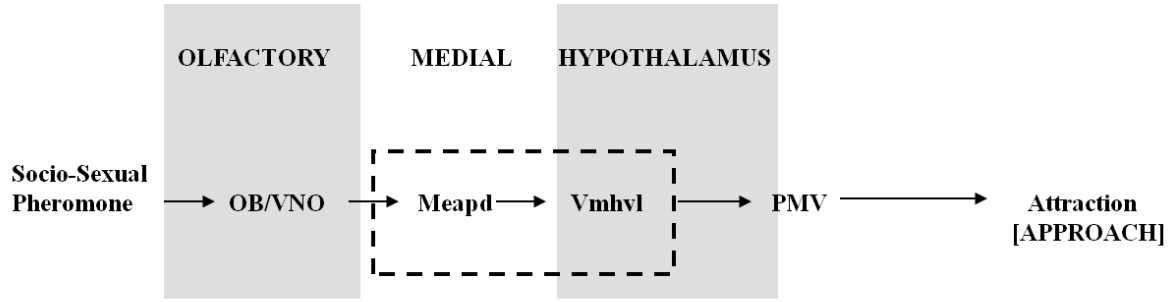



**Figure B: Sexual neural network**

The nodes of the social behavior network receive projections directly from the olfactory centers in rats [22, 23]. In mammals it consists of the MEA, bed nuclei of the stria terminalis (BNST), lateral septum, preoptic area, anterior hypothalamus, ventromedial hypothalamus (VMH) and midbrain [70]. All except the midbrain lie in the limbic region of the basal forebrain. It must not be mistaken that SBN consists of brain regions that are *exclusively* involved in social behavior; instead these nodes are *essential* for social behavior [71]. The SBN has been adapted by researchers to suit their model organisms. For example, studies on songbirds have led to adoption of an altered SBN that includes mesolimbic ventral tegmental area in this case [72].

The second degree of flexibility is imparted by the action of neuropeptides. Neuropeptide vasopressin (also referred to as arginine vasopressin or AVP in mammals and vasotocin in birds) has extensively been studied for its role in male sexual behavior [73]. AVP and its receptors are found distributed across the SBN [74, 75]. AVP and related nonapeptides (nine amino acid containing peptides) can be traced in several invertebrate taxa such as earthworms and leeches [76, 77], suggesting a conservation of its role through a long phylogenic history [78, 79]. For example, signaling through an AVP-like system is essential for reproductive behavior in nematode *Caenorhabditis elegans* [80]. Disruption of this signaling negatively affects mate searching and mate recognition in males [81-83] without diminution of non-reproductive sensorimotor behaviors. Studies in socially diverse estrildid finches are particularly instructional for the role of vasotocin (bird homologue of the AVP) in social behaviors. In this group of birds, vasopressin neurons in medial BNST selectively respond to sexual stimuli like sexual opportunity but not to aversive or anxiogenic stimuli [84]. This observation is congruent with experiments in male mice where neurons immunoreactive to AVP are recruited selectively by sexual behaviors like copulation, but not during social interactions devoid of sexual valence [85]. Vasopressin has also been implicated in approach-avoidance conflicts [5]; but only when animate social stimuli are involved. It is therefore be involved in reproductive behavior such as approaching a mate but inconsequential in foraging oriented approach behavior.

Another important strand of evidence for role of vasopressin system in sexual behavior comes from study of voles (reviewed in [6]). Activation of the AVP system post-mating is necessary and sufficient for pair-bonding of monogamous male prairie voles to mated partner. Thus, central infusion AVP in unmated male is sufficient in this case to sustain pair-bonding [86, 87]. Interestingly, infusion of AVP in the brain of a typically polygamous male montane vole does

not result in pair-bonding [88]. On the flip side, viral-vector mediated expression of AVP receptor V1a (henceforth referred to as AVP V1aR) in target brain regions of AVP can indeed cause male montane voles to become monogamous [89]. AVP V1aR shows strikingly difference in distribution between prairie and montane voles [90, 91]. This suggests that closely related prairie and montane voles show divergent evolution in the distribution of AVP receptors, resulting in divergent sexual behavior. Furthermore, it demonstrates that AVP-mediated sexual behavior in this case is a naturally-selected adaptation to prevalent ecological conditions of prairie voles. The ventral pallidum is known to part of the dopaminergic reward network [92-94]. It is the major output region of the accumbens. Monogamous prairie voles have an increased density of AVP V1aR in the ventral pallidum [90, 95]. This associates a strongly rewarding salience with the stimulus of a mate. This leads to the formation of pair bonding and monogamy. The presence of microsatellites in the AVP V1aR provides a framework for evolution of divergent AVP V1aR types. To assay the role of microsatellites in determining sexual behavior signatures Hammock and Young [96] created two variations of AVP V1aR; containing either a short microsatellite or a long microsatellite. The long alleled animals showed partner preference while those with the short allele didn't. Hence both the ligand and receptor are important in modulating behavioral output.

At this juncture, it is important to ask if the steroid-responsiveness and AVP-responsiveness of SBN are related to each other. Selective implantation of testosterone in ,the AVP expressing, MEA reinstates appetitive sexual behaviors in otherwise castrated male hamsters [97]. Castration of male rats drastically reduces AVP expression in MEA and BNST, while supplementation of exogenous testosterone reinstates AVP levels [98]. Castration of male rats creates a hyper-methylation in promoter sites of AVP, resulting in a lower expression [99]. Exogenous

testosterone in castrated males is sufficient to reverse this epigenetic change and to renormalize AVP expression levels [99]. Thus, testosterone and AVP effects in the SBN are at least linked to each other; and are probably part of a cascade where testosterone affects SBN through its effects on the AVP.

**4. The unified 'extended' social behavior network**

Until this juncture, I have discussed defensive behavior and sexual behavior in isolation. Yet, these behaviors are inter-twined due to the common trade-off between reproduction and survival. Picking up threads from initial parts of this chapter (see page 13), it is crucial to recognize that reproductive pursuit and defensive pursuit must be balanced with each other. In this section, I will attempt to present an integrated view of social behavior.

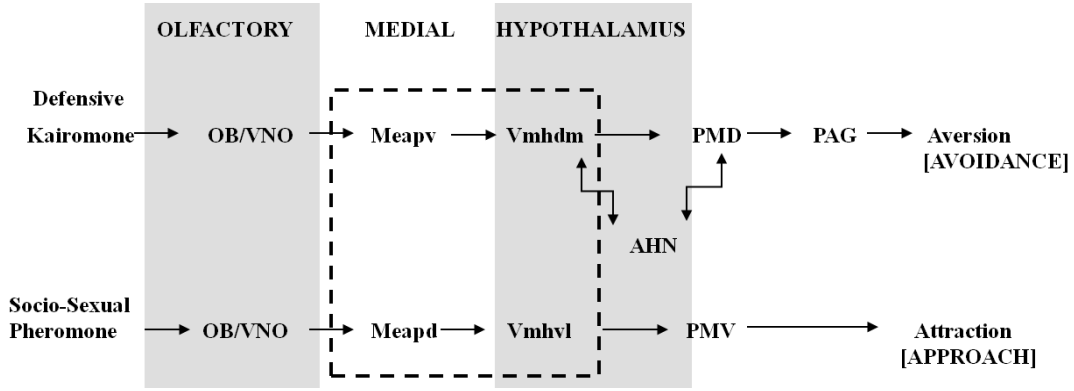



**Figure C: Analogous Defensive and Sexual networks [100, 101](SBN =dotted line).**

The relationship between approach and avoidance has also been extensively studied using immediate early genes (e.g. *fos*) as proxies for neuronal activation. This work complements conceptual observations outlined in preceding paragraph. Anatomical studies using Fos demonstrate considerable parallelism between the processing of attractive and aversive information. This neural congruency starts with the role of the accessory olfactory system in rodents and extends to direct projections to the MEA. Within the MEA, predator cues activate

the postero-ventral part [17, 35] while reproductive cues activate the postero-dorsal part [102, 103]. These sub-nuclei share substantial neurochemical, ontogeny and hodological similarities. The general flow of information after the MEA follows a similar and parallel trajectory through the medial hypothalamic nuclei and later to periaqueductal gray. This parallelism has been intellectually tackled by scientists in two inter-related manner. One view emphasizes spatial segregation of approach and avoidance related behaviors within the general similarity of nodes, very much akin to parallel railway tracks running through the same space. For example, LIM homeo domain genes are differentially expressed in MEA, with *Lhx6* is preferentially expressed in MEApd [100]. Another view, albeit not opposing, posits that nodes of the SBN encode a variety of social behaviors in a combinatorial pattern of the activity [60]. Both views, nonetheless, emphasize remarkable similarity between the neural substrates of innate defensive and reproductive behaviors, suggesting common and co-occurring selection pressures during their evolution. A bulk of these lies in the limbic zone and compose the core SBN. Most of these regions are evolutionarily conserved as is the occurrence social behavior itself.

The similarities in the social brain extend beyond the structural. The neurochemical profiles show a high degree of congruency across species. Three components stand as being highly conserved, i. sex steroid receptors, ii. neuropeptides (AVP and Oxytocin) and iii. neurotransmitters such as dopamine.

As indicated earlier, the ability to respond to sex steroids (testosterone or its metabolites) through receptors is crucial to the function of the SBN. Responding to testosterone allows the SBN to be contextually labile. For example, it is common to see changes in testosterone levels with changes in the environment such as the onset of breeding season or presence of stressors.

Dopamine is a part of the mesolimbic reward system. Dopamine expression can be measured indirectly by quantification of Tyrosine hydroxylase (TH), which catalyses the conversion of L-tyrosine to L-DOPA. TH distribution, despite being conserved, shows a greater variation than other important neurochemical ligands such as AVP and Oxytocin [14]. This along with my emphasis on processing of emotional valence rather than reward is the reason I focused on neuropeptides in this thesis.

AVP and oxytocin have a high degree of structural similarity. Both are nine amino acid containing neuropeptides that differ only at two amino acids [104]. AVP (or vasotocin) has been structurally conserved for almost 450 million years [76, 77]. They were first studied for their role in learning and memory [105]. They garnered interest for their role in social behavior in the 1970s. Central infusions of oxytocin were shown to promote maternal behavior in virgins [106]. The first description of vasopressin in social behavior was the observation that cerebroventicular injection of vasopressin induced a form of social communication; flank marking [107]. The evolutionary origin of AVP in social behavior is unclear. It has a well established role in water homeotstasis. Was this co-opted to have a function in social behavior [108]? Vasotocin has a role in social communication in frogs [109].The role of AVP in social communication in rodents is discussed later. Vasopressin has been more strongly implicated for its role in sexual behavior in males. Henceforth my discussion of neuropeptides will be restricted to AVP.

AVP modulates pair bonding, mate guarding and parental care in monogamous prairie voles [110] .The role of AVP ligand has been extensively discussed on Pg 19. To summarize species specific behavior signature (such as monogamy vs polygamy) are a function of AVP V1aR while the vasopressin ligand modulates temporal changes in behavior. AVP has a role in two other important facets of social behavior. i. Social recognition and social communication.

Social behavior is built upon interactions between individuals. For this the ability to distinguish and recognize each other is essential. AVP influences social recognition at the main olfactory bulb (MOB) via AVP V1aR. This is testosterone dependent [111, 112]. Castrated males recognize juveniles for a longer time. Chronic central administration of AVP in castrates reduced their social recognition skills making them comparable to controls. AVP also influences social recognition its action at the lateral septum. Injection of AVP here lengthens memory[113] . Blocking the V1a receptor activity in the lateral septum inhibits social recognition [114, 115]. Corollary effects were observed on viral vector mediated overexpression of AVP; increase social recognition [114].

The other aspect fundamental to social behavior is communication between individuals. AVP (and its predecessor vasotocin) has a conserved role in social communication (reviewed in [116]). Flank marking is an important form of scent marking in many rodents. AVP has a dose dependant effect on flank marking which occurs via AVP V1aR [117, 118]. Brain regions extending from the medial preoptic area (MPOA) to the anterior hypothalamus (AH) are involved. AVP in the MPOA-AH zone does not appear to be under the direct influence of testosterone. Instead gonadal hormones alter the sensitivity of this zone to AVP. Testosterone enhances the flank marking induced by microinjections of AVP in the MPOA-AH [119]. It causes this by changing the density of AVP V1aR here [120, 121].

AVP is richly distributed in the nodes of the social brain. While discussing AVP special attention must be given to the spatial identity of AVP. The vasopressinergic system can be divided into the hypothalamic and extrahypothalamic networks. The former plays a pivotal role in the stress response. The PVN secretes AVP and CRH (Corticotrophin releasing hormone). The former increases the sensitivity of the pituitary gland to CRH. This potentiates a peripheral stress

response by secreting adrenocorticotrophic horomone which induces the adrenal glands to secrete a class of stress hormones, glucocorticoids. Extrahypothalamic AVP has a prominent role in sexual behavior (discussed on pg 19). Another basis for differentiation of AVP systems is androgen dependency. Castrates show reduced AVP-ir in the MEA, lateral septum, ventral tegmental area, locus coeruleus, vertical limb of the diagonal band of Broca, lateral habenular nucleus, mediodorsal thalamic nucleus, olfactory tubercle, central grey, dorsal raphe nucleus, substantia niagra and the hippocampus. Amongst these the lateral septum, locus coeruleus , vertical limb of the diagonal band of Broca, lateral habenular nucleus, mediodorsal thalamic nucleus, olfactory tubercle ,central grey and dorsal raphe nucleus receive projections from the extended amygdala (BNST in particular). The origin of the VTA and Hippocampal projections is unknown. Hence testosterone primarily affects the extended amygdala associated vasopressinergic networks. This is in agreement with their important role of these regions in sexual behavior. AVP in the PVN is, on the other hand, unaffected by testosterone [98]. Together these comprehensively depict the two vasopressingeric system; the testosterone independent hypothalamic system that mediates stress and the testosterone dependent extra hypothalamic system that mediates aspects of sexual behavior. In this thesis I focus on the latter.

The duality in the influence of testosterone can be explained by differing distribution of androgen receptors (AR) between the hypothalamic and extrahypothalmic zones. In the PVN none of the AVP-ir neurons were immunoreactive for AR [122] . Testosterone might not have any influence on the AVP innervations in the PVN because they are buffered from its action due to the lack of AR. There is an additional explanation to this duality. AVP contains four methylation sites in its promotor. At the MEA, castration increases methylation at two of these sites. The other two remain unaffected. Increased methylation corresponds with the reduced

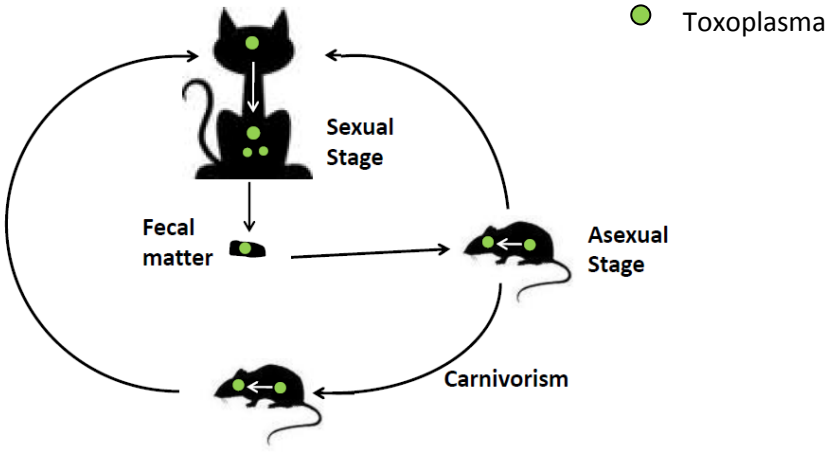
vasopressin innervations in the MEA in castrates. Epigenetic changes can cause sustained long lasting changes in gene expression patterns. This is well suited to address the demands associated with changes in environmental stimuli such as seasonal changes, lack of resources etc. It is possible that testosterone can invoke more rapid changes in AVP expression via its action as a transcription factor. This enables plasticity in the vasopressin–social behavior system to be both long lasting as well as quick.

In summary there is strong support for the existence of an evolutionarily conserved' extended; social behavior network. This will mediate the many facets of social behavior. All of which can be framed as approach vs. avoidance dilemmas. Three components stand out for their importance in the extended social behavior network; i. the limbic zone (especially the extended amygdala and hypothalamus), ii. testosterone and iii. neuropeptides such as AVP and oxytocin. Each of these influences each other bidirectionally. In this thesis I study plasticity in social behavior in this framework.

Neural modulators of defensive and sexual behaviors have been studied extensively. Traditionally techniques such as lesioning, pharmacological disruption and castration have been used to disrupt the neural and gonadal substrates [67]. Such measure causes extreme all or none phenotypes. These extreme responses don't accurately map the plasticity seen in the natural world. It is in this context that I introduce an ethologically relevant, subtle and specific perturbation model for social behaviors, namely the infection of male rats by protozoan parasite *Toxoplasma gondii* (henceforth referred to as Toxoplasma). Rats infected with parasite lose their innate defensive responses toward predator cues and instead develop an attraction [123-125]. Interestingly, felid (cat family) intestines are obligate habitat for sexual reproduction of this parasite [126]. Hence it has been suggested that reduction of fear in infected rats serves to

increase trophic transmission of Toxoplasma from its intermediate rat host to definitive cat host. The behavioral change is shown to be specific and dose-dependent, precluding role of generalized sickness behavior [124, 125]. From the perspective of this thesis, the Toxoplasma-rat model represents an ideal perturbation system to study defensive behaviors because of specificity and ethological scale. Effects of manipulation do not present themselves in all-or-none manner reminiscent of lesion studies, thus allowing study of plastic changes in social behavior. Moreover, I postulate that this perturbation model is also suitable to study approach behaviors in view purported reciprocal relationship between approach and avoidance. It is interesting in this respect that Toxoplasma infection results not only to loss of fear (i.e. loss of function) but also to gain of attraction to cat odors (i.e. gain of function) in at least a subset of animals.

**Box 2: *Toxoplasma gondii* Life Cycle**




**Toxoplasma Life Cycle**

*Toxoplasma gondii* is an obligate parasitic protozoan. It has a two staged life cycle [127]. The asexual stage can occur in all warm blooded animals. Rodents are the predominant intermediate hosts though. Felines are the only definitive hosts where the sexual stage occurs [126]. Oocysts are secreted in their fecal matter. Ingestion of oocyst contaminated food sources facilitates entry in the intermediate host. During the acute phase of the infection tachyzoites disperse through the blood stream causing the infection to spread. A chronic infection occurs wherein parasitic cyst (bradyzoites stage) are presence in immune immuno-privileged organs such as the brain [124] and gonads [128] even after clearance of the peripheral infection. The life cycle is completed only if the animal is eaten by a cat.

# 2. Specific Aims

In this thesis, I aim to study the plasticity in defensive behavior in a larger frame-work of reciprocal relationships between the brain, hormones and behavior. This thesis takes a perturbation approach; much as a molecular biologist will use gene knock-outs to study physiological function of a particular gene. I employ Toxoplasma induced behavioral changes in male rats as a perturbation model.

The specific aims of this thesis are briefly discussed below.

**Specific Aim 1. To characterize Toxoplasma induced behavioral change in male rats.**

Toxoplasma has previously been shown to cause changes in the defensive behavior of rats. An emerging approach considers social behavior as occurring on a single continuum of approach - avoidance conflict. In this specific aim I characterize behavioral changes post-infection on both ends of this continuum by investigating defensive behavior and investment in reproductive pursuits. Both of these are known to have opposing imperatives for the animals (see pages 13 in the introduction), and places opposing demands on the animal behavior. Moreover, I also investigate changes in testicular testosterone due to the infection; in the view of the proposed centrality of the testosterone in negotiating approach-avoidance conflict (see page 13 in the introduction). **[Chapter 3]**

**Specific Aim 2. To validate the role of the medial amygdala vasopressinergic system in sexual behavior in rats.**

The medial amygdala (MEA) has a central role in evaluating social valence of a stimulus (see Pgs 17-25 of the introduction). Moreover, the MEA has been reported to be atypically activated by cat odor exposure post-infection. The MEA contains copious amount of, the neuropeptidepeptide, arginine vasopressin (AVP) in addition to receptors for testosterone and testosterone metabolites. AVP positive neurons in a hodological homolog, the bed nucleus of stria terminalis, are recruited during affiliative social stimuli. In this backdrop, I postulated that MEA-AVP is important for sexual behavior. In this chapter, I investigate this postulate. This specific aim is a precursor of next two chapters. **[Chapter 4]**

**Specific Aim 3. To determine role of the medial amygdala vasopressinergic neurons in approach-avoidance shift in the infected animals.**

Following from the preceding chapter, I determined the role of the MEA-AVP neurons in the observed shift from an avoidance of cat odor to the gain of approach in infected male rats. **[Chapter 5]**

**Specific Aim 4. To delineate molecular proximate mediator of behavioral change pertaining to medial amygdala vasopressinergic system.**

The preceding chapter highlights crucial role of the MEA- AVP system in mediating infection induced changes in defensive behavior. In this chapter, I investigate proximate mechanisms at the molecular level. Specifically I study the testosterone dependent epigenetic mediation of AVP changes in MEA post-infection. **[Chapter 6]**

# 3.*Toxoplasma gondii* infection enhances aspects of reproductive behavior.

**Specific Aim: To characterize Toxoplasma induced behavioral change in male rats.**

Several behaviors are learnt by animals during their individual life histories. For example, a rat can learn to associate neutral auditory tones to anteceding footshock after experiencing a few co-occurrences of both [129-131]. Innate behaviors, on the other hand, do not require learning during life of individual animals. In these cases, stimuli generate behavior because such regulation has been naturally selected for through evolutionary history. In other words, individual rats that do not show innate aversion to cat odors have perished at higher rates in past, such that innate fear has become evolutionarily fixed in the present gene pool. Innate behavior strategies consist of a repertoire such as anti-predatory defenses and sexual responses amongst others. In this thesis I have limited the scope of discussion to include only innate social behaviors.

As indicated in the introduction (Page 26), I have used a behavioral manipulation model as perturbation system in my thesis. Specifically, I use the Toxoplasma-rodent model to study perturbation of innate social behaviors. Toxoplasma is a parasitic protozoan. It has a two stage life cycle [126]. The asexual stage can occur in all warm blooded animals, including rats, birds, livestock and humans. And yet felids (cat family) serve as the only definitive hosts allowing sexual stage of parasitic life (Box2, [126]). In intermediate rat hosts, Toxoplasma forms a chronic infection in the brain and persists in a cystic form even after clearance of peripheral infection [124]. Within the brain, Toxoplasma shows subtle but significant tropism to the

amygdala [124], a component of the social brain network (SBN). Several studies show that Toxoplasma infected male rats lose their innate aversion to cat odor and develop an attraction instead [123-125]; a phenomenon that has been aptly described as a "fatal attraction". It is postulated that such a "fatal attraction" is behavioral manipulation by the parasite to increase its transmission. Parasitic manipulation of defensive behavior has been earlier shown in Long-Evans and Listar hooded rats [124, 125]. I started by extending these observations to the Wistar strain of rats, a strain that is locally available in Singapore.

Male Wistar rats (n=12 per group, 6-8 weeks old) were infected with either 5 million parasite tachyzoites or mock-infected with sterile saline. Avoidance of cat odor was quantified six weeks post-infection, a period consistent with presence of chronic infection and absence of acute peripheral infection. Rabbit urine was used as a neutral non predatory control. Time spent in two opposing arms of a rectangular arena (76 x 9 cm each) containing either cat or rabbit urine was quantified (trial = 20 minute) (Described in detail in Annexe on Pages 107, 114). Infected animals spent significantly greater time in cat bisect (Figure 1[1], exact Mann Whitney test, $^{**}p < 0.005$). It is noteworthy that the median of the infected group lies above the (50%) chance level, demonstrating gain of attraction to cat odor in more than 50 % of the rats. Thus, I was successful in replicating the "fatal attraction" phenomenon is Wistar strain of rats used during this thesis.

According to an emerging conceptual perspective, social behaviors can be integrated in a unitary approach-avoidance continuum [14] (please see Page 13 in introduction for detailed description of this perspective). The Toxoplasma-rodent model is ideally positioned to study plasticity of

[1] Page 33

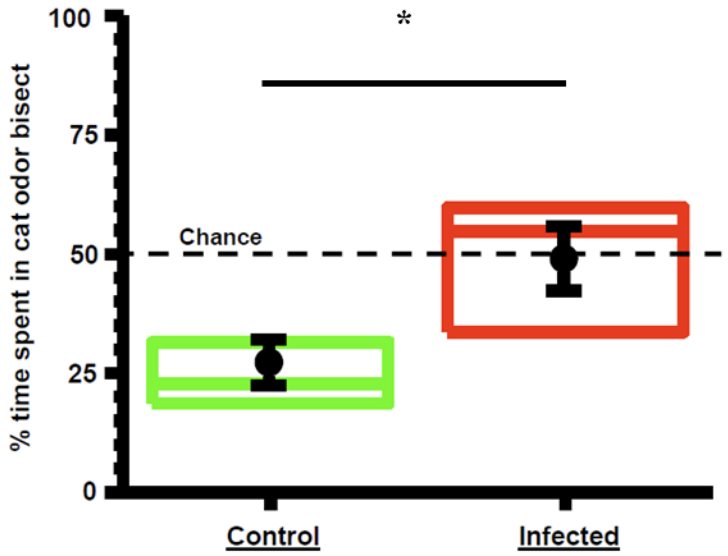



**Figure 1: Infected males show a reduction in fear and even a gain in attraction towards cats**.

Males were infected with 5 million parasites. Seven weeks post infection cat avoidance was quantified. Rabbit urine was used as a control odor. Boxplot with the mean and SEM (in black) are represented. Percentage time spent in cat odor bisect is plotted to the y axis (% time in cat odor bisect= time spent in cat bisect (time spent in cat bisect + rabbit bisect)*100). The dotted line at 50 percent represents chance. Trial duration=1200s; Control mean = 27.70% and Infected mean = 48.73%; Exact Mann Whitney Z=-2.30 $*p < 0.05$. (n = 12 infected + 12 control).

behaviors, I hypothesize that the loss of avoidance in Toxoplasma infected rats will result in the facilitation of behaviors on other end of approach-avoidance continuum, namely sexual behaviors social behavior along this continuum (Page 26 of introduction).

In this backdrop, we[#] studied the investment of control and infected males in secondary sexual characteristics. We used the attractiveness of males to estrus females as proxy to infer their sexual investment. The precise mode of sexual signaling in Wistar rats is unclear. Reports in, closely related, mice, attribute this to urine bound Major Urinary Proteins [132]. These are testosterone dependant genetically coded signals that can accurately reflect the sexual worth of males [12, 132]. Additionally these can bind volatile signals that provide information regarding current status of male such as sickness etc. Collectively olfactory cues broadcast these signals regarding genetic worth and current health status of the male [133]. We used these 'complete' stimuli carried in the urine. Stimulus males were placed in the arena for two hours prior to testing to allow for urine marking. During testing they were confined to chambers at the end of the arena. The assay was performed using 12 unique pairs of control and infected males) (Described in detail in Annexe on Pg 107, 115). Preference of 5-7 estrous females for these 12 pairs was assayed (total of 72 trials; trial duration = 1200 seconds). For 10 out of the twelve male pairs the infected male was preferred over the control. Further in 53 out of 72 trials (i.e. total = 6 females * 12 males infected: control male pair) the female preferred the infected male (506 ± 30 s in infected bisect compared to 308 ± 17 s in control bisect, Figure 2[2] Panel A, paired t-test: $p < 0.00001$; median preference score = 1.54, Figure 2 Panel B).

[#] Co experimentor; Anand Vasudevan

[2] Page 35

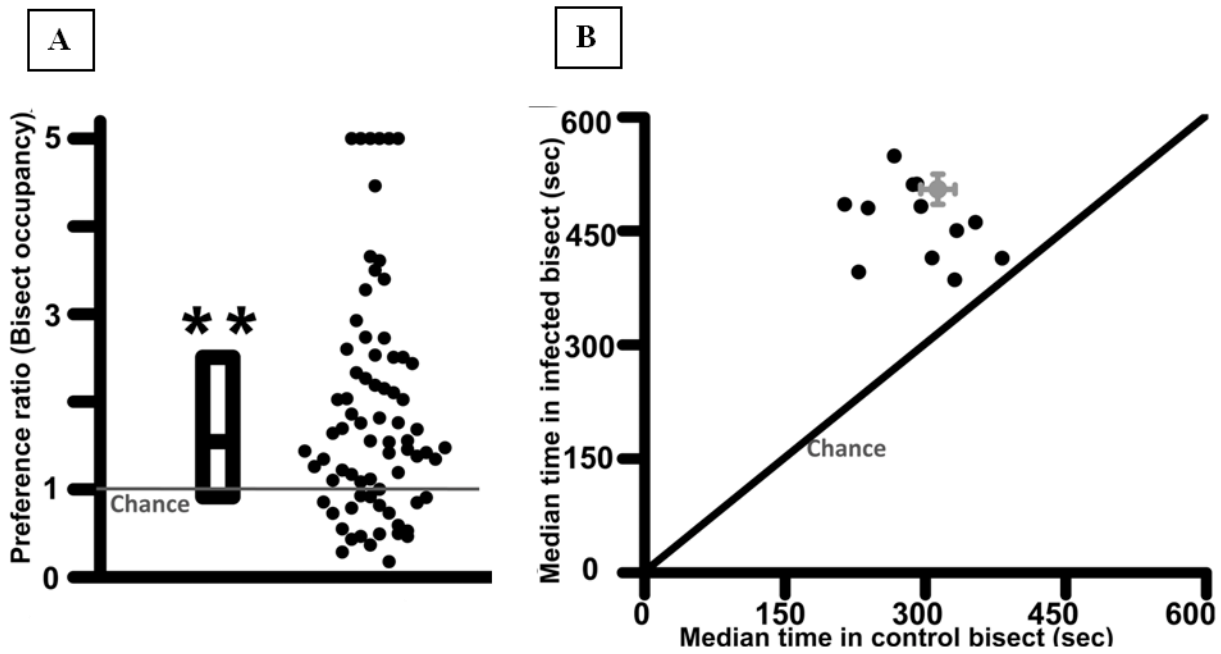



**Figure 2: Females prefer Toxoplasma infected Wistar males**.[#]

Preference was quantified by comparing time spent by an estrus female in two opposing arms of an arena, each arm urine marked by either controls or males infected six weeks earlier (Panel A; trial duration = 1200 s). Ordinate depicts time spent in infected bisect divided by control bisect (ratio >5 assigned arbitrary value of 5). Each dot represents raw data from one female (n=72 trials (6 females x 12 infected: control males pairs), 12 infected: control male pairs). Box plots depict median, 25$^{th}$ percentile and 75$^{th}$ percentile. Preference of females for males in each of the 12 unique pair was calculated by taking median of all females tested for that particular male (Panel B; ordinate and abscissa depicts time spent in infected and control bisect, respectively). Mean and SEM of data used in scatter-plot are depicted in grey color. (Figure and legend taken from [128]). Each dot represents the average preference for a infected: control males pair over 6 females.

[#] Primary experimenter was Anand Vasudevan

We then modified the experimental design such that the female could choose to copulate with either male. Briefly an arena was designed such that females, but not males, could move freely between compartments. Stimuli males were confined to a compartment. Females moved between the compartments and could i. choose mate and ii. pace the mating (Described in detail on Annexe on Pg 107,116). Such an experiment would serve two purposes. It would demonstrate if there are any differences in consummatory aspects of male sexual behavior. It would also show whether information from the previously conducted scent cue based experiment can be extended to an actual mating set up. Mating in rats is paced by the female [134]. Paced mating implies that the female repeatedly enters and exits the male arena, exercising control of choice of male and pace of sexual interaction. Mating follows a well-defined sequence of events. An estrous female first enters the male chamber. This is followed by the female exhibiting a stereotyped solicitation behavior characterized by hopping and ear wiggling. This is then followed by eventual mounting and intermittent dismounting by the male. To allow pacing by females, we constructed an arena of three inter-connect chambers (corner (male) chamber is 30x45cm and center (female) chamber is 15x45cm). Chambers were connected through Perspex partitions containing holes that were big enough for the female (but not the male) rat to pass through (50mm in diameter). A sexual episode in my experiments is defined as mount followed by genital grooming. Trials were recorded for 120 minutes during dark phase. The percentage of the mounts that were sexual episodes (% sexual episodes = sexual episode divided by total mount expressed in %) was also quantified. The paced mating was performed twice with minor alterations. In the first case each female was allowed to mate with only one male; either a control or an infected (Described in detail in Annexe on Pg 116). The infected males showed more sexual episodes than their control counterparts (Figure 3[3] Panel A; paired t test: $p < 0.05$, n = 7 infected and 6 control males). The

[3] Page 37

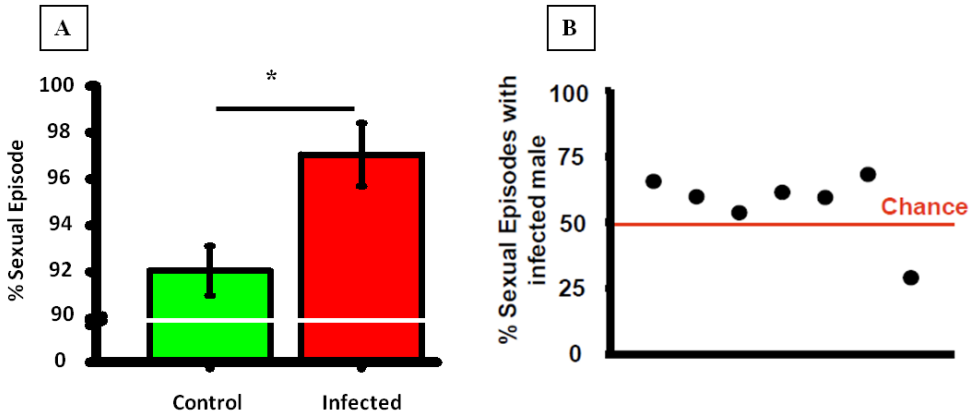



**Figure 3: Boost in sexual activity in Toxoplasma infected Wistar males.**[#]

Sexual episodes is defined as a mount followed by genital grooming (% sexual episodes=sexual episode/ mount * 100). (Panel A) Non Competitive Paced Mating Assay: The infected males showed more sexual episodes than their control counterparts (Infected mean=97.04%, Control mean= 92.05%, independent t test: $p < 0.05$, n = 7 infected, 6 control males) (Panel B) Competitive Paced Mating Assay: Infected males received more mating opportunities. 6 out of 7 females bestowed greater mating opportunities on infected males (n = 7 infected: control pairs; exact Wilcoxon signed ranks test: $Z = -2.21$, $p < 0.05$; one-sample t-test against chance i.e. 1: $t = 4.36$, $p < 0.01$)

[#] Experiments performed with Anand Vasudevan

experiment was performed again to introduce an element of competition. In this case, the female had the choice of moving back and forth and mating with both an infected and control male during a single trial. Preference of seven females of infected or control male pairs were scored. In 6 out of 7 case females exhibited greater than chance levels of sexual episodes with the infected male. In the previous case the comparison between control was indirect (as both mated with different females). Here a direct comparison between sexual performances can be drawn as both males mating with the same female. This eliminates any variation caused by the females themselves. In accordance with the data from the noncompetitive assay, here too, the infected males secured more reproductive opportunities. Infected males showed greater number of sexual episodes in 6 out of 7 unique male pairs assayed (Figure 3 [3]Panel B).

Observations detailed until now demonstrate that Toxoplasma infection reduces avoidance of predator odors and enhances sexual investment in reproductive attraction. Interestingly steroid hormone testosterone both reduces fear and enhances sexual investment. This led me to measure testosterone levels in control and infected animals. Commercial competitive enzyme-linked immunoassay kits were used (Described in detail in Annexe on Pg 116). Testicular testosterone, 3 days post cat exposure, was measured both at basal conditions and after exposure to a stimulus (20 minutes exposure to bobcat urine) that enhanced testosterone in prior pilot study(Described in detail in Annexe on Page 117). At the basal condition, testes from infected animals contained > twice the amount of testosterone in control animals (Figure 4[4]; $p < 0.05$, LSD post-hoc test or exact Mann-Whitney test). Testosterone levels significantly increased in control animals upon cat odor exposure ($p < 0.05$). In contrast, infected animals did not exhibit an increase in testosterone after stimulation, compared to basal infected group ($p > 0.5$; $p$ type 2 = 0.06). The

[3] Page 37
[4] Page 39

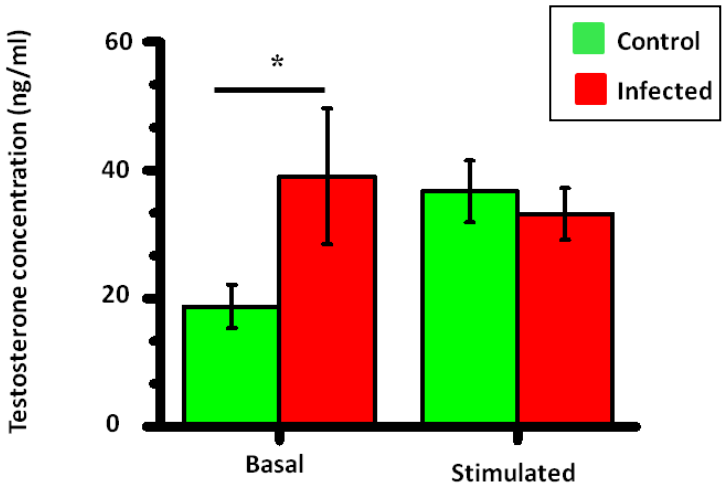



**Figure 4: Infected males showed heightened levels of testosterone**

Testosterone levels were quantified using commercially available kits. Androgen levels were measure in basal and 3 days post cat odor stimulation. One testis was crushed and testosterone extracted using diethyl ether. This was dissolved in 1 ml of buffer. Infected males showed almost double the levels of basal testosterone (control mean = 18.74 ng/ml, infected mean= 39.04 ng/ml, * $p < 0.05$, LSD post hoc). Upon stimulation by cat odor there was no significant difference between testosterone levels in these two groups (control mean = 36.67 ng/ml, infected mean= 33.19 ng/ml, $p < 0.05$, for basal vs. induced testosterone in control animals; $p > 0.5$, for basal vs. induced testosterone in infected animals (Figure and legend modified from[135]).(n=10 Basal control, n=11 Basal infected, n=12 stimulated control and n=12 stimulated infected).

lack of stimulation effect on infected animals suggests that basally high levels of testosterone did not spare significant physiological ceiling to allow for further enhancement.

Data presented till this juncture suggest that the parasite enhances sexual investment by males concomitant with increase in endocrine mediator of sexual signaling. This presents a tantalizing possibility. Toxoplasma has been reported to be sexually transmitted in livestock animals [136, 137]; although it was not known if similar horizontal transmission occurred in rodents. If Toxoplasma is sexually transmitted in rats, it is likely that the parasite benefits directly from the enhanced attractiveness of infected males. In order to examine this possibility, we dissected epididymis from infected males (6-8 weeks post-infection). Epididymis is a tubular structure postliminary to the testes that stores sperms prior to ejaculation. We visualized Toxoplasma cysts here using immunohistochemical staining (Described in detail in Annexe on Pg 118) (Figure 5A[5], estimated burden=520 cysts per animals). The Toxoplasma strain employed here contains a GFP reporter; hence parasites were stained using anti-GFP antibodies. Cell walls were highlighted by binding it with DBA-FITC conjugate. Horizontal transmission was confirmed by presence of Toxoplasma in vaginal lavages of females (Figure 5B, in 4 out 4 mated females) who have mated infected males[$]. Presence of cysts in the brain homogenate of the offspring[$] (Figure 5C, in 43 out of 69 pups) of the previously mentioned females confirmed vertical transmission. This demonstrates that Toxoplasma in transmitted vertically and horizontally through sexual intercourse in rats. It is likely that enhanced attractiveness of infected males results in greater transmission of the parasite through sexual route.

[5] Page 41

[$] Co-experimentor: Deborah Dutta

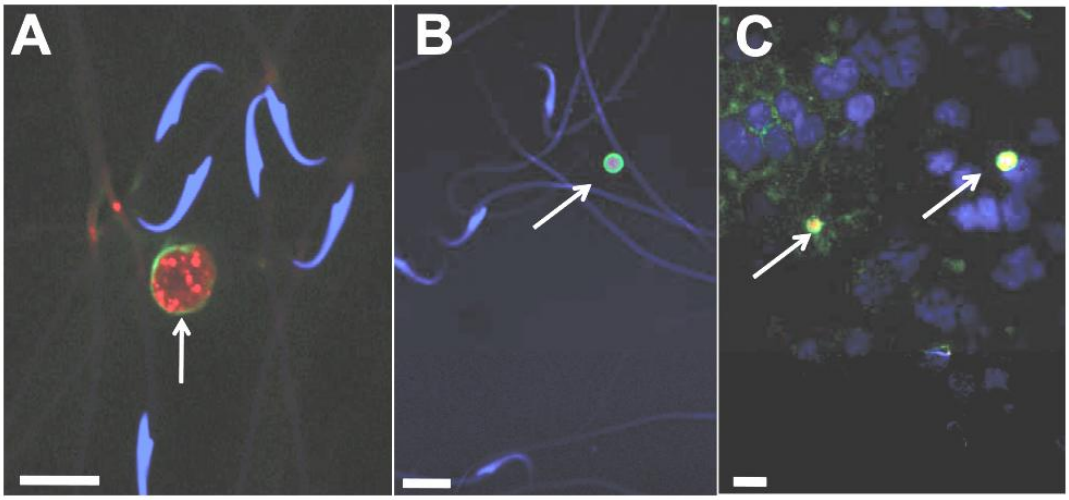



**Figure 5: Toxoplasma can be transmitted horizontally and vertically.**[$]

Scale bar = 10 µm. Toxoplasma cyst visualized in (A) Epididymal tissue, (B) Vaginal lavages of females who have mated infected males and (C) Whole brain homogenate from the offspring. Parasites were engineered to contain a GFP insert. Cyst wall was stained with a fluorescein (green) conjugated Dolichos Bifluorous Agglutinin and bradyziotes signal is amplified with a TSA-cy3 conjugated against the GFP protein. Nuclei are stained blue with DAPI.

[$] performed with Deborah Dutta

As mentioned earlier, testosterone reduces fear [138-140] and enhances sexual signaling [59]. Next I investigated the necessity of testicular testosterone for parasitic behavioral manipulation.

Innate aversion to cat urine was investigated in both infected and mock-infected animals, injected after either castration or sham surgery[&] (Described in detail in Annexe on Pg 111,107,114).

A two-way analysis of variance (ANOVA) demonstrated a significant interaction between infection and exposure to bobcat odor ($F_{(1,41)} = 3.9$, $p = 0.05$). Main effects of infection or induction by odor did not reach statistical significance ($F_{(1,41)} < 2$, $p > 0.15$). In agreement with previous observations (Figure 1), innate aversion was significantly reduced in infected animals compared to control (both groups with sham surgery; Figure 6[6]). In contrast, the infection did not result in loss of fear when castrated animals were employed (Figure. 6; $p = 0.49$). This observation demonstrates that intact testes are required for infection-mediated effects on defensive behavior. I did not examine the effects of castration on male attractiveness because prior studies showed that castration results in complete loss of sexual signaling in male rats making inter-group comparison between controls and infected meaningless [59]. Observations described in this chapter demonstrate that Toxoplasma infection results in a shift of social behavior, moving the negotiated balance away from avoidance of predators and towards approach and sexual signaling. The shift in aversion and sexual behaviors could increase parasite transmission through trophic and sexual routes, respectively. Furthermore, infection mediates an increase in testicular testosterone that could serve as trigger for both reduced fear and enhanced sexual signaling. Parasites and their host engage in continuous coevolution. This continual struggle can be rhetorically seen as an arms race with both partners, developing never ending

[&] Castrations were perfomed by Donna Tan

[6] Page 44

artillery to fuel their battle; an interaction imaginatively described as red queen dynamics. In context of such a coevolution, “love potion” parasites that the enhance reproductive advantage to the host are postulated to have a selective advantage [141]. This is because redeemed reproductive success (i.e. more access to females in case of Toxoplasma) trades-off against cost of parasitism for the host (i.e. greater possibility to being eaten by cats), eventually blunting the selection pressure exerted by the host on the parasite. In case of Toxoplasma, sexual transmission offers an added dividend to this strategy.

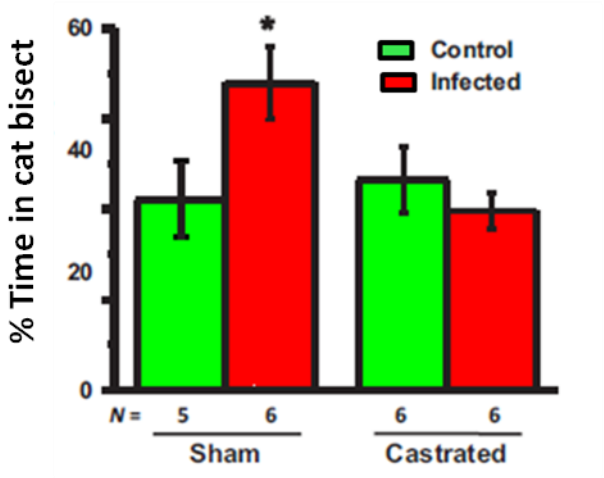



**Figure 6: Castration abolishes Toxoplasma infection induced changes in defensive behavior[&]**

Behavioral effects of infection on innate fear were dependant on intact testes. Infection attenuated innate fear in intact, but not in castrated, males. The ordinate depicts relative occupancy in the bobcat bisect relative to total occupancy in the bobcat plus rabbit bisect. *$p$ < 0.05 for comparison between control intact and infected intact. (Figure and legend modified from [135]**)**

[&] Castration performed with Donna Tan

# 4. MEA AVP is involved in the Processing of Sexual Stimuli

**Specific Aim : To validate the role of medial amygdala vasopressinergic system in sexual behaviors.**

The MEA is an essential brain region for chemosensory processing during reproduction. For example, MEA lesion in male hamsters eliminates mating behavior [142]. Likewise, MEA lesion in male rats drastically reduces penile erections in response to an inaccessible estrus female [143]. Interestingly, such lesion does not affect reflexive erections in response to retraction of penile sheath. This suggests that the MEA involvement is restricted to pheromonal processing and not to downstream initiation of mating. Pheromonal information from the olfactory system gains access to the MEA via a polysynaptic pathway [22, 144]. The MEA relays this information after processing to the bed nucleus of stria terminalis, medial preoptic area and ventromedial hypothalamus. Amongst the sub-nuclei of MEA, the MEApd is specifically involved in processing of reproductive pheromones [103, 145, 146], while the posteroventral part is selectively activated by predator kairomones [17, 147, 148].

Despite a prominent role of the MEApd in processing of reproductive pheromones, the neurochemical identity of the pertinent cell groups is yet undetermined. Yet, several lines of evidence suggest that the relevant neurons are androgen responsive. For example, flank gland secretions in hamster recruit androgen receptor containing cells in MEApd, suggesting that androgen receptors play a role in pheromone detection [149]. The essential role of androgen is also supported by observations that antagonism of androgen receptors in MEA inhibits

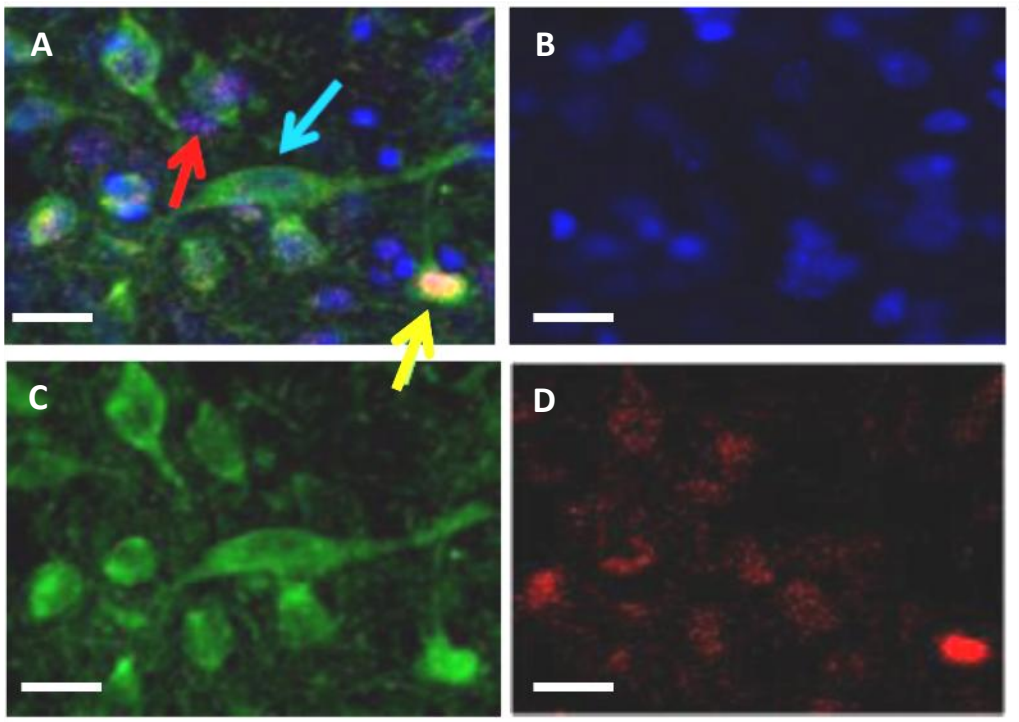



**Figure 7: Immunohistochemical colabelling of AVP and Fos.**

Animals were exposed to stimuli for 30 minutes. Two hours posts the beginning of stimulus exposure animals were sacrificed. Brain tissue were perfused with 4% PFA, flash frozen and sectioned at 40µM. AVP-ir neurons (green) are stained with and anti-AVP antibody (Panel A and C). Fos (red) depicts recent neuronal activation (Panel A and D). Nuclei (blue) are stained with DAPI (Panel A and B). Sections were imaged using a 40X objective and 1.2X digital zoom at a confocal micoscope (Carl Zeiss, LSM 710). Fos positive neurons (Red Arrow) are those that are recruited by the stimulus. AVP positive neurons (Blue arrow) are those that play a role in social affiliation. AVP neurons that are recruited by the stimulus are colabeled (AVP+Fos-ir; (yellow arrow).

erection in male rate in presence of estrus females [150]. Moreover, testosterone implants within MEA of castrated males are sufficient to restore erections in response to inaccessible females [97]. Such testosterone supplementation does not reinstate mating behavior, again suggesting that androgen responsive neurons within MEA are involved in detecting reproductive pheromones and not mating per se.

In addition to androgen receptors, many MEApd neurons also express arginine vasopressin (AVP) [11-13]. The expression of AVP in MEA is dependent on testicular testosterone, diminishing in weeks after castration and reinstated by testosterone supplementation [151-153]. In view of the androgen-dependent expression of the MEApd-AVP and the role of androgen receptors in pheromonal processing, I hypothesized that reproductive pheromones selectively recruit AVP producing neurons in the MEApd. I tested this hypothesis by quantifying co-labeling of AVP and cFos(henceforth denotes protein product of *Fos* gene) an immediate early gene product that marks recently activated neurons, post-exposure to rabbit odor or odor from a sexually receptive estrus female. Briefly, animals were exposed to stimuli (estrous female / rabbit urine) for 30 minutes and sacrificed 2 hours after start of stimulation. Brain tissue was fixed using 4% PFA, frozen and sectioned at 40µm. Immunohistochemical labeling of Fos and AVP was performed on free floating sections. A cocktail of rabbit anti GFP and guinea pig anti AVP were used. Fos signal was developed using a cy3 bound secondary. AVP signal was amplified using a flurescein tyramide signal amplification system. The percentage (cell count/ total DAPI count *100) of AVP-ir, Fos-ir and AVP+Fos-ir neurons was calculated by an unbiased experimenter (Described in detail on Annexe on Pg 118,119).

ANOVA was utilized to analyze activation of MEApd neurons, with odor exposure as between-subject (exposed to either rabbit, n = 6 animals; or estrus odor = 5 animals) and neurochemical

identity (Fos-ir or AVP-ir) as within-subject sources of variance. ANOVA revealed significant main effect for both between-subject ($F_{(1,9)}$ = 8.09; $p$ = 0.019) and within-subject comparisons ($F_{(1,9)}$ = 39.03; $p$ < 0.001). The interaction between odor exposure and neurochemical identity was not significant ($F_{(1,9)}$ = 0.006; $p$ > 0.9).

Exposure to estrus odor significantly increased density of Fos-ir neurons (Figure 8A[8]; independent sample student's t-test; $p$ = 0.019). In addition estrus odor significantly enhanced the density of AVP neurons in the MEApd (Figure 8B; $p$ = 0.042; 27% increase).

Exposure to estrus odor significantly increased the number of colabelled neurons (Figure 9A[9]; $p$ = 0.019; 51% increase). The majority of recruited neurons post-estrus odor exposure concomitantly expressed AVP (% of Fos-ir neurons co- expressing AVP; rabbit odor: 24.3 ± 3.2 %; estrus odor: 64.5 ± 4.3%; $p$ < 0.001). Similarly, a greater proportion of AVP-ir neurons were recruited after the exposure to the estrus odor (% of AVP-ir neurons co-expressing Fos; rabbit odor: 15.5 ± 1.0 %; estrus odor: 45.6 ± 2.8%; $p$ < 0.001). In keeping with these observations, significant correlation was observed between Fos and AVP cell counts in animals exposed to estrus odor (Pearson's correlation; $R^2$ = 0.993, $p$ = 0.001), but not in animals exposed to rabbit odor ($R^2$ = 0.093, $p$ = 0.861). A representative image of the neuronal activation in the MEApd post exposure to rabbit urine and estrous female is included in the Annexe on Pg 104).

Next, I analyzed if enhanced co-labeling of Fos and AVP was merely a probabilistic result of simultaneous increase in density of both Fos and AVP neurons. We calculated expected density of co-labeled neurons (Described in detail in Annexe on Pg 120).

Expected density of Fos+AVP-ir neurons = Probability of Fos-ir x Proabality of AVP-ir

[8] Page 49
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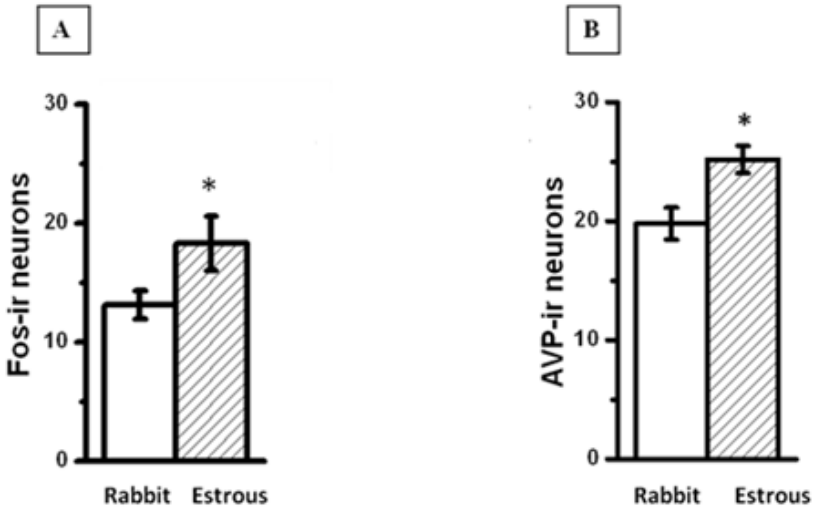


**Figure 8: Changes in the MEApd caused by sexual stimulus**

AVP has previously been described as a marker of social affiliation in the extended amygdala. I extended this result to the reproductive node of the MEA [i.e MEApd]. Males were exposed to either olfactory cues from an inaccessible estrous female (stripes; reproductive/ social affiliatory stimuli). A control group of males were exposed to rabbit urine (white; non affiliatory cue). Neurons expressing AVP and Fos were quantified (Fos-ir= Fos single labeled + colabeled neurons, AVP-ir= AVP single labeled + colabeled neurons). As expected affilitative cues such as estrous female activated more neurons in the MEApd as opposed to rabbit urine (Panel A Percentage Fos-ir ; Rabbit urine mean=12.14±0.82, Estrous female mean = 18.34±2.29 Copulation mean = 17.94±0.68 Independent sample t test $t_{(10)}$ =-2.793 * $p$ = 0.019). Exposure to estrous female caused a rapid (within 2 hours) increase in number of AVP neurons (Panel B Percentage AVP-ir ; Rabbit urine mean=19.78 ±1.34 , Estrous female mean= 25.17 ±1.15 Copulation mean= 22.85±1.69 Independent sample t test $t_{(11)}$ =-2.302 * $p$ = 0.042). n = 6 per treatment.

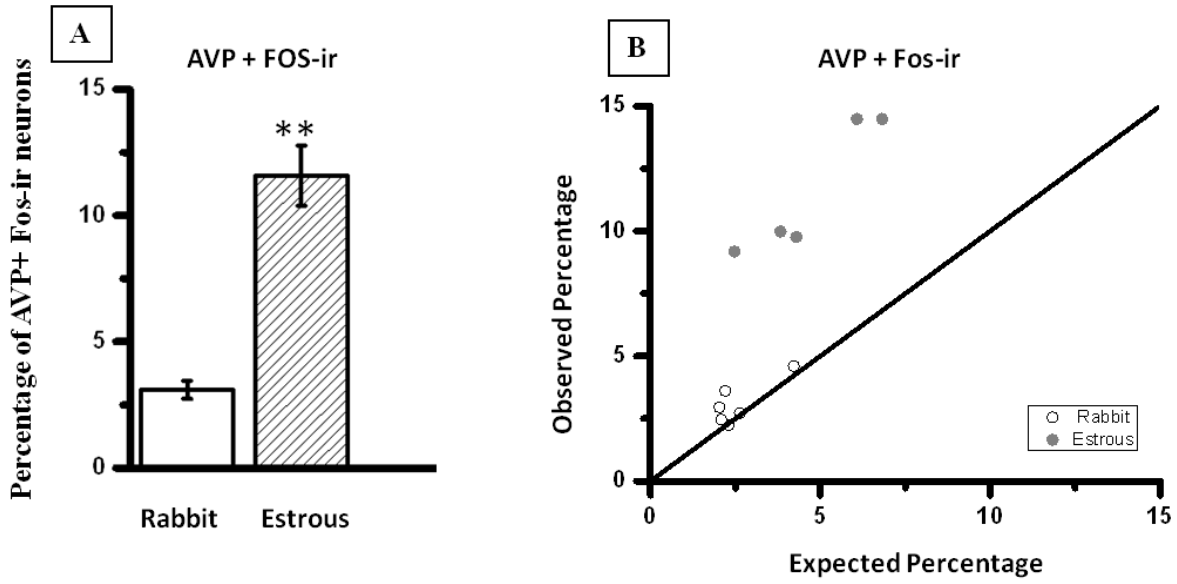


**Figure 9: AVP in the MEApd is activated by sexual stimulus**

AVP has previously been described as a marker of social affiliation in the extended amygdala. I extended this result to the reproductive node of the MEA (i.e MEApd). Males were exposed to either estrous female (patterned) (reproductive/ social affiliatory stimuli). A control group of males were exposed to rabbit urine (white) (non affiliatory cue). Colabeled neurons were quantified (colabeled = AVP+Fos-ir). Affiliative cues (estrous female and copulation) activated more AVP neurons in the MEApd as opposed to rabbit urine (Panel A Percentage AVP+Fos-ir ; Rabbit urine mean=3.09±0.35, Estrous female mean= 11.57±1.19 Independent sample t test $t_{(11)}$ =-8.239 ** $p < 0.001$). Expected frequency of colabeled neurons was calculated (Expected frequency= percentage AVP* percentage Fos). Such colabel is a probabilistic function of frequency of AVP-ir and Fos-ir neurons observed. Estrous (grey) stimulated samples showed a central divergence from the central blue line (Panel B). There is an exposure as between-subject sources of variance). The ANOVA revealed significant main effects and interaction ($F_{(1,9)} > 30$; $p < 0.001$). The expected and observed density of co-labeled neurons (n=6 per treatment) significantly greater than expected (Figure 9b; $p < 0.001$). Thus, recruitment of AVP neurons in the MEA post-exposure to estrous odor was selective and specific.

If Expected density= observed density; the occurrence of Fos+AVP-ir (colabelled neurons) can be attributed to a probabilistic event. The expected probability was compared with observed probabilities using a repeated measure ANOVA. Probabilities as within-subject and odor was not statically different when animals were exposed to rabbit odor (Figure 9B[9]; paired sample student's t test: $p = 0.08$). In contrast, observed co-labeling after exposure to the estrus odor was active biological mechanism driving the activation of AVP neurons. It is not a mere probabilistic occurrence.

Rodents use pheromones to communicate reproductive information like sexual attractiveness and receptivity. Pheromonal information is processed by both main and accessory olfactory system, leading to downstream activation of the amygdala. Amongst various nuclei of the extended amygdala, the MEA is especially important because it is the initial site during pheromonal processing where main and accessory olfactory information intersects [22, 97, 142, 143, 147-149, 154, 155]. Moreover, MEA robustly expresses androgen receptors and is sexually dimorphic in structure [97, 122, 149]. The androgen responsiveness suggests that medial amygdala has access to information and can plausibly integrate both reproductive status and pheromonal environment. In agreement with the important role of the MEA, lesions of this structure ablate penile erections in male rats in response to olfactorily available but physically inaccessible females, while leaving reflexive erections intact [143]. This suggests that medial amygdala is specifically involved in assessment of reproductive pheromones. Within the MEA, the MEApd selectively responds to pheromones of reproductive salience [17, 147, 148]. The information from MEApd is subsequently relayed to hypothalamic regions important for sexual behaviors. Despite the importance of this brain structure in sexual pheromone processing, the

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neurochemical identity of recruited neurons remains undetermined. Here we show that sexual stimuli selectively recruit AVP neurons in the MEApd.

The brain contains two distinct pools of the AVP neurons found in the hypothalamic and extra-hypothalamic regions. The hypothalamic AVP system regulates stress response and diurnal rhythmicity. For example, increased recruitment of AVP neurons in paraventricular nucleus of hypothalamus facilitates stress hormone secretion during challenging environment. In contrast to hypothalamic pool, the extra-hypothalamic AVP system is involved in affiliative behaviors in a variety of paradigms, for example monogamy in prairie voles [156], flocking in birds [157] and copulation in mice [85, 158]. Consistent with reproductive nature of these behaviors, extra-hypothalamic AVP is exquisitely responsive to the testosterone [74, 75, 122, 151-153]. Thus, castration drastically reduces AVP positive neurons and axons in extra-hypothalamic pool; an effect that is reversed by exogenous supplementation of testosterone post-castration. The effect of testosterone on AVP production is mediated by androgen-dependent suppression of DNA methylation on AVP promoters [99]. In this backdrop, we show that exposure to pheromones itself is able to recruit AVP neurons in MEApd, the actual act of copulation not being necessary. This is consistent with prior observations that testosterone acting within MEA promotes sexual arousal to the odor of female rats, without apparent effect on mating behavior itself [97]. It is thus plausible that AVP within MEApd serves as integrative node between reproductive status of the animal (signaled by testosterone) and availability of reproductive opportunities (signaled by recruitment of AVP neurons by sexual pheromones).

We demonstrate a rapid increase in the density of AVP neurons in the MEApd. This suggests that AVP in the MEApd is dynamically regulated by the pheromonal environment. Yet, this increase in AVP density is not sufficient to explain the full extent of MEApd-AVP activation.

This is borne out of the fact that greater a number of active neurons expresses AVP and the greater fraction of AVP neurons become active. Thus, AVP neurons are selectively recruited during exposure to sexually salient environmental signals. This is consistent with prior observations in desert finches expressing vasotocin, a neuropeptide homologous to the AVP [159]. These birds are opportunistic breeders similar to rats. In this species testosterone selectively enhances recruitment of the pre-existing vasotocin neurons in bed nucleus of stria terminalis.

Data presented here show that AVP neurons in MEA are selectively recruited during pheromonal processing. It is likely that these neurons play critical role in mediation of pheromone-directed reproductive behaviors. In addition this report provides further support to the role of extra-hypothalamic AVP neurons in reproductive and affiliative behaviors.

# 5. MEA Vasopressinergic System is Atypically Activated in Toxoplasma Infected Male Rats by Predatory Cues.

**Specific Aim :To determine role of medial amygdala vasopressinergic neurons in approach-avoidance shift in the infected animals.**

As indicated in the introduction (page 26), I propose Toxoplasma infection in male rats as a valuable perturbation model of social behavior. Interestingly, infected male rats lose the innate aversion to cat odors and instead develop an attraction [123, 124]. This gain of attraction in a subset of infected animals (see Figure 1, Page 33) is a rather interesting puzzle. It suggests that neural mechanisms of the infection cannot be explained merely by parasitic damage to limbic circuit. Such damage will fail to institute gain in attraction. Lack of demonstrable effects on olfaction, conditioned fear and learning support this notion [124]. Much of the neural machinery used for innate aversion to kairomones is also shared by olfactory and mnemonic behaviors [17, 101], thus it would be expected that mere damage to limbic circuits will be accompanied by a more generalized behavioral change. Related to this, parasite is known to be widely distributed in the brain [124].

So what is the nature of proximate mechanism if it is unlikely to be a direct anatomical damage? Comparison of Fos expression in control and infected animals present an intriguing possibility. As mentioned earlier, Fos expression has been used as a proxy for neuronal activity in many studies of innate fear. Cat odor activates a well-delineated brain circuit that pivots around MEApv [17]. The MEApv receives information from olfactory system [22, 23] and relays it to

hypothalamic nuclei [27]. Interestingly, anatomically contiguous and architecturally homologous MEApd exhibits robust Fos activity after exposure to reproductive pheromones (Figure 8 Panel A,[103, 145, 146]). In agreement with this parallel organization, control animals exhibit preferential activation of MEApv over MEApd upon cat odor exposure. Infected animals do not exhibit a significant difference in Fos activation of MEApv, but show elevated number of Fos immunoreactive neurons in MEApd. In other words, a sexual part of the MEA is hyper-activated in infected animals without much demonstrable difference in defensive part of the same brain region.

In the introduction, I have presented a case for the integration of approach and avoidance in a unified model of social decision making (see pages 18). Despite apparent divergence in the behavior itself, defensive and sexual behaviors likely represent extremes of an archetypical continuum between approach and avoidance. Placing Fos study described above in the context of this unified conceptual framework, I postulated that *Toxoplasma gondii* infection leads to a shift in balance or trade-off of approach and avoidance continuum. In other words, the infection likely mediates a switch in the valence associated with cat urine. This could be manifested as a shift towards approach which is then observed as an attraction towards cat odor. In the previous chapter, I demonstrate that AVP in MEApd is selectively recruited by sexual stimuli such as exposure to an estrous female. AVP is also up-regulated by testosterone [98, 160], a testicular steroid that is enhanced post-infection [135]. In this context, I hypothesized that behavioral change in innate fear by Toxoplasma is caused by an increased activation of the MEApd AVP system.

Male rats of six-eight weeks age were used (n = 6 mock-infected controls, n = 10 infected with 5 X $10^6$ Toxoplasma tachyzoites). They were exposed to cat urine for 30 minute (7 weeks post-

infection) and sacrificed 90 minute after termination of exposure. Basal levels of neuronalactivation were quantified in animals not exposed to any stimuli. Brain tissue was transcardially perfused with 4% paraformaldehyde, cryopreseserved and sectioned at 40 µM. Immunohistochemical labeling for AVP and Fos was performed by staining with guinea pig anti AVP and sheep anti Fos primary antibodies. AVP signal was amplified using a tyramide signal amplification kit with a FITC flurophore while the Fos signal was processed using a red flurophore bound to secondary antibody. Sections were imaged at the confocal microscope with images acquired at successive 4 µM intervals. Number of total neurons (using DAPI counter-stain) and neurons immunoreactive to AVP or Fos were quantified. A subset of neurons was found to be immunoreactive to both Fos and AVP simultaneously; these were also quantified separately. Caution was taken not to count the same neurons across multiple z stacks. Imaging was performed using 40X objective with a 1.2 X digital zoom. Three fields each were counted and averaged from the MEApd and MEApv of each animal. Counting was randomized for laterality. (Described in detail in Annexe on Pg107,118,119).

Percentages were calculated using total DAPi counts such that

$$\% \text{ Fos-ir} = \frac{\text{total number of Fos-ir neurons}}{\text{Total DAPI Count}} * 100$$

Basal levels of neuronal activity in the MEApv and MEApd of control and infected animals was quantified. This was done in animals that were exposed were not exposed to any stimulus.

Repeated measure ANOVA showed that there was no significant effect of brain region or treatment (Figure 10[10]; $t_{(13)} = 0.671$ $p = 0.514$, independent sample t-test).Repeated measure ANOVA showed that there was no significant effect of brain region (MEApd or MEApv) on Fos -ir counts ($F_{(1.14)} = 0.399$, $p = 0.538$). There was, however, a significanteffect of interaction between the brain region and infection ($F_{(1.14)} = 7.092$ $p = 0.019$). Infected animals exhibited greater expression of Fos-ir neurons on MEApd compared to MEApv (Figure 11[11]; $t_{(7)} = 2.805$ $p < 0.05$, paired sample t-test; MEApd > MEApv in 7 out of 10 animals). The difference between the MEApd and MEApv did not reach statistical significance in the control group, with 1 out 6 animals exhibiting a greater Fos-ir in MEApd ($p = 0.2$). The number of Fos-ir neurons in MEApv was not significantly different between control and infected animals, confirming previously published research. Infected animals did not exhibit a greater number of Fos-ir neurons in MEApd compared to control (Figure S1[S1]; $t_{(13)} = 1.420$ $p = 0.179$ independent sample t-test).

The MEApd is crucial part of extra-hypothalamic parvocellular AVP system, containing robust expression of AVP in neurons. In agreement, I observed greater number of AVP immunoreactive (AVP-ir) neurons in MEApd compared to MEApv. Number of AVP-ir neurons in MEApv was not statistically different between control and infected groups (Figure 12[12]; $t_{(13)} = 1.208$ $p = 0.248$ independent sample t-test). Number of AVP-ir neurons in MEApd also did not reach statistical significance for the difference between control and infected groups (Figure 12; $t_{(11)} = -0.270$, $p = 0.792$ independent sample t-test)

[10] Page 58
[11] Page 59
[S1] Page 103
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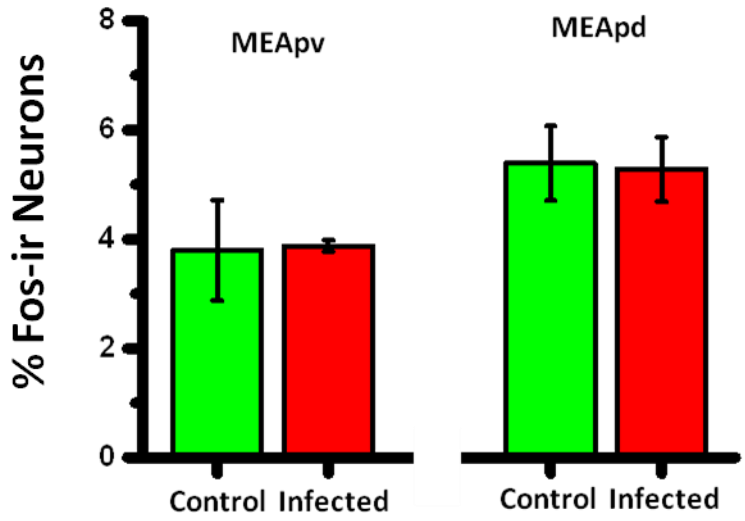


**Figure 10: Basal expression of Fos did not differ between control and infected males**

Males were infected with Toxoplasma. Seven weeks post infection infected and control males sacrificed and tissue were perfused. Fos was used as marker of neuronal activation. Basal levels (without presentation of any stimulus) of Fos-ir neurons were quantified in the MEApv and MEApd. This is a read out of Fos expression merely due to experimental conditions such as handling. There was no difference in the Fos–ir neuron count in the MEApv [Percentage Fos; Control mean =3.79±0.92 Infected mean = 3.87±0.1 Independent sample T test $p$= 0.933]. There was no difference in the Fos–ir neuron count in the MEApd either [Panel B Percentage Fos; Control mean =5.388±0.68 Infected mean = 5.27±0.59 T test $p$= 0.906].

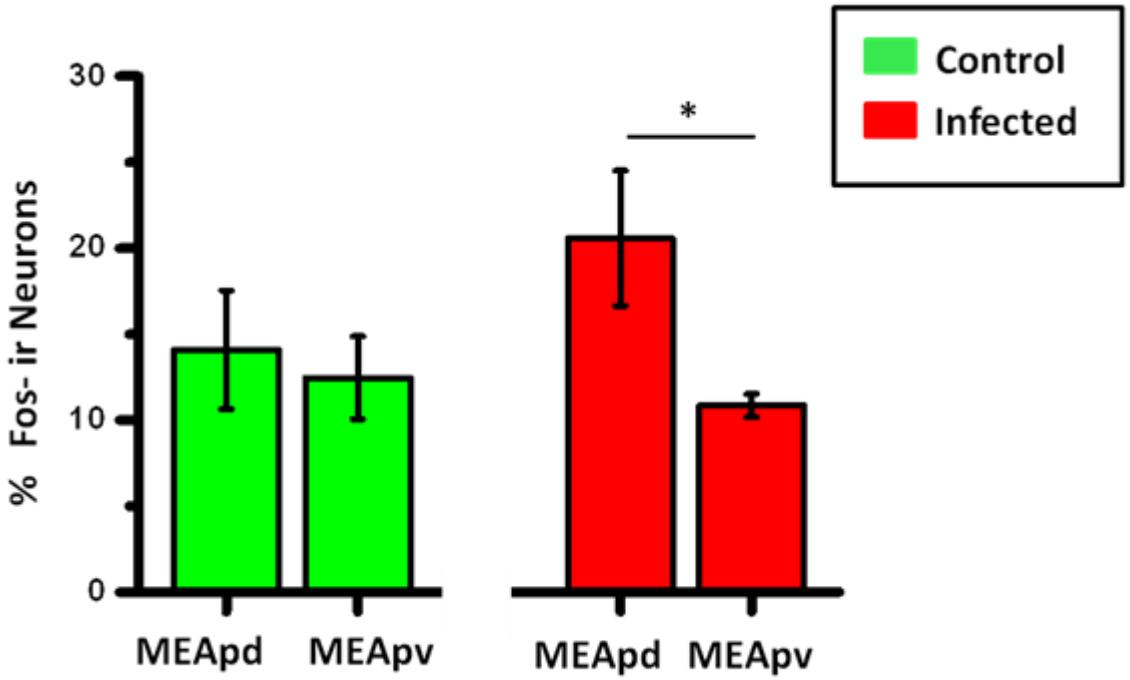


**Figure 11: Toxoplasma alters selectively activation of the MEApd post cat exposure**

Males were infected with Toxoplasma (n = 10 infected, n = 6 control). Seven weeks post infection infected and control males were exposed to cat urine for 30 minutes. Two hours after the start of stimulus exposure animals was sacrificed and tissue were perfused. Fos was used as marker of neuronal activation and Fos-ir neurons were quantified in the MEApv and MEApd (Fos-ir= Fos single labeled + colabeled neurons). All counts were normalized to the total number of DAPI stained nuclei counted. There was no significant difference in Fos-ir counts in the MEApv between control and infected animals ($p$ = 0.2). Infected animals exhibited greater expression of Fos immunoreactive (Fos-ir) neurons on MEApd compared to MEApv ($t_{(7)}$ =2.805 *$p$ < 0.05, paired sample t-test; MEApd > MEApv in 7 out of 10 animals).

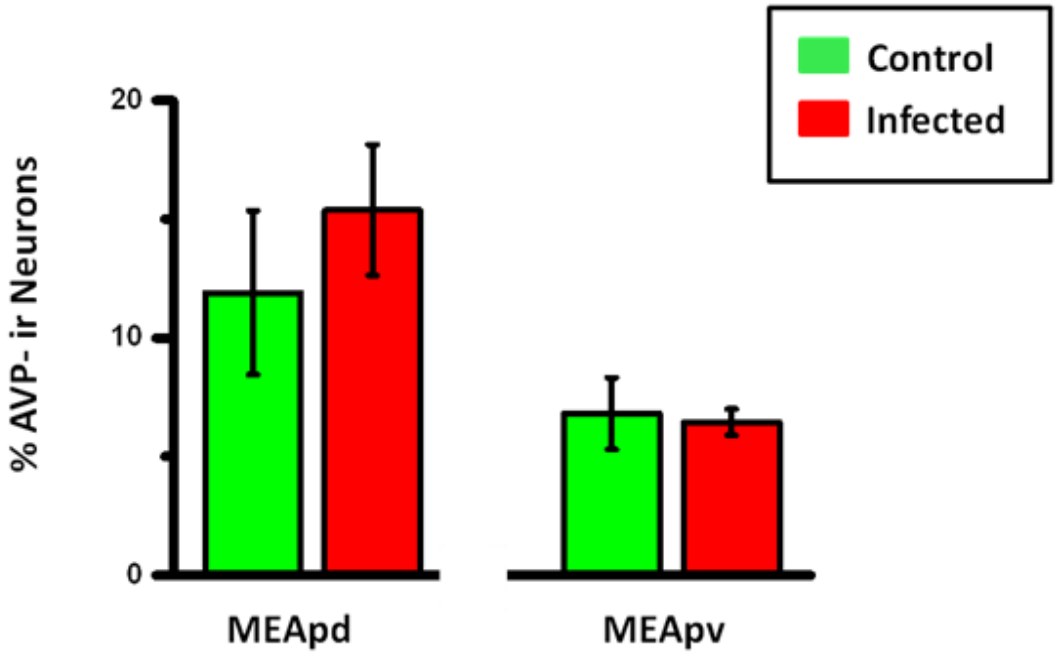


**Figure 12: Toxoplasma does not change AVP-ir populations in either subnuclei of the MEA**

Males were infected with Toxoplasma (n =10 infected, n = 6 control). Males were infected with Toxoplasma. Seven weeks post infection, infected and control males were exposed to cat urine for 30 minutes. Two hours after the start of stimulus exposure animals were sacrificed and tissue was perfused. AVP-ir neurons were quantified in the MEApv and MEApd (AVP-ir = AVP single labeled + colabeled neurons). All counts were normalized to the total number of DAPI stained nuclei counted. There was no significant difference in AVP-ir counts between control and infected animals in the MEApd ($t_{(11)} = -0.270$, $p = 0.792$ Independent sample t-test) or the in the MEApv ($t_{(13)} = 1.208$, $p = 0.248$ independent sample t-test)

Data presented in preceding two paragraphs pertains to the number of Fos-ir and AVP-ir neurons respectively, regardless whether same neurons expressed both AVP and Fos. This later group of (colabeled) neurons is of particular interest because it refers to AVP-ir neurons that were recruited during cat odor exposure. ANOVA revealed that incidence of such co-labeled neurons was greater in MEApd compared to MEApv ($F_{(1,12)}$ = 8.594; p = 0.013); marginal means; MEApd = 8.157, MEApv = 4.290). Further examination revealed that the infection did not significantly affect number of co-labeling neurons in MEApv ($t_{(11)}$ =-2.42 p = 0.813 independent sample t-test; Figure 13[13] Panel A). In contrast, infection robustly enhanced co-labeled neurons in MEApd ($t_{(13)}$ =2.535 p = 0.024 independent sample t-test; Figure 13).In fact, median of infected group was placed considerably elevated over the 75th percentile of the control group (Figure 13 panel B). This difference in MEApv and MEApd activation in infected males is represented in Figure S4 on Pg 106).

It is pertinent to ask if the increase in co-labeled neurons with respect to brain region or experimental treatment is merely a reflection of increase in probability when Fos-ir and/or AVP-ir neurons are increased. In other words, the algebraic probability of co-labeled neurons is mathematical product of the individual probabilities of Fos-ir and AVP-ir. Thus increase in Fos-ir and/or AVP-ir neurons will increase co-labeled neurons without any biological underpinning. In view of this confounding factor, I analyzed observed incidence of co-labeled neurons with expected probability derived by multiplication of probability of finding Fos-ir and AVP-ir neurons. Figure 14 depicts this comparison (Described in detail on Annexe on Pg 120). In case of MEApv, observed incidence of co-labeling was smaller than expected (Figure 14; 15 out of 16

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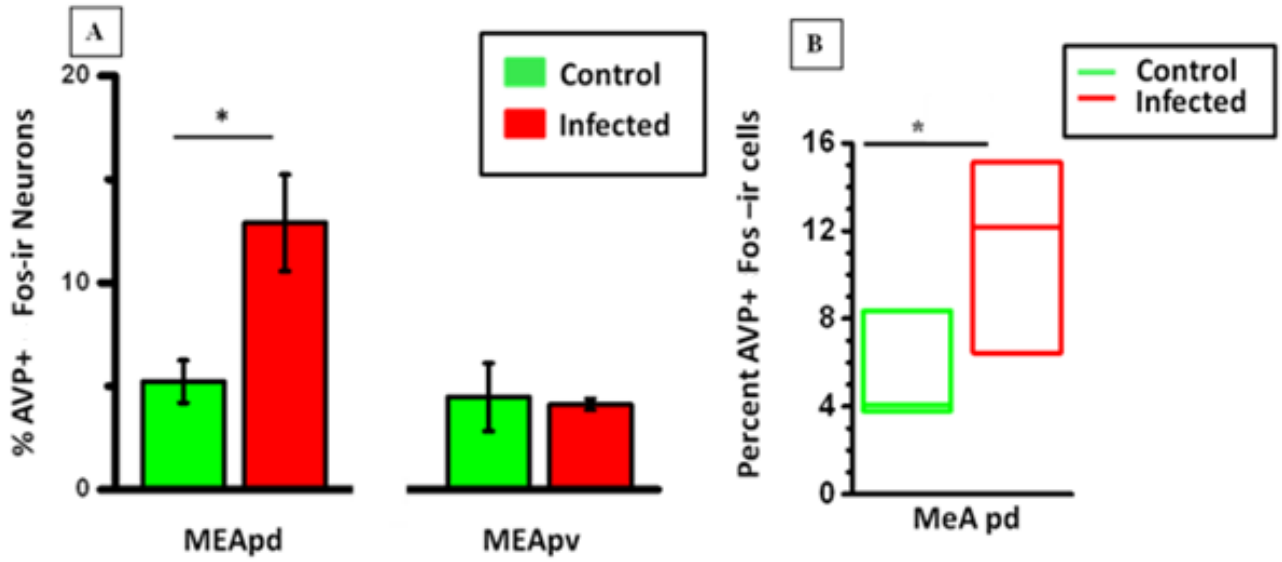


**Figure 13: Toxoplasma infection induced atypic activation of the MEApd post cat exposure is specific to the vasopressinergic system**

Infected and control males were exposed to cat urine for 30 minutes. Two hours post the start of stimulus exposure animals was sacrificed and tissue were perfused. AVP and Fos colabeled neurons were quantified in the MEApv and MEApd (colabeled neurons = AVP+Fos-ir / DAPI, %). (Panel A) The number of co-labeled neurons was greater in MEApd compared to MEApv (Repeated Measure ANOVA $F_{(1,12)} = 8.594$; $p = 0.013$). Infection causes an increase in colabeled neurons in the MEApd (Independent sample t-test $t_{(13)} = 2.535$ *$p = 0.024$). This effect was not seen in the MEApv ($t_{(11)} = -2.42$ $p = 0.813$ independent sample t-test). The difference between percent of colabeled neurons in the MEApd of control and infected animals is so marked that median of infected group was placed considerably elevated over 75$^{th}$ percentile of the control group (Panel B).

animals, control and infected inclusive; $p$ = 0,001, exact binomial test for null hypothesis of random chance between expected > observed and observed > expected). In contrast, expected probability of co-labeling did not account for over-abundance of co-labeled cells in the MEApd (Figure 14[14]; 16 out of 16 animals; $p < 0.0001$). Amongst experimental groups, infected animals exhibited substantially more co-labeling compared to control in MEApd (148% increase mean over mean; $t_{(13)} = 2.535$ $p = 0.024$ independent sample t-test). In fact, 8 out of 10 infected animals had greater number of co-labeled neurons than maxima of control group (Figure 13).

In addition to the MEA, I also quantified Fos-ir, AVP-ir and co-labeled neurons in downstream targets of the MEA; the medial bed nucleus of stria terminalis and sub-divisions of venteromedial hypothalamus. Infection did not produce significant alterations in any of these brain regions (Table 1 page 66). Similarly, basal Fos expression in absence of cat odor stimulus also did not differ significantly between control and infected animals (Figure S1 in Annexe page 103).

Toxoplasma induced behavioral changes have long fascinated scholarly pursuits. Yet, the underlying machinery has been under explained (reviewed in [161]). Data presented in the present chapter provides greater clarity about how Toxoplasma infection leads to both loss of function and gain of function. Pertinently, my previous work (preceding chapter) and work of others demonstrate that MEApd co-labeled neurons are selectively increased upon exposure to affiliative stimuli [103, 145, 146]. This suggests that extra-hypothalamic AVP neurons are preferentially recruited during sexual behavior. It is consistent with requirement of testosterone for sustained AVP production in extended amygdala [98, 160]. In this formulation, non-hypothalamic AVP in the brain can be viewed as interface between physiological milieu

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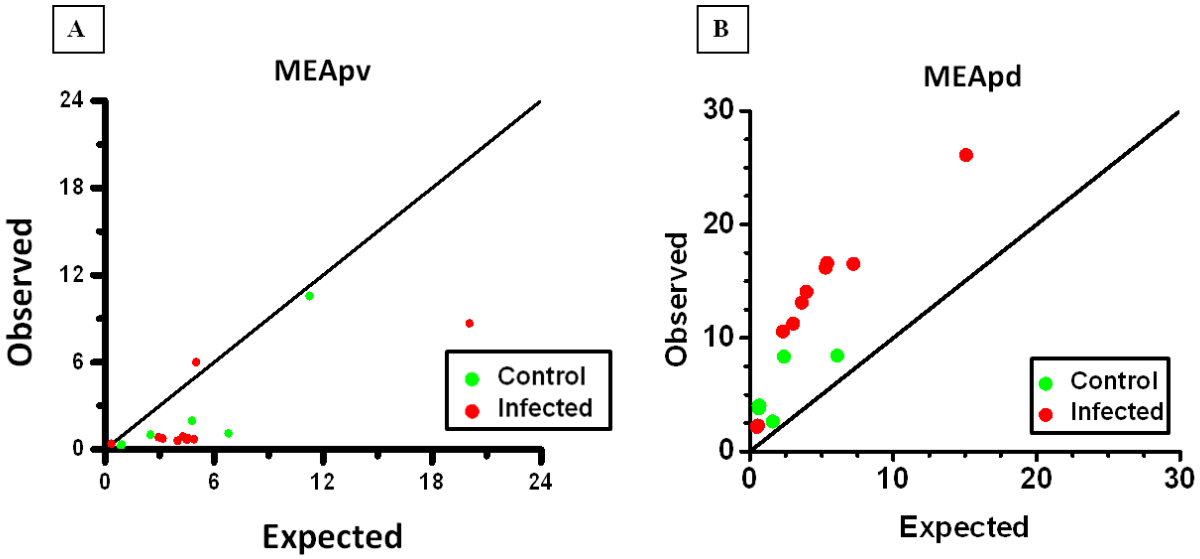



**Figure 14: Increased colabeled neurons in the MEApd of increased animals is driven by an active biological**

Expected frequency of colabeled neurons was calculated based on frequency of AVP-ir and Fos-ir neurons observed (Expected frequency = % AVP-ir X % Fos-ir). Comparison of observed and expected frequency can dissect the involvement of a biological process as opposed to combinatorial probability. In the MEApv, observed incidence of co-labeling was smaller than expected (Panel A 15 out of 16 animals, control and infected inclusive; $p = 0.001$, exact binomial test for null hypothesis of random chance between expected > observed and observed > expected). In contrast, expected probability of co-labeling did not account for over-abundance of co-labeled cells in the MEApd (Panel B 16 out of 16 animals; $p < 0.0001$). This demonstrates that there is an active biological mechanism driving the activation of AVP neurons. It is not a mere probabilistic occurrence.

(testosterone) and sexual behavior. Interestingly, Toxoplasma infection increases testosterone production in infected male, in a coordinated manner by its effect on the steroidogenic machinery of the testes [135]. Thus I suggest that greater testosterone post-infection initiates leakiness between neural substrates of defensive and sexual behavior. In this manner, kairomones that usually release defensive behavior in control animals start to activate sexual brain, resulting in blunting of aversion in infected group and even gain of attraction in a subset of animals.

What does it say about organization of defensive and sexual behavior beyond the idiosyncratic changes in an infected brain? These findings suggest the existence of a coordinated biological system that negotiates approach and avoidance in the ambiguous and ambivalent environment of most animals. Close parallelism of the defensive and reproductive system [100], and the fact that it is possible for a co-evolved parasite to produce leakiness in these parallel circuits, demonstrates that approach and avoidance are not unitary isolated behaviors. Rather these behaviors, in dynamic negotiation, are mediated by pliable neural substrates that show the capacity to change according to endocrine and environmental ambience (and to be manipulated).

| | **Control** | **Infected** | *p* |
|---|---|---|---|
| **BNSTm** | | | |
| Number of Fos –ir cells | 18.9 ± 3.2 | 18.1 ± 2.7 | 0.84 |
| Number of AVP –ir cells | 15.21 ± 2.5 | 15.6 ± 3.7 | 0.93 |
| Number of colabeled cells | 9.4 ± 2.1 | 6.9 ± 1.9 | 0.41 |
| **VMH dm** | | | |
| Number of Fos –ir cells | 22.1 ± 3.3 | 29.0 ± 3.7 | 0.22 |
| Number of AVP –ir cells | 10.8 ± 1.4 | 12.7 ± 3.4 | 0.65 |
| Number of colabeled cells | 7.2 ± 1.2 | 8.3 ± 2.5 | 0.74 |
| **VMH vl** | | | |
| Number of Fos–ir cells | 28.8 ± 5.2 | 36.8 ± 3.4 | 0.20 |
| Number of AVP –ir cells | 12.4 ± 4.3 | 18.4 ± 3.0 | 0.27 |
| Number of colabeled cells | 9.1 ± 3.7 | 12.1 ± 2.8 | 0.51 |

**Table 1: Cell counts in other nodes of the SBN**

Males were infected with Toxoplasma. Seven weeks post infection infected and control males were exposed to cat urine for 30 minutes. After 90 minutes animals were sacrificed and tissue were perfused. Fos-ir, AVP-ir and AVP+Fos-ir (colabeled) neurons were previously quantified in the MEApv and MEApd. There was a significant increase in the percentage of Fos-ir and colabeled neurons in the MEApd of infected animals. This study was extended to the downstream regions the VMHdm (normally processes predatory cues), the VMHvl (normally processes sexual cues) and associated region the BNSTm. The table above shows that there was no significant difference between the any of the neuron populations in the BNSTm, VMHdm or VMHvl. This shows that infection induced changes in activation on AVP are restricted to the MEApd.

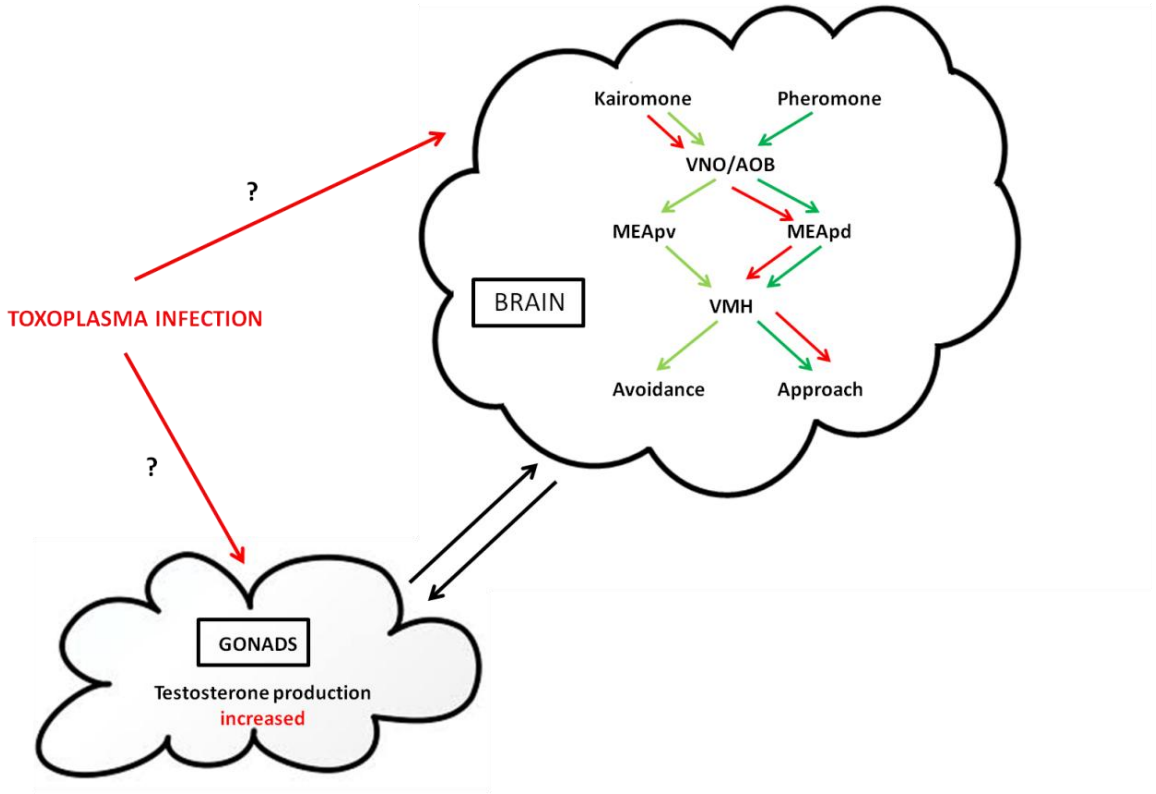



**Figure 15: Model of Toxoplasma induced changes in defensive behavior**

Toxoplasma infected males are attracted to cat odor. My results suggest that this is due to atypical activation of the MEApd (light green = defensive circuit in control, dark green = sexual circuit in control, red= defensive circuits in infected males). Atypical activation is restricted to the AVP neurons in the MEApd. The MEA is important in evaluating valence of stimuli. Additional recruitment of sexual neurons here might cause a shift towards attraction in output behavior. Toxoplasma infection also causes an increase testosterone. Testosterone and MEA reciprocally regulate each other. Chronic parasitic infection is observed in both the brain and testes. It still needs to be elucidated whether gonadal infection causes the changes seen in the brain or vice versa

# 6. Epigenetic changes in MEA AVP are necessary and sufficient for Toxoplasma infection induced attraction to cat odor.

**Specific Aim: To delineate the molecular proximate mediator of behavioral change pertaining to medial amygdala vasopressinergic system.**

AVP was initially studied for its role as an antidiuretic hormone in the periphery [162]. It subsequently became clear that central AVP had an additional role in regulation of physiology and behavior (reviewed in [6, 108, 163]. Amongst its role in central nervous system, hypothalamic magnocellaular AVP neurons mainly reside in periventricular hypothalamus [42, 164]. This pool of AVP neurons play crucial role on stress hormone secretion through its facilitatory action on hypothalamus-pituitary-adrenal axis in concert with corticotropin releasing factor [164, 165]. AVP expressing neurons are also found in extra-hypothalamic sites, especially parts of extended amygdala including MEA and BNST [85, 98, 151]. This pool of AVP neurons mainly consists of parvocellular neurons. These target (see figure below) nodes that have established roles in social and reward behavior such vertical limb of the diagonal band of Broca, olfactory tubercle , lateral septum, mediodorsal thalamic nucleus, central gray, dorsal raphe nucleus and locus coeruleus [98].

Vasopressinergic fibers originating from the MEA and BNST require testosterone for sustained production of AVP. Thus, castration abolishes AVP production in these brain structures; and castration plus exogenous testosterone reinstate it. The drop in AVP levels post-castration is due

to lower production and not enhanced release [160, 166]. This is borne out of the observation that co-administration of colchicine, an axonal transport blocker, does not rescue effects of testosterone on AVP levels [166]. Interestingly, testosterone mediates AVP production through an epigenetic change in DNA methylation in BNST, a part of extended amygdala homologous to MEA [167]. Briefly, AVP is transcribed from a gene that also codes for neurohypophysin and copeptin. Post translational modification of the transcript cleaves away the AVP. This gene has at least four sites in promoter region (GenBank no: AF112363.1) that exhibit CpG islands, potential locations of DNA methylation. Castration creates hypermethylation at two of these four CpG sites, as revealed by higher protection to methylation-specific restriction enzymes [160].

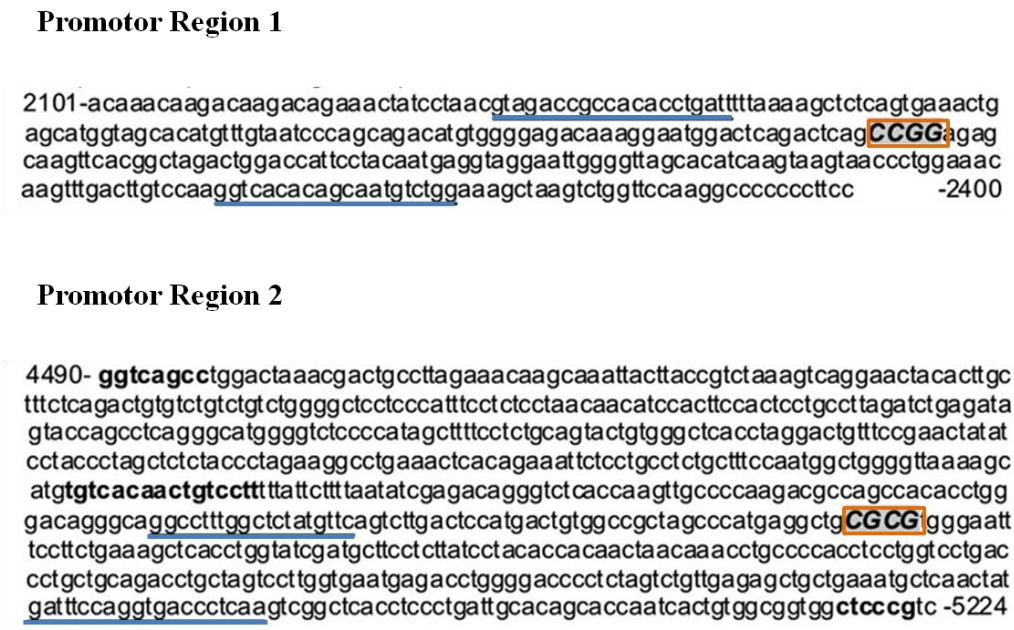



**Figure D: Methylation and primer binding sites in promoter region 1 and promoter region 2 of AVP promoter (GenBank no: AF112363.1). *Adopted from [160]**

Castration simultaneously reduces transcription of the AVP gene. Supplementation of exogenous testosterone along with castration reverses the hypomethylation (induced by castration) and transcriptional changes [160]. This demonstrates that testosterone and/or its metabolites cause epigenetic change in the promoter of AVP genes, leading to enhanced transcription.

This brings us back to the question of the proximate mechanisms of Toxoplasma induced behavioral changes in rats (see chapter 3 for behavioral change). In chapter 3, I describe increase in testicular production of testosterone post-infection. In chapter 4, I describe role of amygdalar AVP in sexual behaviors. In chapter 5, I then show enhanced recruitment of AVP neurons in MEApd post infection, likely creating leakiness between defensive and sexual behaviors. In view of these observations, I hypothesized that Toxoplasma infection leads to hypomethylation in MEApd-AVP. I further hypothesizes that an epigenetic change is necessary and sufficient to create change in defensive behaviors.

One of the most commonly used methods to detect local DNA methylation changes is the bisulfite sequencing technique. This involves the differential conversion of cytosine or methylated cytosine to uracil upon treatment with sodium bisulfite. qPCR amplification and sequencing can detect and quantify this differential conversion. While methylated cytosines are sequenced as cytosines, unmethylated cytosines are sequenced as thymines. However bisulfite treatment is harsh and can cause a reduced DNA yield. Micropunches of the MEApd yield very sparse starting material. Any further reduction in yield is undesirable. This can be overcome by pooling tissue from multiple animals. Such an averaging event might cause group inter differences to get diluted. Hence I chose an enzyme based strategy. Quantification of promoter methylation was performed by methylation specific restriction enzyme (MSRE) digestion followed by quantitative PCR, using previously published methods and primers[160]. Genomic

DNA was isolated from the MEApd using the Phenol choloroform isoamyl alcohol method. The two androgen dependent promoters contained sites for binding of methylation-specific restriction enzymes HpaII (promoter region 1 in Figure 16) and BstUI (promoter region 2). Controls without application of restriction enzymes were run simultaneously. 600ng of gDNA was treated with either restriction enzyme or buffer for 1-3 hours. Subsequently, a quantitative PCR was used to quantify extent of cleavage of template DNA (Detailed description in the Annexe in page 124, 125, 126, 128). Briefly described, increased methylation would protect template DNA from cleaving. Such a sample would require fewer cycle numbers to cross threshold fluorescence during a qPCR reaction. If the promoter site was completely methylated there would be no cleavage of the DNA and hence Ct value of enzyme treated and sans-enzyme samples would be equal. Such a sample would lie on the central line in Figure 16[16] and 17[17]. Divergence from the central line towards the ordinate depicts increasing amount of hypomethylation.

Treatment with HpaII increased Ct value in infected animals, relative to no-HpaII samples (Figure 16[16] Panel A; 8 out of 8 animals; $p = 0.008$, exact binomial test, null hypothesis being random chance of enzyme > no-enzyme and no-enzyme > enzyme). Seven out eight control animals also exhibited increase in Ct value due to HpaII application, although the data did not reach statistical significance in this case (Figure 16; $p > 0.05$). Taken together these observations suggest that promoter site 1 is not completely methylated in rat MEApd. An arbitrary index of departure from chance ($x = y$; blue line in Figure 15 panel A) was calculated as $(x - y)^2$ divided by $(x + y)^2$, where x and y are Ct value without HpaII and with HpaII, respectively. Amongst experimental groups, infected animals exhibited greater departure (mean ± SEM: control =

[16] Page 73
[17] Page 74

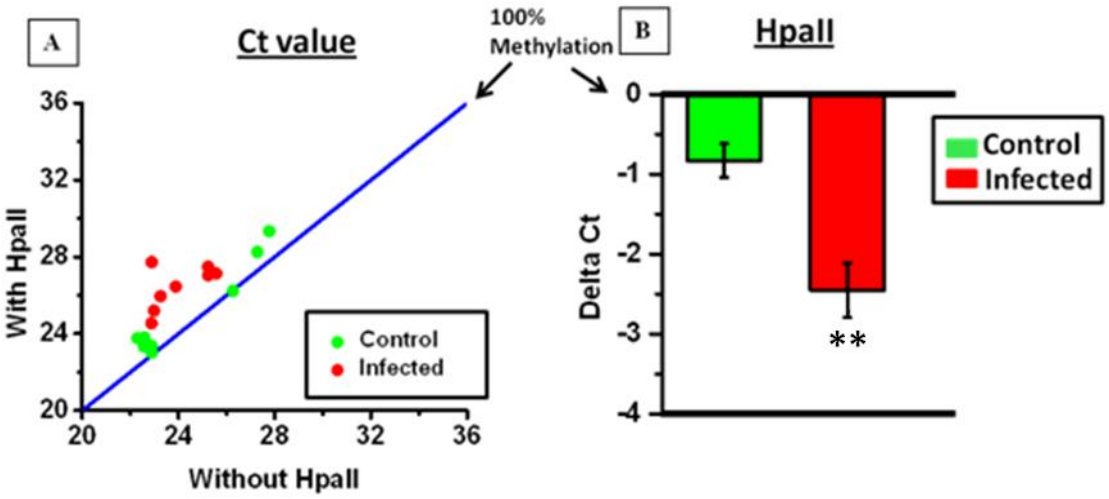



**Figure 16: Infection cause epigenetic hypomethylation of AVP Promoter Region 1**

AVP Promoter region 1 contained a CCGG site. Genomic DNA was isolated from the MEApd. Methylation specific restriction digestion by HpaII followed by qPCR was used to quantify methylation status. No enzyme treatment controls were run for each sample. If Ct enzyme=Ct no enzyme the DNA is completely methylated and lies on the central blue line [Panel A]. Amongst experimental groups, infected animals exhibited greater departure from the central (Divergence from line (arbitrary units)= $(x - y)^2$ divided by $(x + y)^2$, where x and y are Ct value without HpaII and with HpaII, respectively Mean ± SEM; control = 0.0004 ± 0.00013, infected = 0.0028 ± 0.00095; $t_{(14)} = 2.45$, $p < 0.05$, independent sample t-test). Threshold cycles (normalized to non-restriction enzyme treatment samples) were plotted for both control (green) and infected (red) males. Delta Ct= Ct No Enzyme treatment sample – Ct for Enzyme treated samples. A greater delta Ct value implies increased cleavage of template DNA and therefore reduced methylation. The delta Ct values were lower for infected samples when compared with their controls [Panel B]. [Promoter region1; Delta Ct; Control mean = -0.83±0.21, Infected mean = -2.45±0.34 ** $p$ = 0.002 (REST)]. This shows that in infected males methylation of promoter region 1 was 3 times lower than in the controls. (n = 8 infected and 8 control).

0.0004 ± 0.00013, infected = 0.0028 ± 0.00095; $t_{14} = 2.45$, $p < 0.05$, independent sample t-test). Consistent with this, infected animals demonstrated greater magnitude of ΔCt values (Ct value for sans-enzyme substrate from Ct value for corresponding enzyme-treated samples; Figure 16 Panel B; $p = 0.002$, calculated using randomized significance testing in REST). This inter-group difference was striking in that 75$^{th}$ percentile of ΔCt values in infected group was placed below 25$^{th}$ percentile of control animals. These observations suggest that *Toxoplasma gondii* infection creates hypomethylation at the promoter site 1 of AVP gene in MEApd.

Congruent results were observed in case of promoter site 2 after treatment with BstUI. All animals in both control and infected groups exhibited increased Ct values after treatment with the restriction enzyme (Figure 17[17] Panel A; $p < 0.008$ for both control and infected, exact binomial test). Thus, similar to promoter site 1, the present site is also incompletely methylated in gonad-intact male rats. Amongst experimental groups, infection amplified the divergence between observed and chance Ct values (chance being x = y, see preceding paragraph; divergence: control = 0.0009 ± 0.00019, infected = 0.0067 ± 0.00249; $t_{14} = 2.39$, $p < 0.05$). Infected animals also demonstrated greater magnitude of ΔCt values compared to controls (Figure 17 Panel B; $p < 0.001$, REST). 75$^{th}$ percentile of ΔCt values in infected group was placed below 25$^{th}$ percentile of control animals, an observation that was similar to that observed for promoter site 1 (Figure 16).

Data presented in preceding two paragraphs demonstrate that Toxoplasma infection creates an epigenetic change by reducing DNA methylation of AVP promoter in MEApd. Methylation of promoter likely creates a stearic hindrance to the transcriptional machinery, thereby reducing AVP mRNA synthesis. Hence, I hypothesized that hypomethylation in the infected animals enhances AVP transcription. In order to investigate this, I extracted mRNA from the MEApd of

[17] Page 74

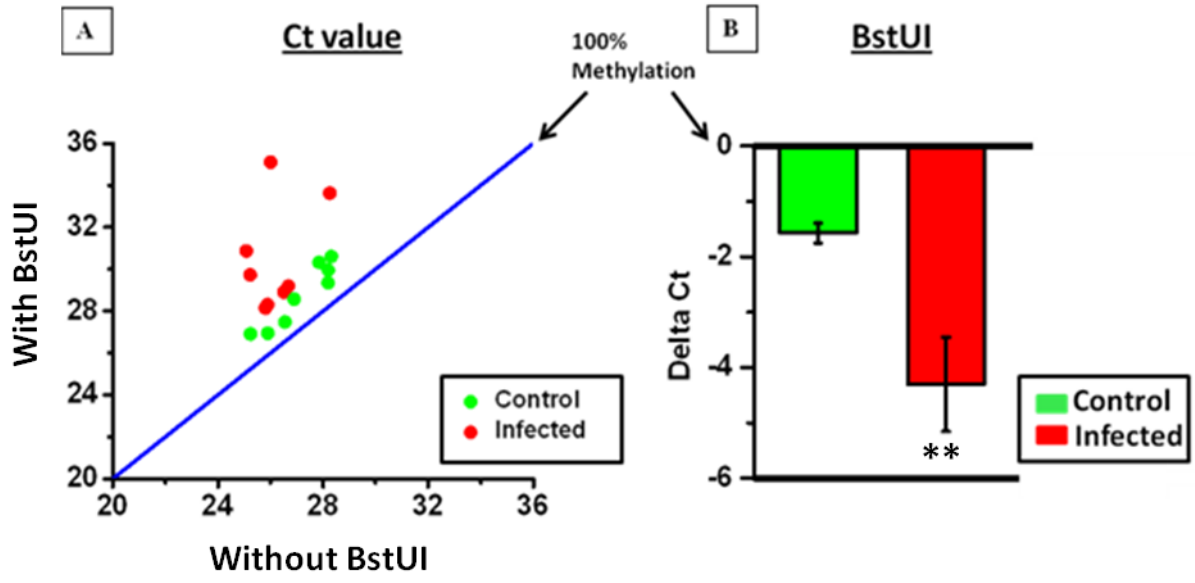



**Figure 17: Infection cause epigenetic hypomethylation of AVP Promoter Region 2**

AVP Promoter region 2 site contained a CGCG site. Genomic DNA was isolated from the MEApd. .Methylation specific restriction digestion by BstUI followed by qPCR was used to quantify methylation status. No enzyme treatment controls were run for each sample. If Ct enzyme = Ct no enzyme the DNA is completely methylated and lies on the central blue line [Panel A]. Amongst experimental groups, infected animals exhibited greater departure from the central (Divergence from line (arbitrary units) = $(x - y)^2$ divided by $(x + y)^2$, where x and y are Ct value without BstUI and with BstUI, respectively Mean ± SEM; control = 0.0009 ± 0.00019, infected = 0.006 ± 0.00249; $t_{14} = 2.39$, $p < 0.05$)**.** Threshold cycles (normalized to non-restriction enzyme treatment samples) were plotted for both control (green) and infected (red) males. Delta Ct = Ct No Enzyme treatment sample – Ct for Enzyme treated samples. The delta Ct values were lower for infected samples when compared with their controls (Panel B Promoter region 2; Delta Ct; Control mean= -1.57±0.18, Infected mean= -4.3±0.85 ***p* = 0.001 REST). control and infected animals (7 weeks post-infection) using the Trizol method. This was

followed by cDNA synthesis using a commercially available kit (from Invitrogen). Quantitative PCR was used to compare transcript levels of AVP and a house-keeping gene HPRT (Described This shows that in infected males methylation of promoter region 2 was 6 times lower than in the controls. (n=8 infected and 8 control).

in detail in Annexe Pg 122 126 128). Difference between AVP and HPRT Ct values was used as index for AVP transcription (ΔCt value). cDNA from infected animals required fewer PCR cycles to reach a pre-determined threshold (ΔCt; Figure 18[18]; $p < 0.05$, REST), suggesting enhanced MEApd-AVP transcription. Level of cDNA in PVN was not different between control and infected animals (Figure 19[19]; $p > 0.05$). This suggest that infection-mediated increase in AVP transcription was specific to MEApd, being absent in PVN. This is consistent with prior observations that hypothalamic AVP, in contrast to AVP in extended amygdala, is not responsive to testosterone; and that hypothalamic AVP is involved in stress regulation and not in sexual behaviors.

Necessity of hypomethylation in infection-induced behavioral effects was further tested by preventing it using systemic administration of L-methionine. Systemic L-methionine creates global hypermethylation of the DNA (previously used in [168, 169] . The systematic route of administration was chosen over localized administration to MEApd because of the concern that immunological upheaval during intra-cranial surgery would initiate recrudescence of cystic Toxoplasma in the brain. Wide-scale recrudescence might interfere with behavioral measurement by regressing the infection to acute phase rather than chronic infection associated with Toxoplasma induced behavioral changes. A two-by-two experimental design was employed (control or infected; injected with L-methionine or placebo; 10 animals each group, total 40

[18] Page 76
[19] Page 78

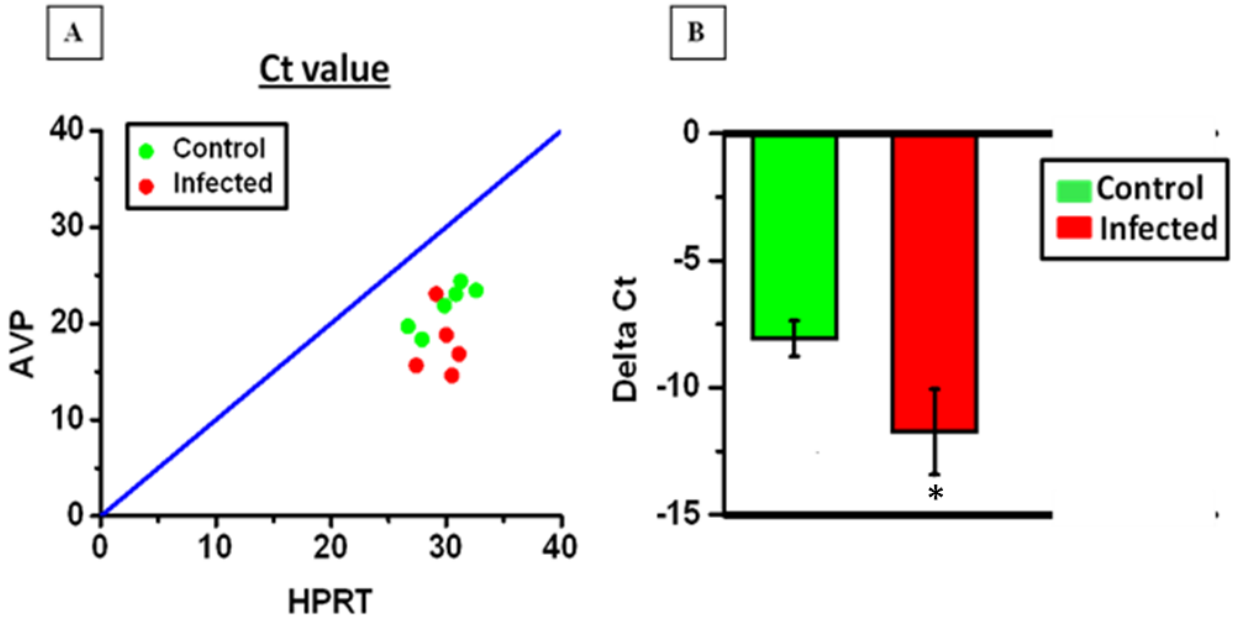



**Figure 18: Infection induced increase in AVP mRNA in the MEApd.**

AVP mRNA levels were quantified from the MEApd of control and infected males. In accordance with the previously observed epigenetic status, AVP mRNA levels were higher in infected males. Housekeeping gene HPRT controls were run for each sample. Threshold cycles (normalized to housekeeping gene HPRT) were plotted for both control (green) and infected (red) males. Delta Ct = Ct for AVP – Ct for HPRT. The delta Ct values were higher for infected MEApd samples when compared with their controls (Panel B Delta Ct; Control mean =-11.74±1.68, Infected mean= -8.066±0.702, * $p$ = 0.043 REST). This translates to a fold change of 12.78 with mRNA abundance being higher in infected males. (n = 6 infected and 5 control).

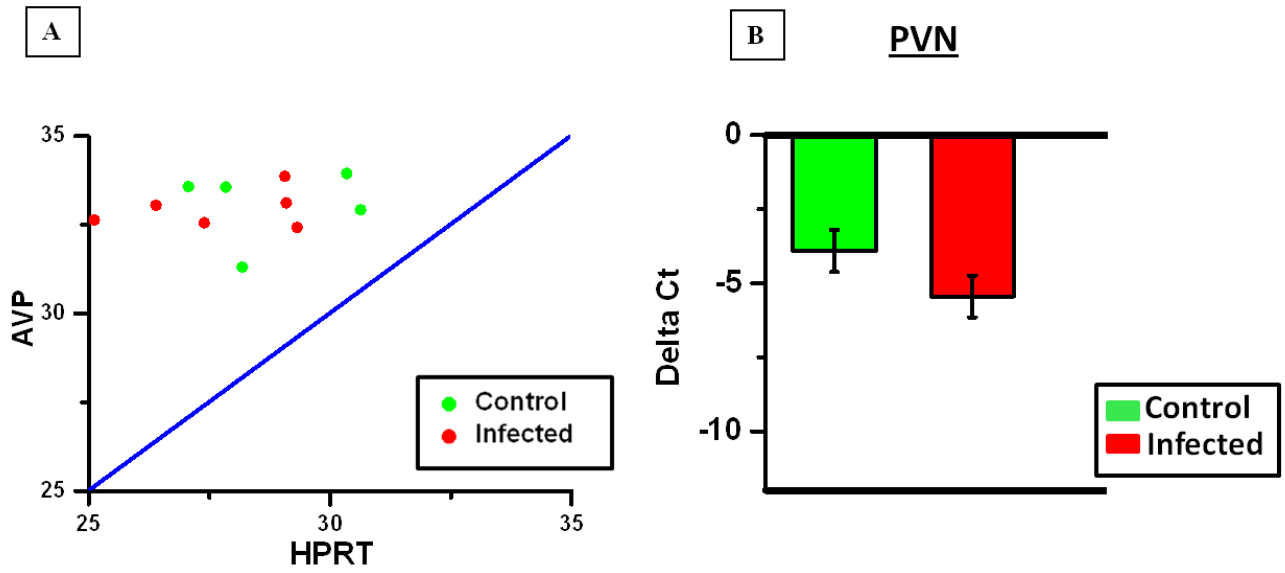



**Figure 19: Infection did not alter AVP mRNA levels in the PVN.**

AVP mRNA levels were quantified from the PVN of control and infected males. Hypothalamic AVP is testosterone independent and not implicated in sexual behaviors. Housekeeping gene HPRT controls were run for each sample. Delta Ct = Ct for AVP – Ct for HPRT. There was no significant difference between the delta Ct values from both groups (Panel B Delta Ct; Control mean =-3.91 ± 0.64, Infected mean= -5.44 ± 0.70 $p > 0.05$ REST). [n= 6 Control males, 5 Infected males]

animals). L-methionine treatment consisted of subcutaneous injection at (dose: 200mg/kg) in sterile saline for ten successive days, twice a day starting 6 weeks post-infection. On the twelfth day after start of injection, I quantified innate fear by measuring aversion to bobcat odor during a twenty minute trial (independent measure being occupancy in cat odor bisect divided by sum of occupancy in cat and rabbit odor bisect, %) (Described in detail in Annexe Pg 107, 109, 114). Analysis of variance (ANOVA) revealed significant main effects of the infection status and methionine treatment ($F_{(1,32)} > 15$, $p < 0.001$). Effects of interaction also reached statistical significance $F_{(1,32)} = 8.2$, $p = 0.007$).

Toxoplasma infection resulted in reduction of defensive response in placebo-treated animals (Figure 20[20]; $p = 0.001$, LSD post-hoc test). Consistent with earlier data (chapter 3) and published reports [124], roughly half of infected animals exhibited gain of attraction to cat odor (Figure 1, chance = 50%). Methionine treatment did not induce significant changes in the behavior of control animals ($p = 0.124$). Furthermore, methionine treatment completely abolished gain of attraction to cat odor in infected animals and renormalized defensive behavior to the level observed in control animals (Figure 20; $p < 0.001$ for comparison between infected placebo and infected methionine; $p = 0.348$ for comparison between control placebo and infected methionine). These observations demonstrate that a hypomethylation event is necessary to induce behavioral effects of the infection on defensive behaviors.

I further investigated if a localized hypomethylation within MEApd was sufficient to recapitulate behavioral effects of the infection. RG108 is an inhibitor of DNA methyltransferase enzyme; its application results in a hypomethylation of DNA [168, 170]. Using osmotic pumps and intra-cranial surgery directed at the MEApd, I delivered RG108 (Dose: 1mg/ml, total volume pumped

[20] Page 82

= 200µl) for 10 days in brain of uninfected male rats. Behavior testing was performed on the twelfth day after start of the infusion (Described in detail in Annexe on Pg 110, 114). Cannula localization was confirmed post-mortem and off target samples were removed from the analysis. RG108 infusion significantly reduced innate fear to cat odor, compared to placebo (Figure 21[21], 12% increase in cat odor occupancy; $t_{(17)} = -2.517$, $p = 0.022$, one-way Independent sample t test). These observations suggest that hypomethylation in MEApd is sufficient to reduce defensive behavior, recapitulating behavioral effects of infection sans infection.

It is pertinent to note here that placebo animals in this experiment exhibit more robust aversion to cat odor compared to control animals in preceding experiments even though same cat odor dose was used as the stimulus. I speculate that stress of intracranial surgery and long-term subcutaneous placement of osmotic pump generated substantial stress, accentuating aversive behaviors.

Previous results in this thesis highlight the importance of testosterone in Toxplasma infection induced changes in defensive behavior. Firstly I have shown that infection causes both a change in the innate fear response (Figure 1) as well as an, uncharacteristic, boost in testicular testosterone (Figure 4). Further I demonstrate a hypomethylation event at two testosterone sensitive methylation sites in the upstream promotor region of AVP (Figure 16 and 17). To confirm the importance of testosterone, I tested the necessity of testosterone in the MEApd in driving changes in defensive behavior post Toxoplasma infection. This was performed by supplementing testosterone specifically to the MEApd of castrates. Wistar (6-8 weeks old) were castrated. Animals were allowed to recuperate for 10 days. This also provided sufficient time for clearance of endogenous testosterone. Animals then underwent a sterotactic surgery to implant

[21] Page 83

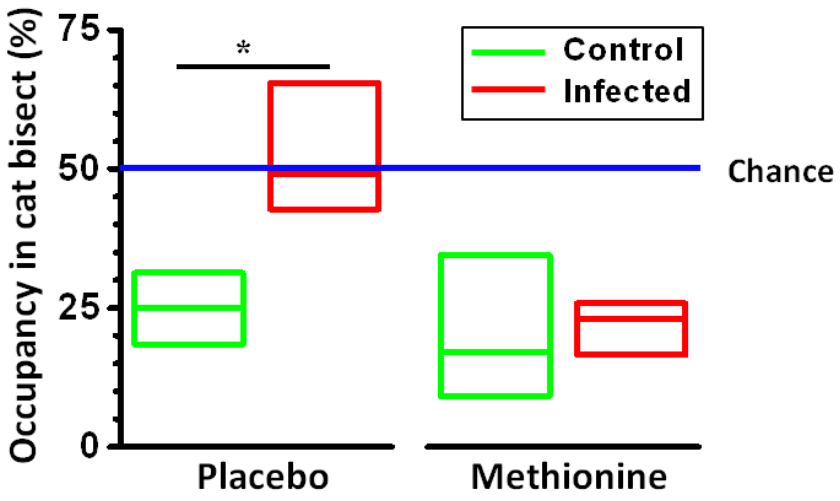
cannulas bilaterally that targeted the MEApd. These were connected to osmotic pumps filled with either testosterone (200 µl; concentration 25mM) or vehicle (3% ethanol in artificial cerebrospinal fluid). Twelve days post start of infusion of testosterone to the MEApd cat odor avoidance was assayed (Described in detail in Annexe Pg 110,114). Testosterone infusion significantly reduced innate fear to cat odor, compared to placebo (Figure 22, 23% increase in cat odor occupancy; $t_{(17)}$ = 6.769 *p=0.046 Independent sample T test). These observations suggest that supplementation of testosterone in MEApd is sufficient to reduce defensive behavior, recapitulating behavioral effects of infection sans infection.

To culminate the scientific pursuit of this thesis, I assessed whether increased AVP in the MEApd was sufficient to recapitulate infection like changes in defensive behavior. AVP was overexpressed in the MEApd using adenoassociated viral (AAV) vectors. Viral vectors were constructed to contain AVP (full gene) plus GFP inserts. Control vectors contained only GFP inserts. Viral vectors (3µl of $10^{11}$ infectious particle/ml solution) were injected using a Hamilton syringe connected to cannula targeting the MEApd (Described in detail in the Annexe on Pg 112). A one month incubation time was selected to allow for stable expression of viral inserts. After this cat odor avoidance assay was performed. Animals in which AVP was over expressed showed a significant reduction in innate fear to cat odor, compared to placebo (Figure 23, 102% increase in cat odor occupancy; $t_{(17)}$ = 1.973 *p=0.025 Independent sample T test). These observations conclusively demonstrate that an increase in AVP in the MEApd is sufficient to reduce defensive behavior, recapitulating behavioral effects of infection sans infection

In short, present chapter argues that Toxoplasma infection creates an epigenetic change in the MEApd of male rats, inducing a hypomethylation at AVP promoter and increasing AVP transcription. Furthermore these epigenetic changes are necessary for behavorial effects of the

infection. The behavioral effects of the infection can be sufficiently recapitulated on pharmacological mimicking of MEApd hypomethylation. In culmination; the action of testosterone in the MEApd is necessary and the increase of AVP in the MEApd is sufficient to cause infection like changes in defensive behavior.

In the preceding chapter, I argued that Toxoplasma infection creates a leakiness between defensive and sexual behaviors (Figure 13 Pg 62), thus causing loss of fear and gain of attraction on cat odor exposure. This chapter extends these observations by suggesting a possible molecular mechanism for this leakiness. I postulate that enhanced testosterone production in testes of infected animals reduce methylation of AVP promoter in the MEApd. This results in an up-regulation of AVP in the vasopressinergic neurons. Such an up-regulation increases probability of their recruitment and creates leakiness between parallel but inter-connected circuits of the MEA.




| Interaction | F | P-value |
|---|---|---|
| treatment | 19.583 | .000 |
| infected | 15.134 | .000 |
| treatment * infected | 8.229 | .007 |

**Figure 20: Artificial hypermethylation reversed infection induced changes in defensive behavior**

Animals were infected with Toxoplasma. 7 weeks post infection infected and control males were injected twice a day for ten days with either L- Methionine or placebo (physiological saline). Methionine is a substrate in the methylation of DNA. Treatment with excess of this substrate increases the methylation reaction. Placebo treated infected and control males show the classically described infection induced changes in behavior. Control males spent 27.04 ± 2.65 percent time in the cat urine bisect as opposed to infected males that spent 50.64 ± 4.22 percent (* $p < 0.05$, LSD post-hoc test). The 75$^{th}$ percentile lies above the chance line showing that many animals showed an active attraction. Post treatment with methionine there was no significant difference between the time spent in the cat bisect by infected and control males [Control (methionine) mean= 21.60 ± 3.77. Infected (methionine) mean= 25.17 ± 3.11]. A two ANOVA was performed and there was a significant effect of methionine treatment $F_{(1, 32)} = 19.583$, $p < 0.001$` and infection $F_{(1,32)} = 15.134$, $p < 0.001$. The interaction of the two factors was also significant (Methionine*infection $F_{(1,32)} = 8.22$, $p = 0.007$).

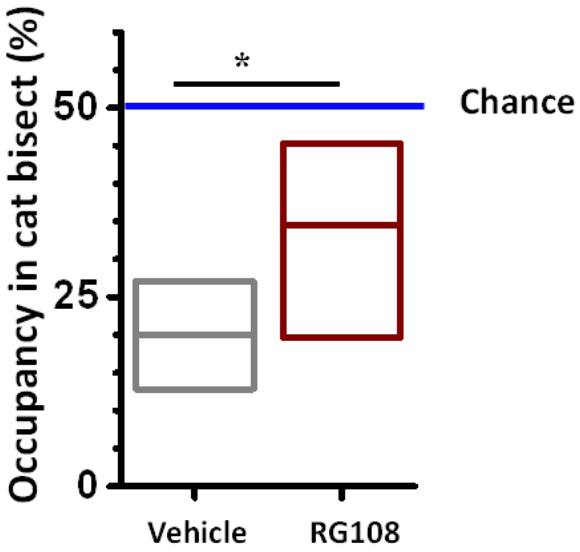



**Figure 21: Infusion of a DNMT inhibitor in the MEApd partially mimicked infection like changes in defensive behavior**

Stereotactic surgery was performed on uninfected males and cannula targetted to the MEApd. Osmotic pumps containing either a DNMT inhibitor (RG108) or vehicular control were connected to the cannulas. Localized infusion to the MEApd was performed for 10 days. On the twelfth day cat odor avoidance assay was performed. Rabbit urine was used as control stimulus. Vehicle treated males spent 20.70±2.87 and RG108 treated males spent 32.99±3.83. RG108 treatment significantly increased the amount of time spent in the cat odor bisect (Independent sample t test $t_{(17)} = -2.517$ * $p = 0.022$). This is similar to the decrease in defensive behavior caused by Toxoplasma infection.

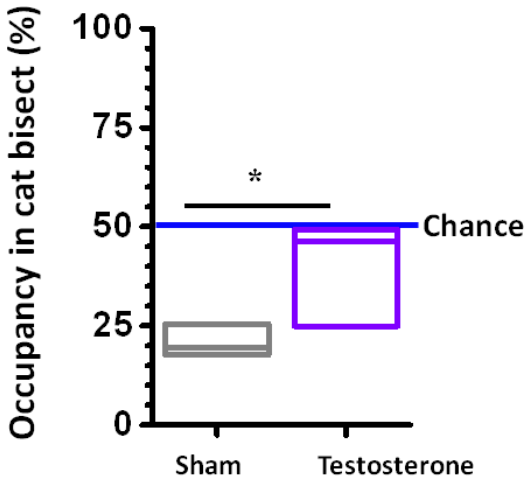



**Figure 22: Infusion of a testosterone in the MEApd partially mimicked infection like changes in defensive behaviour**[Π]

Stereotactic surgery was performed on castrated males and cannula targeted to the MEApd. Osmotic pumps containing either a testosterone (200µL 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 MEApd was performed for 10 days. On the twelfth day cat odor avoidance assay was performed. Rabbit urine was used as control stimulus. Vehicle treated males spent 25.20±5.64 and Testosterone treated males spent 31.12±4.54. Testosterone treatment significantly increased the amount of time spent in the cat odor bisect (Independent sample T test $t_{(17)} = 6.769$ $*p=0.046$). This is similar to the decrease in defensive behavior caused by Toxoplasma infection.

[Π] Secondary experimenter: Dhiraj Singh

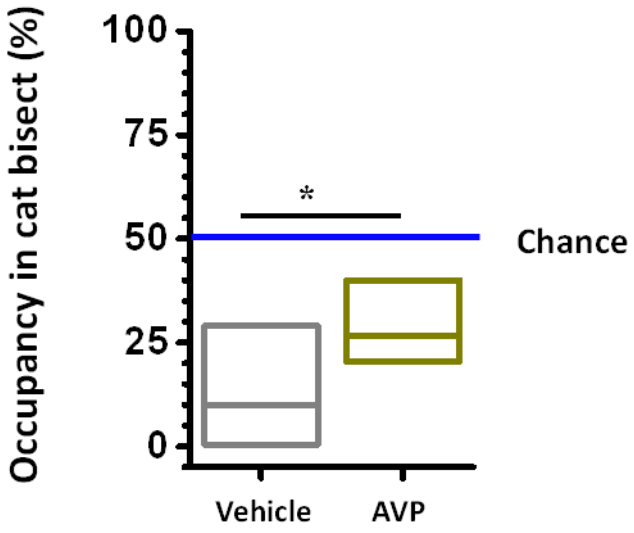



**Figure 23: Overexpression of AVP in the MEApd was sufficient to cause infection like changes in defensive behaviour**[◊]

Stereotactic surgery was performed on castrated males and cannula targeted to the MEApd. Adeno associated viral vectors containing AVP or control inserts were pumped into the target using a Hamilton syringe (rate 3ul/10 min, Dose; $10^{11}$ infectious particles/ml). One month post infusion cat odor avoidance assay was performed. Rabbit urine was used as control stimulus. Males infused with control viral vectors spent 15.68±6.19 and males infused with AVP containing viral vectors spent 31.74±3.48. AVP overexpression treatment significantly increased the amount of time spent in the cat odor bisect (Independent sample T test $t_{(17)} = 1.973$ *$p$=0.025). Overexpression of AVP in the MEApd was sufficient to drive an infection like reduction of innate fear.

[◊] Adenoassociated viral vectors constructed by Vineet Kumar

# 7. Discussion

Connection between genotype and phenotype is usually referred in relation to *individuals*. Yet there are atypic examples such as natural selection acting on genes of the wasp, increasing frequency of wasp alleles that cause non-orb web weaving by the spider. In other words, genotype of the parasite has had an "extended phenotype [171]" reflected in the spider (another individual) and web (outside individuals). Parasitic manipulation of host behavior and physiology is a classic candidate of extended phenotype [172, 173], Toxoplasma infection in rats being particularly well-studied example. Toxoplasma genes not only cause changes in behavior of its host (attraction to predators) but cause an extreme extended phenotype by altering mate choice behavior in uninfected females.

Several possibilities have emerged during prior work to explain the mechanisms of the Toxoplasma effect. One line of work suggests a role of tropism to particular brain regions on part of the parasite. Brain regions such as the amygdala and nucleus accumbens have been suggested to harbor increased proportion of parasites [124, 174]. However no specific set of brain regions have been consistently observed to be preferred. Alternatively interaction of Toxoplasma with resident astrocytes has also been proposed to be involved through up-regulation of kynurenic acid potentially resulting in a disruption of the NMDA channels [175]. Yet another intriguing possibility is potential involvement of two parasitic genes with a remarkable homology to mammalian tyrosine hydroxylase [176, 177]. This enzyme catalyzes the rate-limiting step in dopamine synthesis [178], a neurotransmitter involved in the signaling of reward and motivation. In relation to this, drugs that interfere with dopaminergic transmission rescue behavioral effects of the infection [179]; and the infection enhances dopamine release in mouse brain slices [177].

Data presented in this thesis argues for a novel mechanism of mediation of the behavioral change. I propose that the up-regulation of testosterone, after parasitic invasion of the testes, initiates DNA hypomethylation in the AVP promoter once testosterone crosses the blood-brain barrier and binds to its receptors in MEA. The MEA is situated in broader neuroendocrine system that has evolved to mediate negotiation between defensive and sexual behaviors [100, 180]. Greater AVP involvement in MEA results in shift of this balance away from defensive behaviors and towards sexual behavior. This evolved plasticity will be useful, for example, to titrate behavioral response to varying physiological and environmental situations. Toxoplasma infection taps into this system by shifting balance away from defensive behaviors through a more potent vasopressinergic tone. This interpretation is further strengthened by the observations that Toxoplasma infected male rats enhance investment in reproductive advertisement [128, 135].

Parasitic manipulation of host behavior is often invoked as rhetorical tool to argue that natural selection acts on genes and not necessarily individuals [49]. I subscribe to the view that these examples also can be used as a perturbation models. For example, disruption of orb weaving in the preceding example can be used to study spider webs in much the same way as a molecular biologist would compare control animals with gene knock-out, or a pharmacologist will compare receptor antagonist with placebo. This is because parasitic manipulations are result of a long-drawn coevolution culminating is remarkable specificity. For example, Toxoplasma changes defensive behavior in rats without affecting olfaction, learning or conditioned fear [124]. If we can understand proximate mechanism of this phenomenon, we can hopefully also gain greater clarity about the organization of defensive behavior itself in the brain [181].

This thesis use perturbation approach to broaden present knowledge about social behavior in two important ways. Firstly, the thesis argues for an integrated framework for approach-avoidance

behavior by showing that defensive and sexual behaviors are intricately linked at the level of proximate mechanisms (Page 21). Secondly, these results suggest that brain, hormones and external environment are part of a trinodal circuit with reciprocal influences. In previous sections of the thesis and here, I refer to this as the 'extended social behavior network'. My observations are not the first instance when these ideas have been articulated. In the two subsequent paragraphs, I discuss prior intellectual footing of these ideas and place my observations in into their context.

I now briefly turn to approach-avoidance conflict. Several papers have argued that social behavior should be seen as a decision-making construct that negotiates between approach and avoidance in an ambivalent and uncertain environment [14]. More details about this line of argument have been previously presented in the introduction (Pg 13). It then follows logically that the biological substrates for approach and avoidance are in an eternal negotiation, co-existing in parallel in terms of neural mediators. In chapters 1 and 3, I show that Toxoplasma infection is able to create a leakiness between defensive and sexual behaviors. This leakiness is coupled to hormonal changes reminiscent of greater sexual approach and lesser defensive avoidance [135, 182]. The fact that it is possible for a coevolved parasite to produce such a leakiness demonstrates that approach and avoidance are not unitary and isolated behaviors. Rather these behaviors exist in a dynamic continuum. And they are mediated by plastic neural substrates, changing in tandem with endocrine and environmental ambience.

Testosterone is necessary for male reproductive behavior. Testosterone-responsive behaviors can be roughly divided in consummatory sexual behaviors (e.g. penile erection and mounting); secondary sexual characteristics (i.e. investment to influence mate choice of females, e.g. scent marking in rodents); and, behaviors required for mate search and/or acquisition (e.g. intra-sexual

aggression and territoriality). Castration abolishes consummatory behaviors and secondary sexual characters [183]. Re-supplementation with exogenous testosterone reinstates these behaviors in castrated rats [184, 185]. Greater testosterone leads to relatively more robust expression of these behaviors [59]. Thus, testosterone is necessary for primary sexual behaviors and secondary features of mate choice. The relationship between testosterone and aggression or territoriality is more mixed [186]. In male birds of some species, these behaviors require testosterone; while the exact same behaviors seem to be quiet independent of testosterone in other phylogentically proximal birds [186]. An apparent resolution of this paradox lies in the observations that the same behavior is often expressed in response to varying ecological needs in different species [186-188]. For example, territoriality can be sexual (i.e. to gain greater opportunity to mate with females) or platonic (e.g. to gain exclusive opportunity to forage [186]. Similarly, a pair-bond between male and female can be sexual (i.e. raising progeny with joint reproductive stake) or platonic (e.g. joint defense of a resourceful territory [186]. In these examples, selection has been acting on disparate levels of survival (natural selection) or reproduction (sexual selection) despite the observed behavior being non-disparate. Testosterone seems to be involved whenever the behavior is sustained by reproductive motives, sexual or parental. Testosterone is a costly chemical in terms of metabolic and opportunity costs. These costs exist because testosterone is immuno-suppressive [63, 189], promotes metabolically expensive secondary sexual characters and reduces fear thus increasing predation. Thus, it is not surprising that incipient environment and behavior affects testosterone, constraining its production when it is not required. For example, stressful circumstances reduce testosterone ("if a lion is chasing you now, mate tomorrow, if there is tomorrow"). Testosterone is also reduced during subordination in a social hierarchy [190, 191], in non-breeding seasons and [192] when

reproductive opportunities are not available [186, 192]. These observations make a case that testosterone and behavior influence each other, forming a loop.

The bidirectional framework discussed above can be easily extended to a trinodal model through inclusion of brain. For example, prenatal stress can cause growth and behavioral changes sustained through adult life [193-195]. These changes are initiated by maternal stress hormones and perpetuated by epigenetic programming of the brain creating a long-term facilitation of stress hormone secretion in progeny [196-198]. Hormones, brain and behavior form a functional

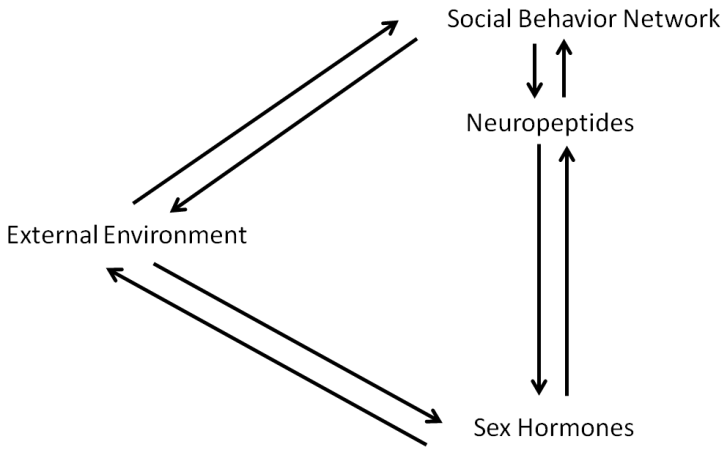



Fig E: Proposed extended social behavior network

unit in this case. Similarly, Toxoplasma effects in rats involve hormonal change that creates an epigenetic signature in specific parts of the brain, thereby sustaining long-term behavioral changes. It can be postulated that changes in the MEA of infected male can facilitate the hypothalamus-pituitary-gonadal axis through anatomical connections between medial amygdala and medial parts of the hypothalamus [70]. Although a speculation at this point, it could represent a positive feedback loop whereby gonadal testosterone promotes an epigenetic upregulation of AVP in the MEA and the MEA in turn enhances gonadal testosterone. This will

perpetually sustain the behavioral change until a disruptive physiological event terminates this feedback loop. The argument that I am attempting to develop is that Toxoplasma effects on the host behavior provides evidence for a trinodal relationship between brain, hormones and behavior. Another vantage point of viewing this is from the action of testosterone on AVP system. I have demonstrated that reproductive pheromones activate the AVP neurons in posterodorsal MEA (MEApd). This activation is a proverbial neural footprint of reproductive opportunity in the environment. Testosterone epigenetically regulates AVP transcription [160, 199], creating a reflection of endocrine milieu in the brain. In other words, the AVP neurons in MEApd provides an interface between external reproductive opportunities and internal hormonal milieu; an interface that is situated within brain. This interface is plastic, as demonstrated by the ability of the Toxoplasma to potentiate it via enhanced testosterone. The parasite in this case co-opts trinodality inherent in the host evolution.

"Although many studies of insects, birds, and mammals have documented the functional significance of single interactions such as fights, the reproductive benefits of long-term social bonds are less immediately obvious" Cheney et al. 1986 [200]. Social behavior will evolve in the context when the adaptive value exceeds the cost it imposes such as competition for resources, increased predation and disease transmission. The discussion of adaptive value and plasticity of social behavior are intertwined. In this thesis I haven't explicitly addressed the former. However my data advocates a model for plasticity in social behavior. Key components of this model are conserved across species; AVP and nodes of the limbic system dates back 450 million years. This permits my findings to be extrapolated beyond the lab rat model used here.

Several previous studies have discussed the epigenetic reprogramming of host cells to benefit the pathogen or parasite [201]. For example, *Mycobacterium leprae* promotes de-differentiation of

the infected Schwann cells to a more stem cell like progenitor state, allowing for greater opportunity for the bacterium to spread [202]. In the case of the Moloney murine leukemia virus the proviral insertion of this retrovirus causes DNA hypermethylation in the flanking sequences of the host genome [203]. The resultant transcriptional silencing contributes to the latent maintenance of the provirus in the host genome. Thus, parasites can influence host metabolism through DNA methylation and exploit pre-existing epigenetic mechanisms of the host to change their behavior.

Data in this thesis highlights that epigenetic status of AVP in the SBN (specifically MEA) informs variation in social behavior phenotypes. A similar paradigm has been extensively studied by Michael Meaney's group (for example in [204]). They describe the effect of maternal care on stress reactivity in offsprings. Changes in stress reactivity are long lasting and caused via gluococorticoid receptors in the hippocampus. These studies were pioneer in their description of the long lasting (yet reversible) changes in behavior that were caused by epigenetic changes in the brain. Their findings argue for a stable yet dynamic epigenome that can regulate phenotypic plasticity [205]. Parallels can be drawn between our studies with Toxoplasma infection being analogous to maternal stress, social behavior being targeted instead of stress and AVP in the MEA being the effecter instead of GR in the hippocampus. DNA methylation can be linked to mental health; especially disorders affecting social behavior. MeCP2 (methyl CpG binding protein 2) is strongly expressed in mature neurons. It is involved in suppressing transcription by associating with methylated DNA. Mutations or reduction in MeCP2 expression are seen in autism and Rett syndrome [206, 207]. Taking a step back, does variation in epigenetic status of AVP account for variations on social behavior observed? Continuing on the unified theme of social behavior; does a decreased methylation of AVP promote a generalized increased in

approach behavior such as increased ricky behavior, heightened sociability and sexual behavior and vice versa.

In this study I have specifically modeled variation in defensive/innate fear system. Phobia is the unbridled/excessive fear response. These could be a result of hypermethylation of AVP in the social brain. Systemic manipulations can influence epigenetic status of AVP in the medial amygdala. Naturally, this is demonstrated by the testosterone dependency of methylation status of AVP in the MEA. My experiments show that peripheral administration of methionine was sufficient to rescue the runaway decrease in fear associated with Toxoplasma infection in rodents. This proffers a tractable model wherein peripheral manipulations might be able to restore appropriate fear responses by modulating epigenetic changes in the SBN/ associated nodes.

Insights into the mechanism of testosterone action would bolster this model. It would also enable it to be expanded to females. Expression of enzymes involved in DNA methylation such as DNMT3a and MeCP2 are testosterone sensitive. Both of these influence social behavior. Testosterone could indirectly act via these. Alternately it could also act directly via its receptors (AR and ER). These are transcription factors. The presence of an androgen or estradiol responsive elements associated with AVP would strengthen this hypothesis.In summary, I present proximate hormonal, anatomical and molecular substrates of Toxoplasma induced behavioral change in defensive and sexual behavior of male rats. My observations provide mechanistic insights into regulation of behavior through continuous interactions between brain and behavior, and into interaction of approach and avoidance behaviors. I believe this has been a satisfactory scientific pursuit.

# 8. Annexe

**Annexe 1: Abbreviations; Brain Regions**

| | |
|---|---|
| AHN | Anterior Hypothalamic Nucleus |
| AOB | Accessory Olfactory Bulb |
| BLA | Basolateral Amgdala |
| BNST | Bed Nuclei Stria Terminalis |
| BNSTm | Bed Nuclei Stria Terminalis, Medial |
| BST | Bed Nuclei Stria Terminalis |
| CEA | Central Amygdala |
| LS | Lateral Septum |
| NaC | Nucleus Accumbens |
| MEA | Medial Amygdala |
| MEAp | Medial Amgdalar Nuclei, Posterior |
| MEApd | Medial Amygdalar Nuclei, Posterodorsal |
| MEApv | Medial Amygdalar Nuclei, Posteroventral |
| MHZ | Medial Hypothalamic Zone |

| | |
|---|---|
| MPN | Median Preoptic Nucleus |
| OB | Olfactory Bulb |
| PAG | PeriAqueductal Gray |
| PMD | Dorsal Premotor Cortex |
| POA | Pre Optic Area |
| PVN | Paraventricular Nucleus |
| SBN | Social Behavior Network |
| SDM | Social Decision Making |
| VMH | Ventromedial Hypothalamic Nuclei |
| VHMdm | Ventromedial Hypothalamic Nuclei, Dorsomedial |
| VMHvl | Ventromedial Hypothalamic Nuclei, ventrolateral |
| VNO | Vomeronasal Organ |
| VTA | Ventral Tegmental Area |

**Annexe 2: Abbreviations; Miscellaneous**

| | |
|---|---|
| ACTH | AdrenoCorticoTropic Hormone |
| AR | Androgen Receptor |
| AVT | Vasotocin |
| AVP | Arginine Vasopressin |
| AVP-ir | AVP immunoreactive neurons |
| cDNA | Complementary DNA |
| CRH | Corticotropin-Releasing Hormone |
| Ct | Cycle Threshold |
| DEPC | Diethylpyrocarbonate |
| DNA | DiNucleic Acid |
| DNMT | DNA methyltranferase |
| ER | Estradiol Receptor |
| Fos-ir | Fos immunoreactive neurons |
| gDNA | genomic DNA |
| GFP | Green fluorescence protein |
| HPA | Hypothalamus-Pituitary-Adrenal |

| | |
|---|---|
| HPG | Hypothalamus-Pituitary-Gonad |
| HPRT | Hypoxanthine-guanine phosphoribosyltransferase |
| IEG | Immediate Early Gene |
| IHC | Immunohistochemistry |
| LH | Luteinizing Hormone |
| LHRH | Luteinizing-hormone- Releasing Hormone |
| MeCP2 | Methyl CpG binding protein 2 |
| mRNA | Messenger Ribonucleic Acid |
| MSRE | Methylation Specific Restriction Enzyme |
| PBS | Phosphate Buffered Saline |
| PFA | Paraformaldehye |
| SEM | Standard error of mean |
| TH | Tyrosine Hydroxylase |
| Toxoplasma | *Toxoplasma gondii* |
| TSA | Tyramide signal amplification |
| qPCR | Quantitative Polymerase Chain Reaction |

**Annexe 3: Glossary**

| | |
|---|---|
| Challenges | Demanding environmental conditions such as competitors, predators, diseases |
| Defensive Behavior predatory defenses | In the scope of this thesis it refers to innate anti |
| Extended Amygdala | Composite structure of the brain that includes the amygdalar nucleus, portions of the substantia innominata, the medial nucleus accumbens, and the bed nucleus stria terminalis. The amygdalar and bed nucleus stria terminalis are key to the discussion of this thesis |
| Extended Social behavior | An extension of the classically described social |
| Behavior Network | work to include the external and internal environments. |

| | |
|---|---|
| External Environment | The physical environment an organization lives in. It includes factors such as abundance of resources/mates, competitors, seasonal variation etc. |
| Fitness Value | Refers to Darwinian fitness, i.e. A relative measure of reproductive success of an organism in passing its genes to the next generation. |
| *fos* | Gene of immediate early gene FOS |
| FOS | Protein of immediate early gene FOS |
| Internal Environment | In this thesis refers to the hormonal status of the animal with A focus on testosterone and corticosterone. |

| | |
|---|---|
| Hodological | Pertaining to hodology, i.e. study of pathways |
| Kairomone | A chemical emitted by an organism which mediates interspecific communication. |
| Limbic Zone | A set of structures that lies on both sides of the thalamus, just under the cerebrum. It includes the hypothalamus, the hippocampus, the amygdala etc.It is primarily responsible for our processing emotions. |
| Neuropeptide | Small proteinaceous substances produced and released by neurons. They act upon neural substrates and are used in communication between neurons. Key examples are oxytocin and vasopressin. The latter has been extensively discussed in this thesis |

| | |
|---|---|
| Opportunities | Appetitive environmental conditions such as mates, food, offspring |
| Reproductive Behavior | Encompasses a behavioral strategies involved in mating, parental care, pair bonding (or lack of it depending on the species), levels of circulating androgens/ sexual signals etc. |
| Sexual Behavior | Same as reproductive behavior. |
| Social Behavior | Usually Social behavior consists of a set of interactions among individuals of the same species. I have extended it to include interspecific interactions such as antipredatory responses. |
| Social Behavior Network | Within the basal forebrain and midbrain . It consists of the medial MEA, bed nuclei of the stria terminalis (BNST), lateral septum, preoptic area, anterior hypothalamus, ventromedial hypothalamus (VMH) and midbrain The nodes of this network are |

| | |
|---|---|
| | reciprocally connected, contain receptors for sex steroid hormones. It is involved in multiple forms of social behavior in mammals |
| Social Competence | Social competence' refers to the ability of an individual to optimise its social behaviour depending on available social information. It increases the Darwinian fitness of social behavior. |

**Annexe 4: Supplementary Figures**

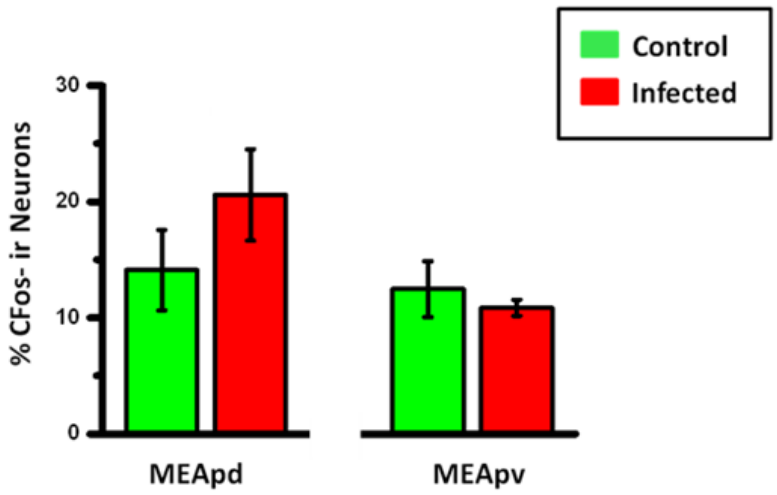



**Figure S1: Post infection the MEApd trend towards increased neuronal activation after exposure to cat stimulus.**

Males were infected with Toxoplasma (n = 10 infected, n = 6 control). Seven weeks post infection infected and control males were exposed to cat urine for 30 minutes. Two hours after the start of stimulus exposure animals was sacrificed and tissue were perfused. Fos was used as marker of neuronal activation and Fos-ir neurons were quantified in the MEApv and MEApd (Fos-ir = Fos single labeled + colabeled neurons). All counts were normalized to the total number of DAPI stained nuclei counted. Infected animals did not exhibit greater number of Fos-ir neurons in MEApd compared to control ($t_{(13)} = 1.420$ $p = 0.179$ independent sample t-test). The number of Fos-ir neurons in MEApv was not significantly different between control and infected animals ($t_{(13)} = 0.671$ $p = 0.514$, independent sample t-test). Hence the defensive function of the MEA is not diminished by Toxoplasma infection even though a fear response in not observed.

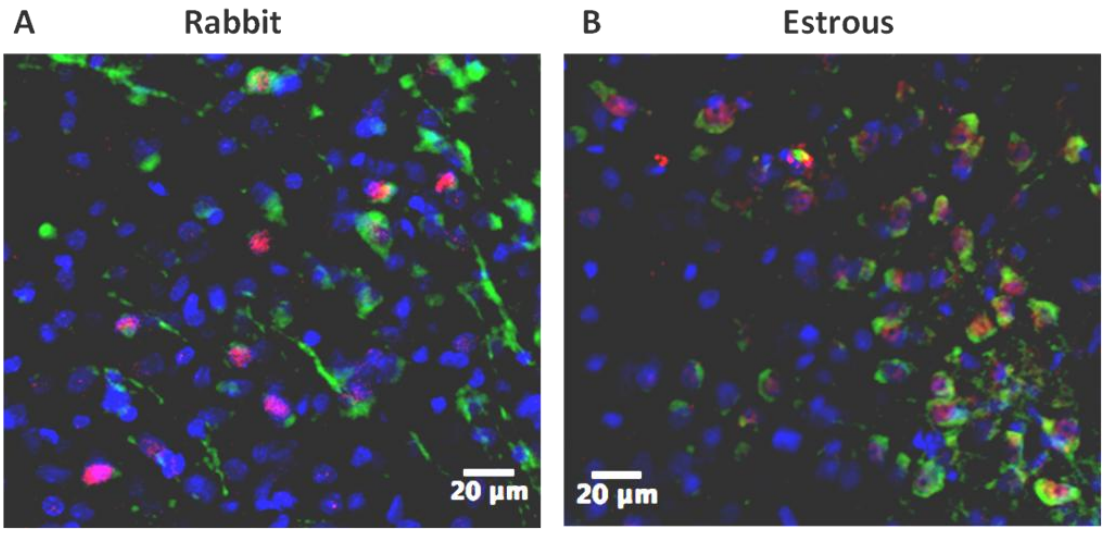



**Figure S2: Differential activation of AVP-ir neurons post exposure to rabbit and estrous urine.**

Animals were exposed to cat urine for 30 minutes. Two hours posts the beginning of stimulus exposure animals were sacrificed. Brain tissue was perfused with 4% PFA. Brain tissues were sectioned at 40µM. AVP-ir neurons (green) are stained with and anti-AVP antibody.Fos (red) depicts recent neuronal activation. Nuclei (blue) are stained with DAPI. Sections were imaged using a 40X objective and 1.2X digital zoom at a confocal micoscope (Carl Zeiss, LSM 710). In control males fewer colabelled neurons (ie AVP-ir (green) +Fos-ir (red) observed (Panel A). In infected males there is a increased observation of colabelled (Panel B). This illustrates the activation of ‘AVP-ir neurons by sexual stimulus in males. Images depicted here are representative of their respective groups.

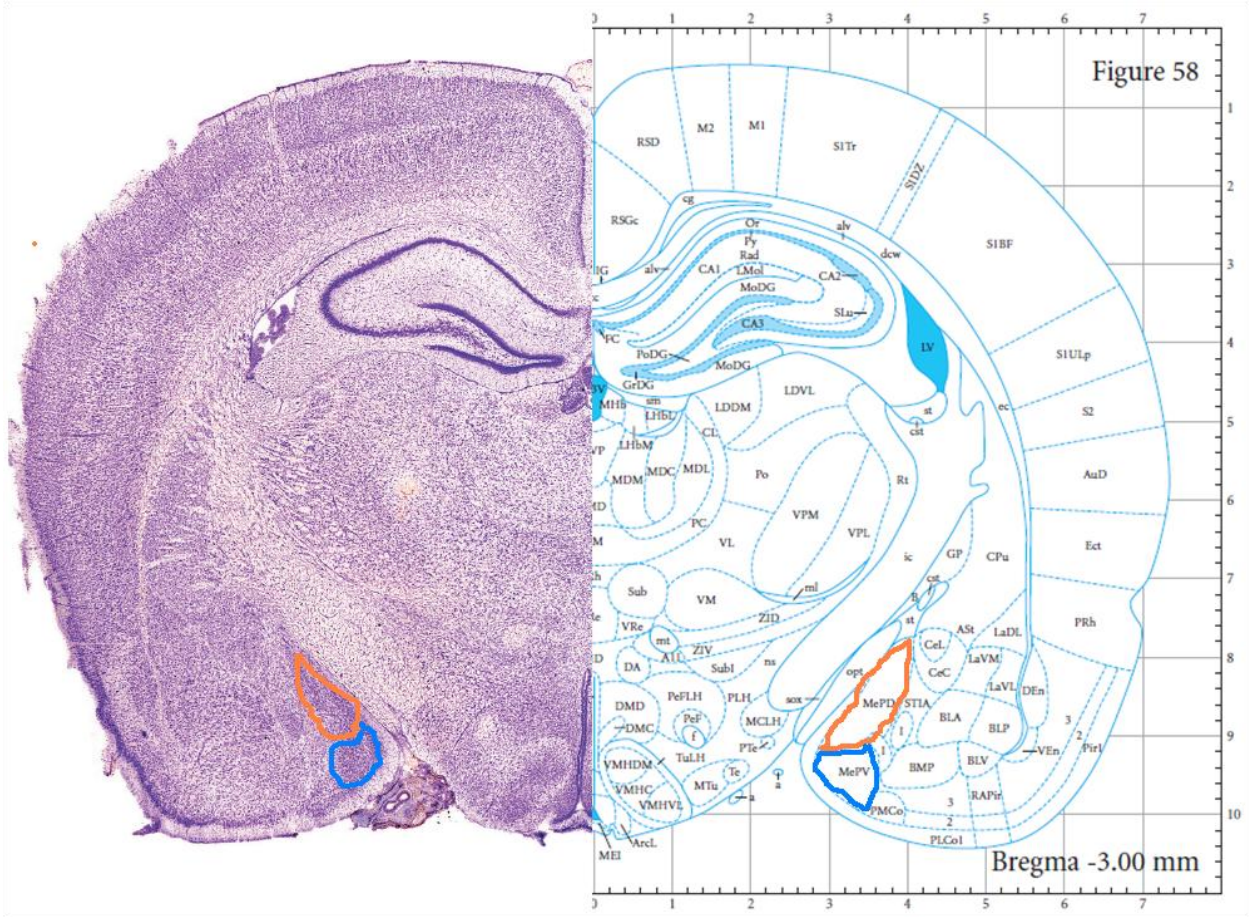



**Figure S3: Spatial localization of the two subnuclei of the MEA.**

Representative images of the MEA. On the right hand side is a Crysl Violet stained brain section (Bregma -3.0).On the left hand side is a diagrammatic representation of the neuroanatony at the same Bregma. The MEApd (referred to in the picture as MePD) is highlighted in orange on both sides and the MEApv (referred to in the picture as MePV) is highlighted in blue. Images taken from [208].

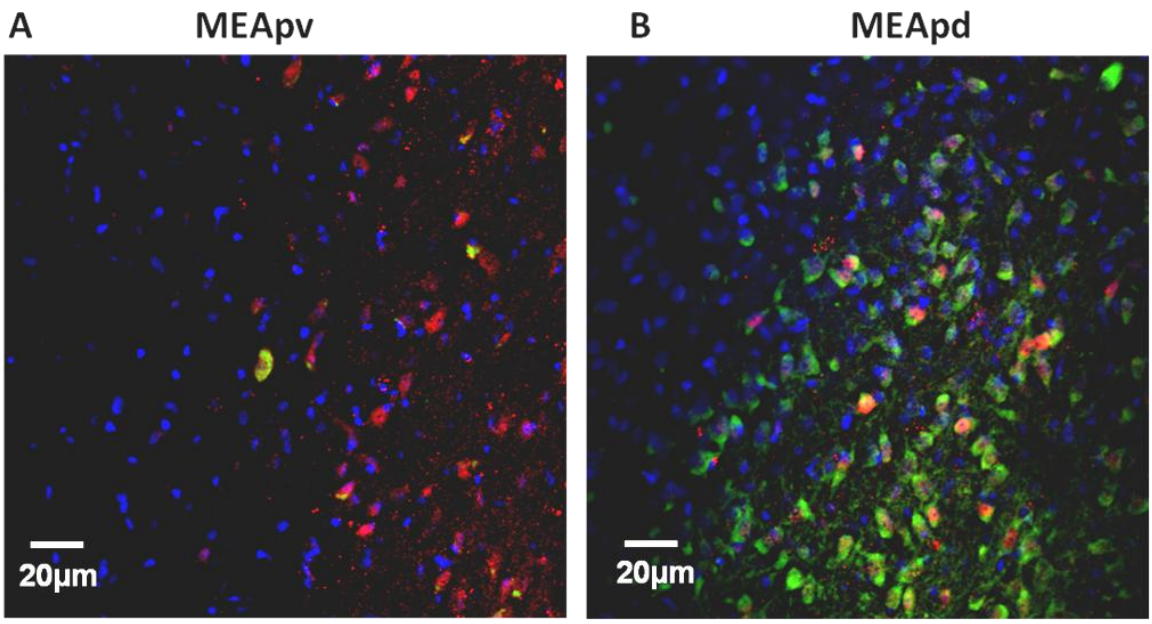



**Figure S4: Differential activation of the MEApv and MEApd post exposure to cat urine in infected males.**

Animals were exposed to cat urine for 30 minutes. Two hours posts the beginning of stimulus exposure animals were sacrificed. Brain tissues were perfused with 4% PFA. Brain tissues were sectioned at 40µM. AVP-ir neurons (green) are stained with and anti-AVP antibody.Fos (red) depicts recent neuronal activation. Nuclei (blue) are stained with DAPI. Sections were imaged using a 40X objective and 1.2X digital zoom at a confocal micoscope (Carl Zeiss, LSM 710). In control males fewer colabelled neurons (ie AVP-ir (green)+Fos-ir(red) observed (Panel A). In infected males there is an increased observation of colabelled (Panel B). Images depicted here are representative of their respective groups.

## Annexe 5: 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 group of twos at NTU vivarium. Males and females were housed in same room. Animals were provided with food and water ad libitum and maintained in 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.

#### 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 needle. Male rats were injected intraperitonially 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. Behavior experiments were conducted >$7^{th}$ 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 achieves chronic infection in more than 95% of cases.

**d. Determination of Estrus**

Female rats were acclimatized in the vivarium for 2-3 days upon arrival. They were lightly restrained and vaginal lavages (~10 µl) collected at 11AM. Relative ratios of cornified, epithelial and leuckocytes in lavage was examined under 20X magnification.

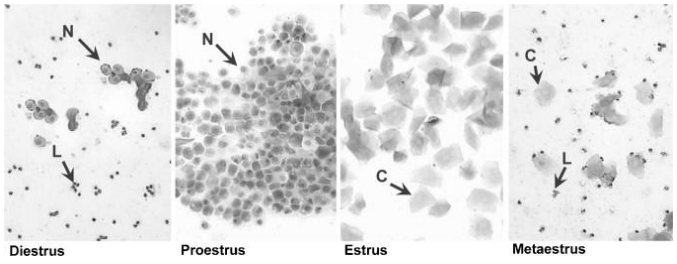



**Figure C : Cells present in Vaginal lavages of females in different stages of sexual cycle.** This figure has been copied from [209](Figure 1). N=Nucleated epithelial cells, L=leuckocytes and C=cornified cells.

Estrus was characterized by extensive presence of cornified cells. Only estrus females were used in behavioral experiments. Females used were uninfected and sexually inexperienced.

**e. 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.

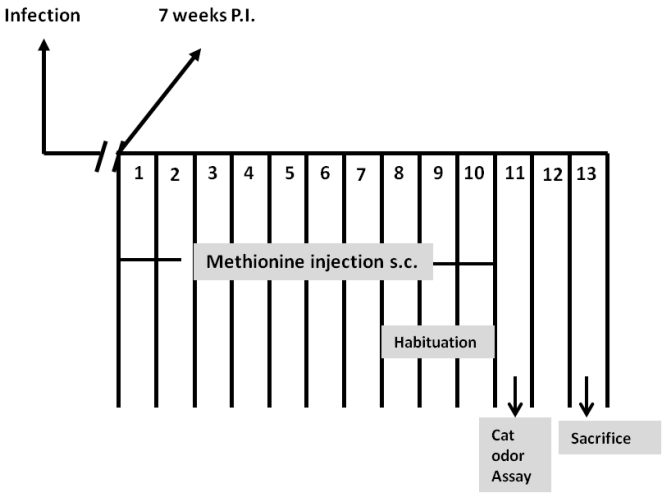



**Figure C: Experimental time line for Methionine treated animals**

**f. 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 cannula (Plastic one) were implanted bilaterally into the MEApd (AP = -3.0, L = +/- 3.8, V = -7.0)[208]. 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 µl) 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 (part of another experiment). On the thirteenth day animals were sacrificed.

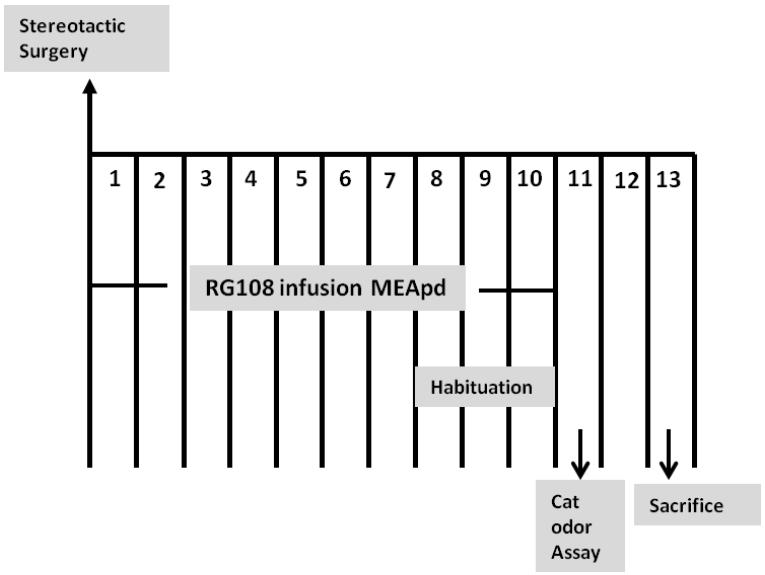


**Figure D : Experimental time line for RG108 treated animals**

* Preparation of Artificial Cerebrospinal Fluid (ACSF)

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

| Solution A | | Solution B |
|---|---|---|
| NaCl | 8.66 grams | NaH2PO4.7H2O 0.214 gram |
| KCl | 0.224 gram | Nah2PO4.H20 0.027 gram |
| CaCl2.2H2O | 0.206 gram | Dissolve in 500 ml sterile water |
| MgCl2.6H20 | 0.163 gram | |
| Dissolve in 500 ml sterile water | | |

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

**g. 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) and Lidocain (0.5ml,1%). A medial

incision was made in the scrotum of heavily anaesthetized animal. Testes and vas deferens of 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.

**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) were implanted bilaterally into the MEApd (AP = -3.0, L = +/- 3.8, V = -7.0)[208]. Testosterone (Sigma, Concentration 25mM, total volume 200µL) was infused into the MEApd using osmotic pumps (Alzet, model 2002). Testosterone was dissolves 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 has been described above. On the eleventh day animals were exposed to cat odor to ascertain fear levels.On the thirteenth day animals were sacrificed.

**g. Infusion of AAV-with AVP insert into MEA**

**Preparation of AAV with AVP insert**[χ]

AVP (Gene ID NM-016992) gene is cloned into multiple cloning sites of pAAV-IRES-hrGFP using EcoRI and XHOI restriction enzymes. Resultant recombinant construct was verified by DNA sequencing using sequencing primer. The recombinant expression plasmid is co-transfected into the AAV-293 cells with pHelper (carrying adenovirus-derived genes) and pAAV-RC (carrying AAV-2 replication and capsid genes), which together supply all of the factors required for AAV replication and packaging .Three plasmids (the helper, the one with rep-cap and the one containing AVP gene of interests cloned between the ITRs). Plasmids were prepared as followed 300 micrograms of each plasmid, 8750 µl of the medium without FBS (IMDM) and filtered 1250 µl polyethyelene imine (Polysciences,Inc.). 1 ml of this to each of the plate. Incubate for 3 days and harvest the cells .Virus packaging and purification was using Adeno Associated Virus (AAV) Purification (Virapur, ViraKit CAT NO. 003063). Virus titration done using plaque assay method from Virapur.

**Infusion of viral vector**

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 viral vector infused bilaterally into the MEApd (AP = -3.0, L

[χ] Performed by Vineet Kumar

= +/- 3.8, V = -7.0)[208]. Special care was taken to remove the dura so as to not clog the cannla. AAV associated viral with AVP and control inserts (Dose, Total volume 3µl infused) was infused into the MEApd via Hamiltion syringe connected to a cannula (Plastic One) (using connector PE tubing).Viral vectors (3µl) was pumped 1 µl at a time (flow rate 0.3µl/min). There was a rest period of 2minutes following the first two microlitres pumped and an 8 minute rest period after the final microlitre. The total time per hemisphere was~25 minutes (pumping + rest periods). The rest periods enabled the solution being infused to diffuse slowly. One month post surgery the animals were exposed to cat odor to ascertain fear levels.

**2. Behavioral Assays**

**a. Cat Odor Avoidance Assay**

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 we 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 , Songapore, cat: Predator  pee, USA). Urine was topped up every 2 hours. Occupancy of male in control and infected arms was quantified over trial duration of 1200s.

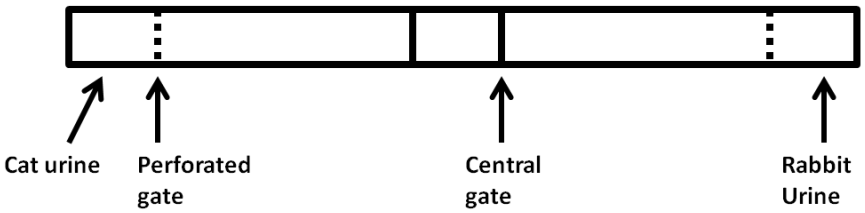



**Figure E : Cat odor avoidance assay arena**

**b.Mate Choice experiment using Infected and control males**

Experiment was conducted in the same arena used for the cat odor avoidance assay.

At the start of experiment the central gate was closed. One control and infected male was introduced and allowed to explore (and urine mark) their respective arms for 2 hour. They were then confined in extremities of the arena by closing the perforated gate. This allowed for olfactory cues to be emitted without possibility of physical contact.

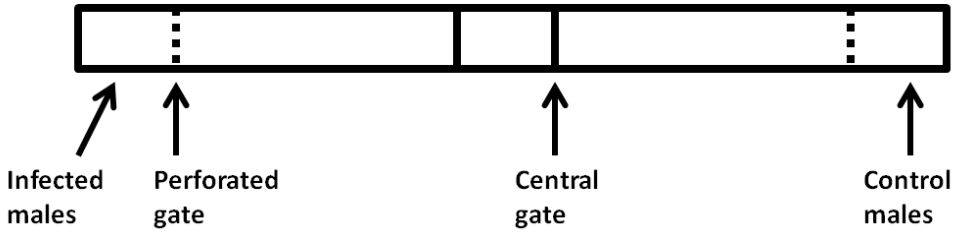



**Figure E: Mate choice assay arena**

An estrus female was introduced in the central hub and the gates opened. Occupancy of female in control and infected arms was quantified over trial duration of 1200s

**c.Paced Mating Assay**

**i.Non Competitve Assay**

A rectangular arena was used. This was divided into two chambers (30 X 45cm) by Plexiglas dividers. The dividers had cuts outs (50 mm diameter). These were big enough for the female to pass through but too small for the males. All experiments were conducted during the dark phase of the circadian cycle. Females were habituated in the arena for three consecutive days (15 minutes each).On the day of the experiment males and estrous females were brought to the behavior room and acclimatized for 10 minutes. Males were introduced in one arm and allowed to urine mark for 15 minutes prior to beginning of experiment. Females were then introduced into the other arm and the trials were recorded for 2 hours. Trials where the females did not show lordosis within the first 15 minutes were aborted. Trials were recorded using camcorder (model Samsung Schneider) under dim light conditions. Trials were timed manually and timeline of sexual events reconstructed.

**ii.Competitive Assay**

This experiment is similar to the above mentioned. The only difference being that the arena is divided into three chambers with two Plexiglas dividers with cut outs. The central small zone is the female chamber.

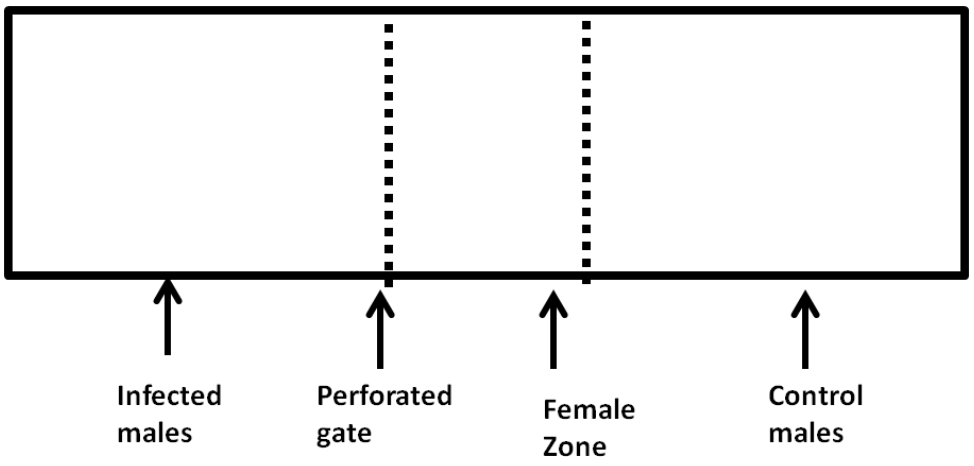



**Figure F : Paced mating arena**

The two terminal chambers house a control and infected male respectively. Female can enter both the male compartments and choose her mate.

**3. Testosterone 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 (Assay Designs).

**4.Immunohistochemistry**

**a. Visualization of Toxoplasma cysts**

Presence of Toxoplasma in testes was visualized by histological staining of Toxoplasma tissue cysts in epididymis (7 weeks post infection). Epididymis tissue was homogenized in PBS. This was smeared on a suprafrost glass slide and air-dried overnight. Toxoplasma was detected using an anti-GFP antibody (1:1000; Millipore) as the Toxoplasma strain used in our study has a GFP insert. This signal was further amplified using a combination of avidin-biotin complex (Vector Labs) and tyramide signal amplification coupled with Cy3 dye (1:75, red color; Perkin Elmer). Cyst wall was visualized by staining with dolichos biflorus agglutinin (20 μg/ml; Vector Labs) coupled with fluorescein (green color). The DBA binds to polysaccharides found in the Toxoplasma cyst wall. Slides were counter-stained with DAPI (blue) (Vector Labs) and visualized in a fluorescent microscope using 40X objective (Carl Zeiss; LSM 710).

**b. AVP and Fos colocaliztion studies**

Animals were exposed to stimuli for 30 minutes and sacrificed 2 hours after start of stimulation. Exposure to cat odor (2ml urine) was in the home cage and estrous female in testing arena. Males that underwent latter treatment were habituated to arena for ten minutes on ten consecutive days. Animals handling was kept minimal during and post-stimulation.

Males (control and infected) were anaesthetized with isoflurane. A cardiac perfusion was performed. Animals were first flushed with ~50 ml of PBS and then with 500 ml of 4% PFA. Brains were dissected and maintained in 4% PFA overnight at 4°C. They were then transferred to 30% sucrose until they sank to the bottom. The brains were sectioned at thickness of 40 µm at room temperature in a vibratome. Sections are stored at -20°C in antifreeze solution. Sections were stained as free floating sections. Prior to staining the sections are washed in 1X PBS (3X10 min). Sections are treated with 3% $H_20_2$ for 15 minutes and washed with PBS (3X10 min). They were blocked in 5% serum for 2 hour at room temperature. Primary antibody treatment: Guinea Pig Anti AVP (Bachem, 1:500), Rabbit Anti Fos (Calbiochem, 1:1000), 5% blocking serum and 0.3% triton X) for 72 hours at 4°C. Wash in 1X PBS (6x10 min) Secondary antibody treatment: Biotinylated anti Guinea Pig Anti rabbit tagged with Dylight549 (1:200, Vector Lab), 3% blocking serum,0.3%Triton X) for 2 hours at RT. Wash in 1X PBS (6x10 min). Sections are placed in pre prepared Enzyme A and B solution (ABC kit vector Labs) for 45 minutes at RT. They treated with TSA solution (1:100, Perkin Elmer), washed and counterstained with DAPI for 1 minute. The sections were mounted on superfrost slides and air dried overnight followed by mounting in permount.

**c. Imaging and counting**

Stained sections were imaged using a 40X objective lens with a 1.2X digital magnification at confocal microscope (Carl Zeiss LSM 710). Sections were optically sliced at 4 µm, three set of stacks per animal. Neurons positive for

DAPI, Fos and AVP were counted. Neurons that were co stained for AVP and Fos were considered to be colabeled. Images were quantified between Bregma -2.76 to -3.24. Caution was taken not to duplicate neuron counts across different optical stacks. Scores were cumulated per animal and percentages calculated using total DAPI counts. Experimenter was blind to treatment groups while scoring.

**d. Calculation of observed and expected frequencies**

I calculated expected probability of encountering colabeled neurons by multiplying individual probabilities of AVP-ir and Fos-ir neurons. Individual probabilities for AVP-ir were calculated by division of number of AVP-ir neurons with total number of DAPI positive neurons counted (i.e. probability that a particular DAPI positive neuron will be also be AVP-ir). Individual probabilities for Fos-ir were also counted in the similar manner. A product of these probabilities defines the baseline expectation of colabeling by mere chance and assuming biological independence between Fos and AVP activation. The observed probabilities of colabeled cells were compared to this expected baseline, with null hypothesis of colabeling being a mere mathematical coincidence adapted from [196]).

**4. Microdisection 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 (between bregma -2.76 to -3.24) was microdissected using autoclaved glass Pasteur pipettes to obtain micropunches that were transferred to 80 µl of lysis buffer in the eppendorf tubes. An additional 40 µl lysis buffer was pipetted into each of the glass Pasteur pipettes to wash down any remaining tissue into their respective eppendorf tubes. 1 µl of RiboLock (Thermo Scientific, Singapore; RNase inhibitor) was added per tube to ensure that the RNA does not degrade.

Paraventricular Nucleus was micro-dissected as a control.

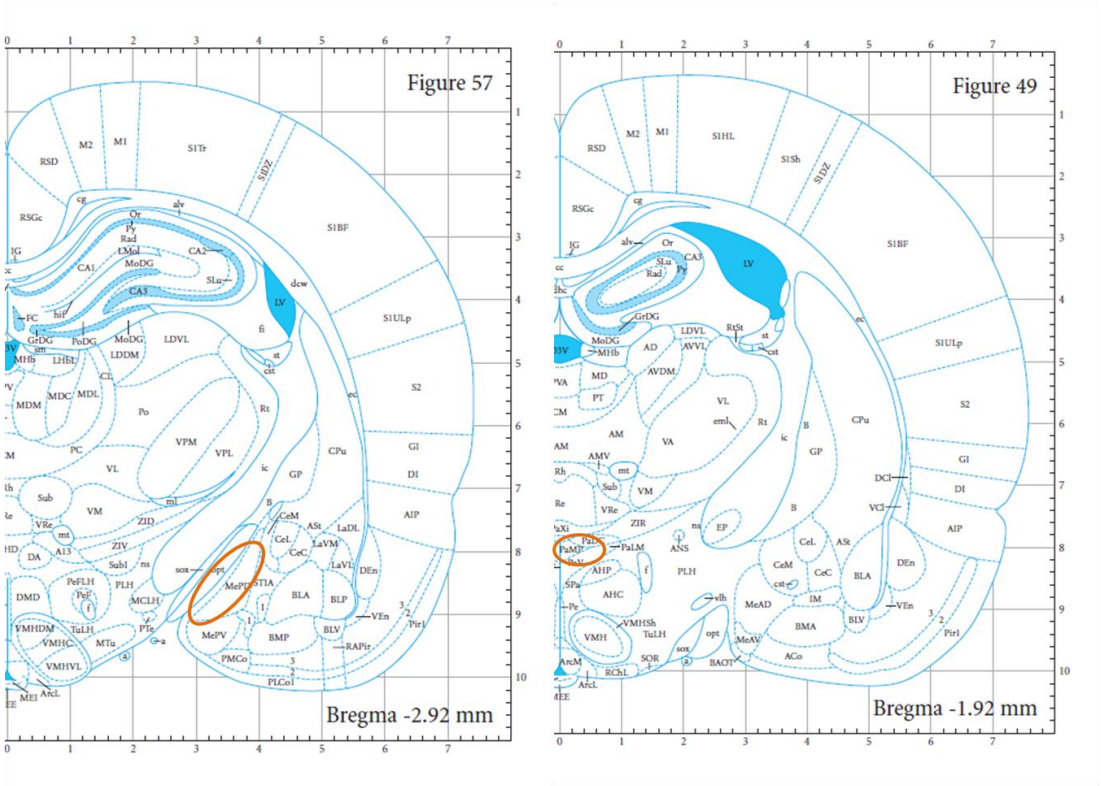



**Figure G: Region microdissected (Adopted from [208])**

**5. 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 µL DEPC water with 1 µL Ribolock. Samples were immediately converted to cDNA.

cDNA synthesis was performed using the RevertAid first strand cDNA synthesis kit from Fermentas (Catalogue number #K1621, #K1622). 20 µL 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

| **Reagent**<br><br>**(Thermo Scientific, Singapore)** | **Volume** |
|---|---|
| **5X reaction Buffer**<br>(250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl2, 50 mM DTT) | **4µl** |
| **Ribolock RNAase inhibitor**<br>(20 u/µl) | **1µl** |
| **Revertaid M-MuLV Reverse Transcriptase**<br>(200u*/µg) | **1µl** |
| **Oligo (dT)18 primer**<br>(0.5µg/µl) | **1µl** |
| **DNTP mix**<br>10Mm | **2µl** |

**5. 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 4C 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.

## 6.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) or BstUI (unmethylated CGCG). HpaII (New England Biolabs, Ipswich, MA) was used to cleave DNA at promoter site 1 and BstUI (Fermentas Inc., Glen Burnie, MD) at promoter site 2 (see Figure 16). 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. For promoter site 2, 600 ng of DNA from each animal was diluted in 1 µl of 'buffer R' in separate tubes. 1 µl of BstUI restriction enzyme was added and mixture incubated at 37 °C for one hour with. Both enzymes were inactivated by treatment at 65 °C for 20 min.

[%] Adapted from [76]

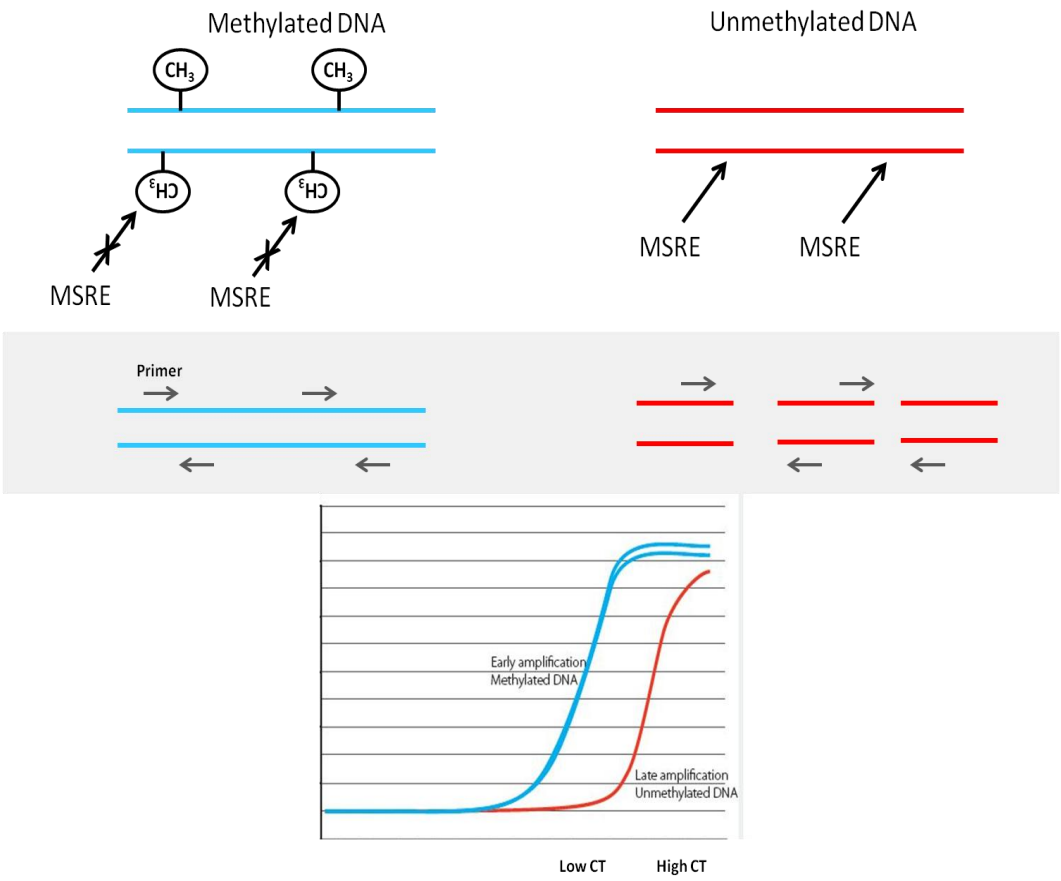


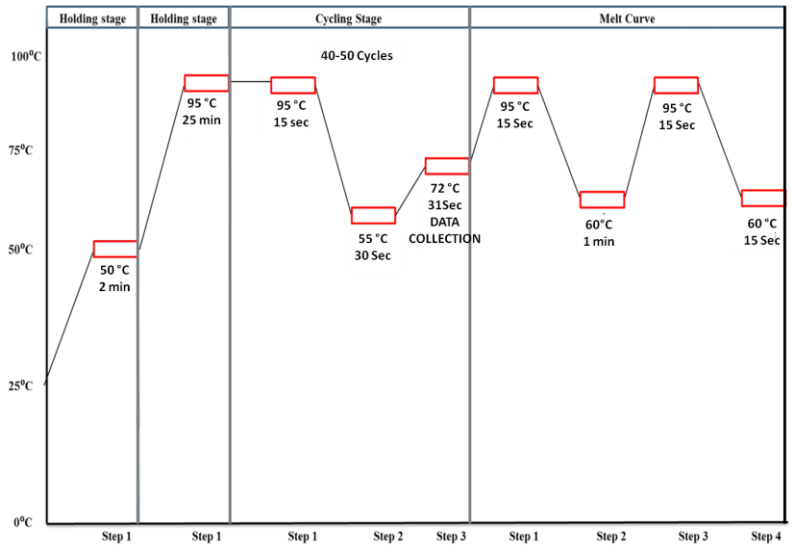

**Figure H: MSRE method of promoter methylation quatification**

## 7. qPCR reaction[%]

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

[%] Adapted from [76]

| **Reagent** | **Volume** |
|---|---|
| **Sybrgreen buffer**<br><br>**(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** |



## 8. Primers Used

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 [76]

## 9. 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/qpcrefficiency/). Relative expression with reference to internal control was quantified using freely available REST software (http://www.REST.de.com)[210]. 10000 randomizations were used. This software uses PCR efficiency-calibrated model and randomization tests to obtain relative expression level.

## Annexe 6: References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**Annexe 7 : Publication List**

- **Dass SA**, Vasudevan A, Dutta D, Soh LJ, Sapolsky RM, Vyas A, 20122 Protozoan parasite Toxoplasma gondii manipulates mate choice in rats by enhancing attractiveness of males. PLoS One. 20122; 6(11):e27229. Epub 2011 Nov 2

- Lim A, V Kumar, **Hari Dass S.A**, Vyas A, 2012 Toxoplasma gondii infection enhances testicular steriodogeneis in rat. Mol Ecol. 2013 Jan; 22(1):102-10

- Copulation or sensory cues from the female augment Fos expression in arginine vasopressin neurons of the posterodorsal medial amygdala of male rats.

  **Shantala Arundathi Hari Dass** and Ajai Vyas (Accepted at Frontiers in Zoology)

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

  **Shantala Arundathi Hari Dass** and Ajai Vyas

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# Protozoan Parasite *Toxoplasma gondii* Manipulates Mate Choice in Rats by Enhancing Attractiveness of Males

**Shantala Arundathi Hari Dass[1☯], Anand Vasudevan[1☯], Deborah Dutta[1], Linda Jing Ting Soh[1], Robert Morris Sapolsky[2], Ajai Vyas[1]***

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## Abstract

Females in various species typically avoid males infected with parasites, while parasite-free males advertise their status through conspicuous phenotypic traits. This process selects for heritable resistance and reduces direct exposure of the female to parasites. Coevolving parasites are likely to attempt to circumvent this obstacle. In this paper, we demonstrate a case of parasitic manipulation of host mate choice. We report that *Toxoplasma gondii*, a sexually transmitted infection of brown rats, enhances sexual attractiveness of infected males. Thus under some evolutionary niches, parasites can indeed manipulate host sexual signaling to their own advantage.

**Citation:** Dass SAH, Vasudevan A, Dutta D, Soh LJT, Sapolsky RM, et al. (2011) Protozoan Parasite *Toxoplasma gondii* Manipulates Mate Choice in Rats by Enhancing Attractiveness of Males. PLoS ONE 6(11): e27229. doi:10.1371/journal.pone.0027229

**Editor:** Gordon Langsley, Institut national de la santé et de la recherche médicale - Institut Cochin, France

**Received** August 5, 2011; **Accepted** October 12, 2011; **Published** November 2, 2011



**Funding:** This research was funded by Nanyang Technological University, Singapore, through its Nanyang Assistant Professorship program; Stanley Medical Research Institute and National Institute of Health, USA (grant AI41014). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Host and parasites coevolve by placing constant demand on each other for adaptations and counter-adaptations. Selection pressures acting on the host can be very severe, in fact severe enough to drive host towards bi-parental reproduction even when this is very costly to maintain non-childbearing males [1]. Hence, it is not surprising that females typically avoid and in fact show aversive response to parasitized males [2–12]. Aversion of females to parasitized males is likely driven by the evolutionary need for females to choose for heritable parasite resistance [13]; and/or avoid direct transmission of contagious diseases during mating [14]. Such female aversion is detrimental for parasites, particularly if they are transmitted by sexual intercourse. We posit that in this situation, parasites have an evolutionary pressure to manipulate host males in a way that overcomes the traditional female aversion.

We tested this hypothesis using a common protozoan parasite of the brown rats, *Toxoplasma gondii* [15]. Recently, *Toxoplasma gondii* has been reported to be sexually transmitted in sheep and dog [16,17]. We first tested if it is a sexually transmitted in rats; we then investigated if chronic *Toxoplasma gondii* infection manipulated female mate choice.

## Results

### *Toxoplasma gondii* is a sexually transmitted infection in brown rats

Parasitic cysts containing bradyzoites could be visualized eight weeks post-infection in epididymis, a tubular structure that collects and stores sperm (estimated burden = 520 cysts per animal, 3–7 μm in diameter, n = 5 animals; Figure 1A). These cysts were viable; feeding them resulted in sero-conversion of uninfected females (4/4 attempts; one infected epididymis/female). Infected males were mated with uninfected females. *Toxoplasma gondii* cysts could also be observed from vaginal lavage of females 12 hours after mating (6 out of 7 mating; Figure 1B), indicating that *Toxoplasma gondii* was successfully ejaculated. Mating with an infected male resulted in transmission of infection to females (4/4 mating). This was demonstrated by successful PCR amplification from female brain of a gene unique to the parasite. Using previously published primer sets, we successfully amplified B1 gene of *Toxoplasma gondii* from genomic DNA prepared from crude lysate of female brains four weeks after mating (see [18] for methods). Identity of PCR product was confirmed using DNA sequencing. Infection was also confirmed by serological tests showing presence of anti-parasite IgG antibodies in mated females (serum dilution 1:1000; 6/7 mating). Serum obtained from mated females contained primary antibodies against laboratory-cultured *Toxoplasma* gondii tachyzoites (visualized using anti-rat IgG coupled with Cy3). Mated females were allowed to give birth, and pups were tested for presence of *Toxoplasma gondii*. Parasite cysts were detected in 43 out of 69 pups that we tested (Figure 1C). Hence *Toxoplasma gondii* is a sexually and vertically transmitted infection in rats.

### Reproductive parameters of control and infected animals were comparable

Despite invading reproductive organs, *Toxoplasma gondii* did not adversely affect sexual behavior or fecundity/fertility of infected males. We tested the attraction of sexually naïve control and

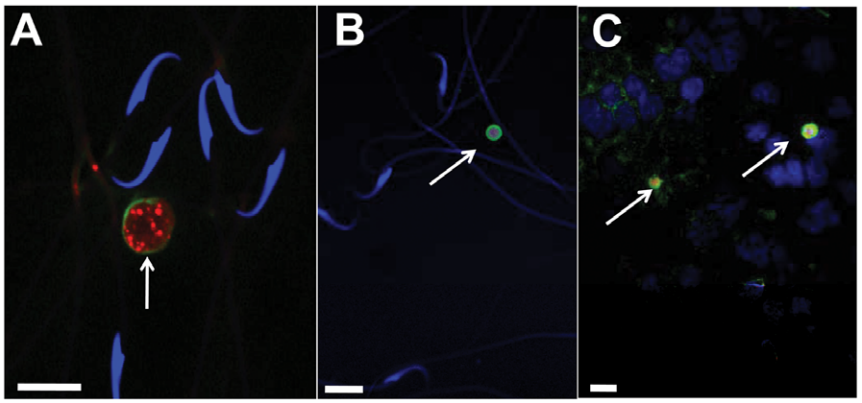


**Figure 1. *Toxoplasma gondii* is transmitted through intercourse.** *Toxoplasma gondii* cysts were observed in epididymis of infected males (panel A), in vaginal lavage of naïve females mated with infected males (panel B) and in brain of pups derived from these mating (panel C). Scale bar = 10 µm. Parasites are stained red (anti-GFP antibody couples with fluorogenic detection using Cy3), cyst wall green (dolichos biflorus agglutinin coupled with fluorescein) and sperm nuclei in blue (DAPI).
doi:10.1371/journal.pone.0027229.g001

infected males to females. Attraction was quantified by comparing time spent by a male in two opposing arms of an arena, each arm containing either an inaccessible estrus female or an unfamiliar male. Both control and infected animals exhibited comparable attraction to females (n = 11 males in each group; exact Mann-Whitney test: $Z = -0.23$, $p > 0.8$; % time spent in bisect containing receptive female = 58±4.8% for control and 59±3.7% for infected animals). Control and infected animals exhibited comparable number of mounts and intromissions during paced mating with uninfected females (n = 6 control and 7 infected males; exact Mann-Whitney test: $Z < -0.43$, $p > 0.7$; Table 1). Likewise, infection did not affect the number of pups, weight of newborns or sex ratio of progeny resulting from mating (Table 2).

## Uninfected females preferred infected males

Next, we tested if uninfected females avoided parasitized males, as would be expected from the extensive literature on this subject [2–12]. Preference of an estrus female for control or infected male was determined during a two-choice preference task (12 pairs of males, 5–7 females per pair; total 72 trials). Contrary to expectation, sexually naïve females spent more, rather than less time in the infected bisect (Wilcoxon signed ranks test: $Z = -4.547$, $p < 0.00001$; 506±30 s in infected bisect versus 308±17 s in control; n = 72 trials). A preference score of females for infected males was computed for each trial by dividing time spent in infected bisect by that in control. During 74% of trials, females spent more time in the infected bisect (Figure 2A; $Chi^2$ test: $Chi^2 = 16.1$; $p < 0.0001$; infected>control = 53 trials, control >infected = 19 trials; median preference score = 1.54).

Time spent in infected and control bisects for each unique male pair was calculated by taking median of all females tested for each male pair. In all male pairs tested, median occupancy in infected bisect was greater than median occupancy in control area (Figure 2B; exact Wilcoxon signed ranks test: $Z = -3.059$; $p < 0.0001$).

The generality of this finding was confirmed using a different strain of rats in a geographically different laboratory (Long-Evans rats in Stanford University; Figure 2C; n = 36 females; $Chi^2$ test: $Chi^2 = 13.4$; $p < 0.001$; infected>control = 29 females, control >infected = 7 females; median preference score = 1.30). In this case, male bedding was used instead of urine marks and males were absent from the arena during testing. Since males were not physically present during this experiment, this precludes a role for male vocalization in our observations.

**Table 1.** Despite invading reproductive tissue, *Toxoplasma gondii* did not affect mating performance of infected animals.

| | Group | N | Mean | Std. Deviation | Std. Error Mean | *p*-value |
|---|---|---|---|---|---|---|
| Mounts | Control | 6 | 129.67 | 65.347 | 26.678 | 0.731 |
| | Infected | 7 | 119.86 | 40.429 | 15.281 | |
| Intromissions | Control | 6 | 110.50 | 51.130 | 20.874 | 0.945 |
| | Infected | 7 | 116.43 | 39.782 | 15.036 | |

Control and infected males were mated with estrus female in a paced mating set-up, whereby female could pace the sexual interaction. Both control and infected animals performed comparably.
doi:10.1371/journal.pone.0027229.t001

## Infected males gained greater reproductive opportunities

In a competitive paced mating set-up whereby female chose to mate between one control and one infected male, females allowed greater number of reproductive opportunities to infected males (Figure 2D). During two-hour trial, infected Wistar males secured more intromissions compared to controls (n = 6 pairs; exact Wilcoxon signed ranks test: $Z = -2.21$, $p < 0.05$; one-sample t-test against chance i.e. 1: $t = 4.36$, $p < 0.01$). Hence, *Toxoplasma gondii* infection altered mate choice of females in a completely unexpected direction, whereby infected male gained in terms of reproductive fitness.

# Discussion

## Further support for manipulation hypothesis

The core of parasitism is the ability of an organism to exploit its host. According to the behavioral manipulation hypothesis, a parasite may be able to alter the behavior of its host for its own selective benefit [19–22]. *Toxoplasma gondii* has been previously described as a classical case of parasitic behavioral manipulation [23–26]. It blocks the aversion of rats for cat urine, instead producing an attraction. This behavioral change is likely to increase the likelihood of a cat predating a rat. This is thought to

**Table 2.** Despite invading reproductive tissue, *Toxoplasma gondii* did not affect number and sex ratio of progeny.

| | Group | N | Mean | Std. Deviation | Std. Error Mean | *p*-value |
|---|---|---|---|---|---|---|
| Body weight of newborn pups | Control | 83 | 6.266 | 0.4374 | 0.0480 | 0.04 |
| | Infected | 76 | 6.414 | 0.5230 | 0.0600 | |
| # of Pups per mating | Control | 6 | 14.17 | 0.983 | 0.401 | 0.47 |
| | Infected | 6 | 13.00 | 2.191 | 0.894 | |
| Sex Ratio (% Male Pups) | Control | 6 | 39.433 | 23.3232 | 9.5216 | 0.69 |
| | Infected | 6 | 48.883 | 9.7183 | 3.9675 | |

Infected animals sired comparable number of live progenies with similar sex ratio. Pups born to infected fathers weighed marginally more, an effect statistically significant but of very small effect size.

doi:10.1371/journal.pone.0027229.t002

reflect an adaptive behavioral manipulation by the parasite because it reproduces sexually only in the gut of the cat [23,24,27]. In this report, we show that *Toxoplasma gondii* is transmitted through sexual intercourse in brown rats, and that the parasite can manipulate mate choice of uninfected females. This behavioral change plausibly enhances transmission of the parasite from males to females and their progeny. Data presented here suggest a novel class of behavioral manipulation by *Toxoplasma gondii*. This runs counter to well-established observations in rodents [4,6] and in other animals [2,3,5,7–11] that females detect and avoid males infected with an array of viruses, bacteria, protozoa and nematodes. For example, female mice avoid males infected

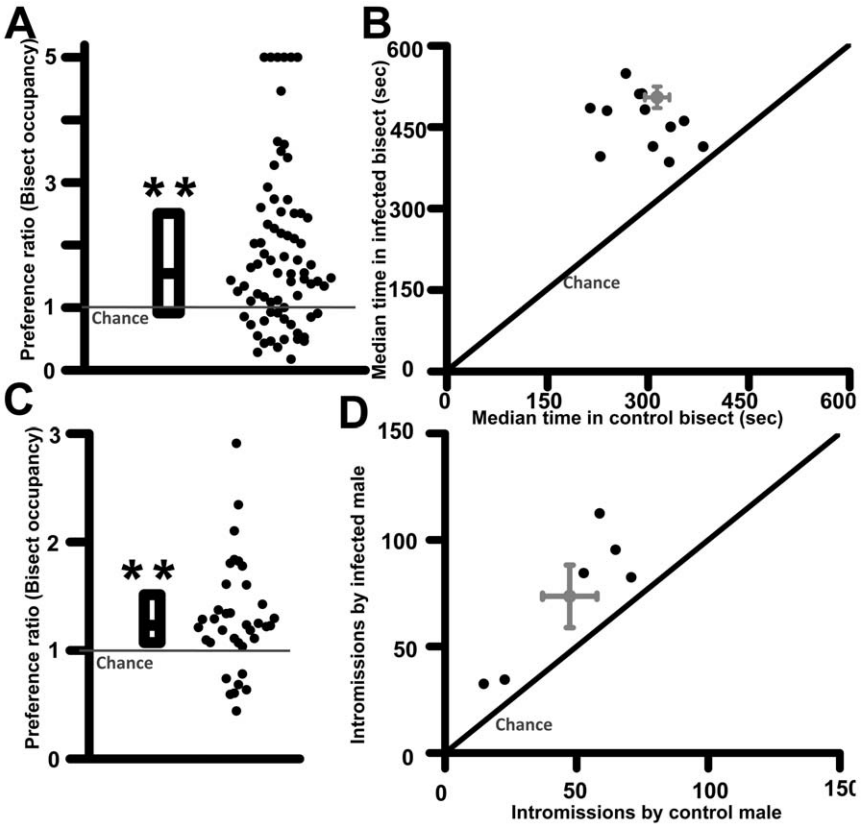


**Figure 2. Uninfected females preferred infected males.** Preference was quantified by comparing time spent by an estrus female in two opposing arms of an arena, each arm urine marked by either controls or males infected six weeks earlier (panel A; trial duration = 1200 s). Ordinate depicts time spent in infected bisect divided by control bisect (ratio >5 assigned arbitrary value of 5). Each dot represents raw data from one female. Box plots depict median, 25$^{th}$ percentile and 75$^{th}$ percentile. Preference of females for males in each of the 12 unique pair was calculated by taking median of all females tested for that particular male (panel B; ordinate and abscissa depicts time spent in infected and control bisect, respectively). Mean and SEM of data used in scatter-plot are depicted in grey color. These results were confirmed using a different rat strain in a geographically distinct location (panel C; Long-Evans rats in SU, USA; in place of Wistar rats at NTU, Singapore). **, $p<0.001$; Chi$^2$ test. In a competitive paced mating, females chose to participate in more mounts and intromissions with infected males (panel D; ordinate and abscissa depicts number of intromissions by infected and control males, respectively). *, $p<0.01$; one-sample t-test against chance, i.e. 1. Intromission data exhibited one outlier that has been removed from the graph. Mean and SEM are depicted in grey color.

doi:10.1371/journal.pone.0027229.g002

with *Eimmeria vermiformis*, a close relative of *Toxoplasma gondii*, and have an aversive physiological reaction to the smell of such males [6]. As a note of caution, our evidence for sexual/vertical transmission is restricted to our examination of seven matings between infected males and uninfected females, four females post-mating and sixty-nine progenies, under laboratory conditions. This is supported by evidence of sexual transmission in sheep and dogs [16,17]. This observation is in need of verification from other research groups and in larger-scale field experiments. This has important implications because of our assumption that sexual transmission is frequent enough to produce selection pressure for a parasitic manipulation.

The notion of extended phenotype refers to the situations where genotype of an organism manifests its phenotype outside the physical confines of its body [28]. Parasitic behavioral manipulation is an elegant demonstrations of extended phenotypes. In this case, genotype of the parasite induces a phenotype in terms of host behavior. The ability of the *Toxoplasma gondii* to not only advantageously alter the behavior and physiology of its host, but to also secondarily alter the behavior of uninfected females presents a striking example of the "extended phenotype" [28].

We suggest that enhanced attractiveness of infected rats is a parasitic manipulation meant to increase chances of parasite transmission. This suggestion should be carefully tested against the ease of erroneously invoking an adaptationist explanation (see [20] for scholarly discussion of the issue). A behavioral manipulation is "too well fitted" to its purpose to arrive by chance. In other words, there needs to be a conformity between the observed change and "a priori design specification" that a engineer might use [20]. For example, both increase and decrease in locomotion of prey can be argued to lead to increased predation; it is not purposive [20]. In this regard, an infection that imparts more attractiveness to male is an "a priori design specification"; an obvious solution to get more parasites travel with semen. Thus, we posit that the behavioral change reported here constitutes a behavioral manipulation rather than a generalized effect of infection. Nonetheless, this view makes an important assumption that sexual transmission plays a sizeable part in life history of the parasite, an assumption that needs to be tested further in large-scale field experiments.

## Exploitation of host sexual selection

Various theories have been proposed to explain the functional significance of mate choice (reviewed in [29–31]); based on direct benefits to the female [32], heritable parasite resistance [13], prevention of associative transmission of infections [14] or runaway selection processes [33]. Regardless of selection pressure that maintains mate choice, it remains beneficial for a sexually transmitted parasite to manipulate it. This possibility has not been studied extensively. On related note, male chironomid midges (*Paratrichocladius rufiventris*) have better success at forming mating pair if infested by a mite (*Unionicola ypsilophora*) [34]. Mating in midges generally involves females forming a swarm and male flying through it trying to capture a female. It is not clear if female mate choice or inter-sexual signaling plays any meaningful role in midges. Thus, possibility of parasitic effects on mate choice has remained unaddressed in this system.

Apart from parasitic manipulation of mate choice, there are two kinds of situations where host sexual signaling and parasites could interact. In one type of examples, parasites invade reproductive tissue of host producing 'parasitic castration' [35], aiming to block host from spending valuable metabolic currency on its own reproduction. In a second type of examples, parasites act as 'illegitimate receivers' of sexual signals in order to locate potential hosts [36]. Both of these situations are distinct from behavioral manipulation of mate choice.

Female mate choice is an important mediator of reproductive success of male rats. Several semi-naturalistic and laboratory studies have demonstrated that female rats strongly pace the sexual interaction through punctuated display of solicitation behavior (reviewed in [37], also see [38–40]). Indeed neuroendocrinological changes essential for initiating pregnancy depend on intermittent nature of coital stimulation in females. These observations suggest that solicitation controlled by females is central to rat sexual behavior. Thus, it is a plausible speculation that a parasitic strategy based on female mate choice will be selected.

Role of *Toxoplasma gondii* infection in rat mating success has been investigated before [41], using a semi-naturalistic setting. It was reported that infection did not alter mating success as defined by number of ejaculations and mounting. Reproductive success of males in this arrangement is a product of both male-male competition and mate choice. One possibility is that females in this arena had low opportunity to pace the interaction, for example by hiding in home-boxes that had an opening small enough to allow only females and not males. As indicated earlier, several studies have established that intermittent solicitation by females is regular feature of reproductive ritual in rats; and it facilitates successful pregnancy. Another possibility is that male-male competition heavily contributed to the mating success in semi-naturalistic setting, overriding influence of female mate choice. This possibility will require further experimentation in form of careful dissociation of both intra-sexual and inter-sexual behaviors. Pending that, it is difficult to ascertain if parasitic manipulation of mate choice will result in significant gain of reproductive fitness of infected males.

## Mechanism

What are the proximate mechanisms? It can be safely assumed that pheromonal communication rather than acoustic signaling is involved. This is because presence of soiled bedding itself is sufficient to show difference between infected and control males. Major urinary proteins play important part in sexual signaling of house mouse [42,43]. It is possible that change in similar proteins in rats is involved in manipulation of mate choice. This possibility is supported by the fact that rat urine contains large amount of major urinary proteins. On the other hand, mouse and rat major urinary proteins have evolved separately after divergence from a common ancestor [44]. Presently, there is no unequivocal evidence that rat major urinary proteins do participate in sexual signaling. Further experiments are needed to delineate pheromonal mechanisms involved in this behavioral change.

## Cost of the parasitism

There are varieties of documented cases where parasites manipulate host behavior in order to gain selective advantage [19–21]. Such behavioral manipulations usually have detrimental consequences for the host. But the atypical effects of *Toxoplasma gondii* on mate choice raise an intriguing speculation. Reproductive success in infected males is likely to be elevated, by virtue of their increased attractiveness. Potentially, this elevation could more than offset the decreased fitness of infected males due to their increased likelihood of being predated by cats. While difficult to test in studies of natural populations, these data raise the possibility that parasitic behavioral manipulation will at least blunt the cost of parasitism for infected males. It must be noted that precise determination of reproductive fitness is a difficult task, in view of insufficient quantitative knowledge about rodent reproduction and predation. Thus possibility that infection raises reproductive

success of males itself is a speculation at this moment, albeit an interesting and plausible one (also see [41] for a contrary view).

Parasitic manipulation of sexual signaling is in contrast to the idea that sexual selection results in discrimination against parasitized males. Under some evolutionary niches, parasites can indeed manipulate host sexual signaling to their own advantage.

## Materials and Methods

### Animals

Wistar rats (48 days old, housed two/cage) were obtained from vivarium of National University of Singapore. The Nanyang Technological University institutional animal care and use committee reviewed and approved all procedures (ARF SBS/NIE- A0106AZ). Long-Evans rats (49 days old, three/cage) were obtained from Charles River laboratories (Willmington, MA). The Stanford University administrative panel for laboratory animal care approved procedures pertaining to Long-Evans rats (**APLAC#11603**). Animals from this source tested serologically negative for *Toxoplasma gondii*.

### Parasites and Treatments

We used a Prugniaud strain genetically modified to constitutively express green florescent protein under GRA2 promoter. Parasites were maintained as tachyzoites by passage in human foreskin fibroblast monolayers [24,25]. Infected fibroblasts were syringe-lysed by using a 27-gauge needle to release tachyzoites. Animals were either infected with tachyzoites ($5\times10^6$, i.p.) or mock-infected with sterile phosphate buffered saline. It is noteworthy here that oral ingestion of oocysts or tissue cysts, and not intraperitoneal entry of tachyzoites, constitutes a more naturalistic route of infection. Yet, we have chosen to use tachyzoites because this produces a more consistent cyst burden in our experience with minimal risk of occupational exposure to laboratory personnel.

All behavioral experiments were conducted between 6 to 8 weeks post-infection, a period know to harbor chronic infection. Infected and control animals gained comparable weight during the experiment.

### Visualization of *Toxoplasma gondii* cysts

Smears from epididymis, post-mating vaginal lavage and brain tissue of fetus were examined for the presence of *Toxoplasma gondii* cysts. Taking advantage of endogenous production of GFP by the transgenic parasite, cysts were observed using anti-GFP antibody (Millipore Cat # AB3080, 1:200 antibody dilution). Interaction between GFP and primary antibody was visualized using tyramide signal amplification coupled with Cy3 dye (red color; Perkin Elmer Cat # NEL744001KT**).** Cyst wall was stained using dolichos biflorus agglutinin (20 μg/ml) coupled with fluorescein (Vector Labs Cat # FL1031, green color). Sperm nuclei were visualized using DAPI (blue color). Stained smears were imaged using a confocal microscope (LSM 710 META, Zeiss).

### Polymerase chain reaction and Serological confirmation of infection status

We utilized polymerase chain reaction to selectively amplify a 35-fold repetitive sequence of the gene B1, a gene that is selectively found in *Toxoplasma gondii*. Primer sets and PCR methods were adopted from previously published protocols (5′-GGAACTGCATCCGTTCATGAG-3′ and 5′-TCTTTAAAGCTTCGTGGT C-3′) [18].

Infection was also confirmed by serological detection of anti-Toxoplasma 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 1 ml of the serum (1:1000) overnight at 4°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 by treating with anti Rat IgG-Cy3 (1:200, Millipore) (red color).

### Mate choice assay

Naturally cycling females were used in all assays.

Wistar: Female mate choice was quantified by comparing time spent by estrus females in two opposing arms of an arena (76×9 cm each; 15 cm high) during a 20 minute trial. Opposing arms were scent marked by control and infected males. Males spent 60 minutes before the trial urine marking their respective bisects. Male were restricted to one end of bisect during actual trial, a perforated plastic sheet separating males and test female. Occupancy of female in each of the arm was quantified.

Long-Evans: Male stimulus consisted of 100 g of 24 h soiled bedding, pooled from 4 cages each housing three males; placed in opposing corners of a plastic box; 92.4×35.3 cm, 40.1 cm high. Occupancy of estrus female in bisects containing either control or infected odor was quantified.

### Paced mating

Non-competitive paced mating: Experiments were conducted in a glass arena with two chambers connected through a 5 cm semi-circular gate. One chamber housed either a control or an infected male (30×45 cm). At the start of trial, estrus female was placed in the arena and observed for duration of 2 hours. Because of difference in body-size, only female could cross the gate connecting two chambers; thus allowing female to pace sexual interactions by withdrawing in chamber not occupied by the male. Intromissions were defined as a reproductive sequence starting with mount and ending in self-genital licking by males.

Competitive paced mating: Arena similar to the one described above was used, but with two terminal chambers holding a control and an infected animals during same trial. Female could thus choose to have paced sexual encounter with either male or to withdraw in middle compartment where neither male was present.

### Statistical analysis

Mann-Whitney test was performed to determine statistical significance when comparing between experimental groups. Wilcoxon signed-rank test were used when comparing paired differences in within-subject parameters. Wherever appropriate, chi-square test was used to test deviation from ratios predicted by random occurrence. In all statistical tests, exact statistics was used to eliminate asymptotic and approximate statistical assumptions.

## Author Contributions

Conceived and designed the experiments: SAHD A. Vasudevan RMS A. Vyas. Performed the experiments: SAHD A. Vasudevan DD LJTS A. Vyas. Analyzed the data: SAHD A. Vasudevan A. Vyas. Wrote the paper: RMS A. Vyas.

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MOLECULAR ECOLOGY

Molecular Ecology (2013) **22**, 102–110 doi: 10.1111/mec.12042

# *Toxoplasma gondii* infection enhances testicular steroidogenesis in rats

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**Abstract**

**The protozoan parasite *Toxoplasma gondii* enhances the sexual attractiveness of infected male rats and attenuates the innate fear of cat odour in infected individuals. These behavioural changes plausibly lead to greater transmission of parasites through sexual and trophic routes, respectively. Testosterone, a testicular steroid, is known to reduce fear and enhance sexual attractiveness in males. Here, we show that *Toxoplasma gondii* infection enhances expression of genes involved in facilitating synthesis of testosterone, resulting in greater testicular testosterone production in male rats. In several species, testosterone mediates trade-offs between sexually selected traits and life history decisions. Augmentation of testosterone synthesis by *Toxoplasma gondii* suggests that parasites may manipulate these trade-offs in rats.**

*Keywords*: anti-predator behaviour, behavioural manipulation, mate choice, parasitism, sexual transmission, testosterone, trophic transmission

*Received 12 March 2012; revision received 19 August 2012; accepted 23 August 2012*

## Introduction

Relationships between parasites, sexually selected traits and predators have been well studied and widely debated. Sexually selected traits evolve to advertise heritable capacity to ward off parasites and/or risk of direct transmission of parasites during sexual encounter (Hamilton & Zuk 1982; Able 1996). At the crux of this formulation is the idea that, in many species, sexually selected traits require sustained production of testosterone, which is expensive in terms of metabolic and opportunity costs (Folstad & Karter 1992; Wingfield *et al*. 1997). Thus, cost of sustaining testosterone-dependant traits confers an honesty whereby only fit males can afford to display. In interest of completeness, it should be noted that some of sexual selection models do not depend on parasites as driving force (Andersson & Simmons 2006).

Sexually selected traits are by necessity conspicuous; they evolve to communicate and advertise. One of the collateral costs of such traits is that predators find it easier to locate conspicuous males, thus setting up a trade-off (Zuk & Kolluru 1998). Related to that testosterone can cross the blood–brain barrier and reduces fear in addition to its role in organizing sexual behaviours (Kavaliers *et al*. 2001; Aikey *et al*. 2002; King *et al*. 2005; Hermans *et al*. 2006). Reduced fear might further increase predation rates of high-testosterone males.

Observations noted in the previous two paragraphs posit a very interesting and as yet unexplored possibility. From the perspective of the parasite, it is highly beneficial to successfully alter sexually selected traits in host. This requires parasite-induced increase in host testosterone synthesis. If the parasite is sexually transmitted, this change will result in greater transmission through sexual and vertical routes. If the parasite is transmitted by predation, it will result in greater transmission through the trophic route (due to reduced fear and greater conspicuousness of high-testosterone males). If the parasite relies on both sexual/vertical and trophic routes, benefits of the change are additive.

The possibility of such 'love-potion' parasitism has been discussed sporadically (Lafferty 1999), but not experimentally tested. In this report, we test this hypothesis using *Toxoplasma gondii–Rattus Norvegicus* association. This is an ideal system to test this hypothesis because *Toxoplasma gondii* is transmitted through both sexual and trophic routes in rats (Dass *et al*. 2011). Moreover, we have earlier shown that males infected

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with *Toxoplasma gondii* become more attractive to sexually receptive females (Dass *et al.* 2011). This phenomenon is in stark contrast to general observations showing females avoidance of parasitized males (Kavaliers *et al.* 2005). Prior reports have also shown that rats infected with *Toxoplasma gondii* exhibit reduced innate fear to cat odours (Berdoy *et al.* 2000; Vyas *et al.* 2007; Vyas & Sapolsky 2010; Webster & McConkey 2010).

In view of the centrality of testosterone to sexually selected traits, we hypothesize that *Toxoplasma gondii* infection enhances testosterone synthesis and that this is an evolved trait of the pathogen characterized by a coordinated change in steroidogenic machinery of the host testes.

Most testosterone is made in testicular Leydig cells. Luteinizing hormone receptor (LHR) is a critical positive regulator of steroidogenesis (Parker & Schimmer 1995; Stocco 2000; Payne & Hales 2004; Azhar & Reaven 2007; Payne 2007). Activation of LHR results in acute facilitation of cholesterol transport to mitochondria through the enzyme StAR (Fig. 1). Cholesterol is then converted to the testosterone precursor pregnenolone through $P450_{scc}$ (Fig. 1). On a more chronic timescale, activation of LHR results in up-regulation of steroidogenic enzymes present in smooth endoplasmic reticulum of Leydig cells (Fig. 1; 3β-HSD, $P450_{c17a}$ and 17β-HSD) (Azhar & Reaven 2007; Payne 2007). We studied effects of infection on mRNA abundance of LHR and steroidogenic genes described above. We also compared testosterone concentration in testes and bloodstream of control and infected animals. Lastly, we investigated effects of castration on host behavioural change postinfection.

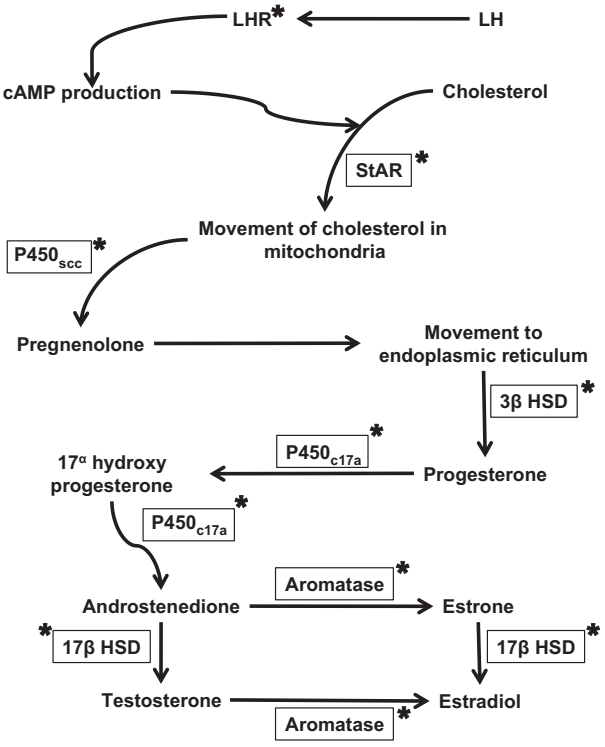


**Fig. 1** Metabolic steps involved in testosterone biosynthesis in rat testes. Testosterone synthesis is regulated by occupancy of LHR in Leydig cells of testes. Leydig cells produce testosterone using series of modification of cholesterol, each involving a distinct enzyme. *Proteins quantified in the present report. (LH, luteinizing hormone; LHR: luteinizing hormone receptor; cAMP: cyclic AMP; StAR: steroidogenic acute regulatory protein; $P450_{scc}$: cholesterol side-chain cleavage enzyme; 3β HSD: 3β-hydroxysteroid dehydrogenase; $P450_{c17}$: C17 hydroxylase/C17, 20 lyase/desmolase; 17β HSD: 17β-hydroxysteroid dehydrogenase.). Testosterone often exerts its action after conversion to alterative neuroactive ligands, dihydrotestosterone and oestrogen.

## Materials and methods

### *Animals and parasites*

Male Wistar rats (48 days old at the start of the experiment, housed two/cage) were obtained from vivarium of National University of Singapore. The Nanyang Technological University institutional animal care and use committee reviewed and approved all procedures.

We used a Prugniaud strain of *Toxoplasma gondii*, similar to prior reports (Vyas *et al.* 2007; Dass *et al.* 2011). Rats were either infected with the parasite ($5 \times 10^6$ tachyzoites, intraperitoneal) or mock-infected with sterile phosphate-buffered saline. All experiments were conducted between 6 and 8 weeks postinfection, a period consistent with observed behavioural changes (Vyas *et al.* 2007; Dass *et al.* 2011). Infection of rats with this strain of the parasite and at the dose used here does not result in illness or weight loss. It is still unclear whether *Toxoplasma gondii* infection is also asymptomatic to rats under wild conditions where food and water may not be available *ad libitum*.

### *Quantitative PCR*

The abundance of messenger RNA in the testes was quantified for LHR and steroidogenic enzymes (StAR, $P450_{scc}$, 3β-HSD, $P450_{c17a}$ and 17β-HSD), using the standard SYBR green-based real-time quantitative PCR. For each sample, the threshold cycle numbers ($C_t$, mean of triplicate) required to reach a predetermined fluorescence value was measured.

Total RNA was extracted from all infected and control testes samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Purified total RNA was subjected to DNase I digestion, followed by column purification using DNA

Free RNA kit (Zymo Research, Irvine, CA, USA). Eluted RNA (1 μg) was reverse transcribed with a first-strand cDNA synthesis kit (Fermentas, Glen Burnie, MD, USA). Each qPCR was initiated by constituting a 25-μl reaction volume comprising 12.5 μL of SYBR green master mix (2X; Applied Biosystems, Carlsbad, CA, USA), 0.25 μL of each primer (10 μM), 0.5 μl of cDNA (50 ng/μL) and 11.25 μL of DEPC-treated water. Real-time fluorescence was determined using the ABI PRISM 7500 system (Applied Biosystems, Foster City, CA, USA).

Primer sets used for various mRNA have been described in Table 1. GADPH was used as an internal reference. The internal reference was included in each plate for each sample.

### *Calculation of mRNA abundance*

Relative expression with reference to internal control was quantified using freely available REST© software (http://www.REST.de.com) (Pfaffl *et al.* 2002). This software uses PCR efficiency-calibrated model and randomization tests to obtain relative expression level with robust statistical testing. 10 000 randomizations were used. PCR efficiency for each primer set was quantified using a series of sequentially diluted pooled samples that were quantified using qPCR in similar conditions (efficiency >90% for all primers). Median and interquartile range of relative expression estimates is depicted in figures. 95% confidence intervals are also stated.

### *Quantification of testosterone levels*

One testes of each animal was homogenized and suspended in diethyl ether (twice the volume of the homogenized testes sample). For determination of the testosterone in plasma samples, 100 μL of plasma was suspended in 200 μL of diethyl ether. The mixture was shaken and layers were allowed to separate. The top layer containing the solvent was pipetted into in a clean test tube. The procedure was repeated three times and solvent layers were combined. Diethyl ether was then evaporated to dryness. Extracted testosterone was dissolved in one ml of PBS. The testosterone concentration was estimated by enzyme-linked immunoassay using commercial kit (Enzo Life Sciences, Farmingdale, NY, USA). This kit exhibits low cross-reactivity to related steroids like dihydrotestosterone, oestrogen and corticosterone (<0.001%, quantification provided by the supplier) and has been previously employed in estimates of testosterone in faeces, tissue and plasma samples (Chang *et al.* 2004; Ezenwa *et al.* 2011; Mitra & Sapolsky 2012). Any absorbance value that exceeded minimum or maximum bounds of standards employed in the assay was discarded.

### *Castration and measurement of innate fear*

Innate fear to bobcat urine was quantified in control and infected animals, both in castrated males and a separate set of sham-operated males. A medial incision was made in the scrotum of heavily anaesthetized animal. Testes and vas deferens of 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.

For quantification of aversion to predator odour, undiluted bobcat urine (2 mL; Leg Up Enterprises, Lovell, ME, USA) and rabbit urine (2 mL, from local vivarium) were placed on two terminal sides of a rectangular Plexiglas arena (two arms of 46 × 9 cm each; 15 cm high, separated by a central chamber). Three habituation trials (10 min each) without odour preceded the testing. The location of the animal was recorded over a trial of 20 min using a top-mounted video camera coupled with tracking system (Any-maze; Stoelting, Wood Dale, IL, USA). Amount of time spent in bobcat and rabbit arm was calculated. Preference to bobcat was quanti-

**Table 1** Primers used for determining mRNA abundance

| | Forward primer | Reverse primer |
|---|---|---|
| LHR | CATTCAATGGGACGACTCTA | GCCTGCAATTTGGTGGA |
| StAR | GGGCATACTCAACAACCAG | ACCTCCAGTCGGAACACC |
| $P450_{scc}$ | CTTTGGTGCAGGTGGCTAG | CGGAAGTGCGTGGTGTTT |
| 3β HSD | TGTGCCAGCCTTCATCTAC | CTTCTCGGCCATCCTTTT |
| $P450_{c17a}$ | CTCTGGGCACTGCATCAC | CAAGTAACTCTGCGTGGGT |
| 17β HSD | GACCGCCGATGAGTTTGT | TTTGGGTGGTGCTGCTGT |
| Aromatase | GCCTGTCGTGGACTTGGT | GGTAAATTCATTGGGCTTGG |
| GAPDH | GGGCAAGGTCATCCCTGAGCTGAA | GAGGTCCACCACCCTGTTGCTGTA |

All primers are in 5′–3′ direction.

fied as % time spent in bobcat arm divided by sum of time spent in both bobcat and rabbit arm.

### *Statistics*

Randomization tests were used for statistical testing of relative expression, free of distributional assumptions for the data ($n$ = 7 control and 8 infected animals). The correlation between mRNA abundance of steroidogenic enzymes and LHR was estimated using Spearman's rank correlation coefficient. Two-way analysis of variance (ANOVA), followed by a LSD post hoc test, was used to analyse the effect of infection and cat odour on testicular testosterone ($n$ = 11 and 10 animals for basal control and infected, respectively; $n$ = 12 each for control and infected post-cat odour). ANOVA was also used to analyse effects of castration on innate fear ($n$ = 6 per group, except five animals for control animals without castration). Normality of experimental data and normality of residuals of the ANOVA were confirmed using Kolmogorov–Smirnov goodness-of-fit ($P > 0.7$ and $P > 0.6$, respectively).

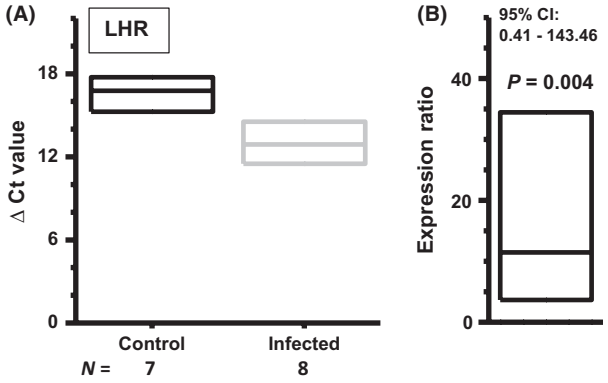


**Fig. 2** *Toxoplasma gondii* infection increased mRNA abundance of Luteinizing hormone receptor (LHR). mRNA isolated from testes of infected animals required lower number of PCR amplification cycles for LHR to reach a predetermined threshold (panel A). Ordinate depicts PCR cycles needed when using LHR primer minus when using GAPDH primer (a housekeeping gene). Box plot depicts median, 25th percentile and 75th percentile. Testicular mRNA from infected animals contained greater abundance of LHR, compared to controls (panel B). Ordinate depicts spread of expression ratio (fold change, infected/control) obtained during 10 000 randomization trials.

## Results

### *Infected animals exhibited greater mRNA abundance of LHR*

We determined expression of LHR by comparing mRNA abundance between control and infected animals. During quantitative PCR, samples derived from infected animals required fewer cycles to reach a predetermined threshold (Fig. 2A and Table 2). Indeed, the 75th percentile of $\Delta C_t$ values for infected animals was well below the 25th percentile of control animals (Fig. 2A, ΔΔ median $C_t$ = 3.9). Infected animals exhibited greater mRNA abundance of the LHR gene by 10.5-fold (Fig. 2B, $P$ = 0.004; expression ratio, REST©, please see methods for calculation of fold change).

### *Infected animals exhibited greater mRNA abundance of steroidogenic enzymes*

In agreement with the notion that LHR regulates levels of StAR and $P450_{scc}$, we observed statistically significant correlation between $\Delta C_t$ values of LHR and that of StAR and $P450_{scc}$ (Table 3). We next studied the effect of infection on these enzymes.

Samples derived from infected testes required fewer cycles to reach the threshold for StAR (Fig. 3A and Table 2, ΔΔ median $C_t$ = 1.3). Infected animals exhibited greater mRNA abundance of StAR gene by 2.65-fold (Fig. 3B and Table 2, $P < 0.001$). Similar to StAR, the $P450_{scc}$ also required fewer cycles to reach the threshold (Fig. 3C, ΔΔ median $C_t$ = 0.7) and exhibited greater mRNA abundance (Fig. 3D and Table 2, 1.65-fold up-regulation, $P$ = 0.006).

We observed significant correlations between $\Delta C_t$ values of LHR and that of 17β-HSD or $P450_{C17a}$, but not of 3β-HSD (Table 3). We next studied the effect of infection on the mRNA abundance of these enzymes. The expression of 3β-HSD was not different between control and infected testes ($P$ = 0.559, Table 2). Testes from infected rats required fewer cycles to reach the threshold for $P450_{C17a}$ (Fig. 4A and Table 2, ΔΔ median $C_t$ = 2.6) or 17β-HSD primers (Fig. 4C and Table 2, ΔΔ

**Table 2** Mean $C_t$ values for GAPDH, Luteinizing hormone receptor (LHR) and enzymes involved in testosterone synthesis

| | GAPDH | LHR | StAR | $P450_{scc}$ | 3β HSD | $P450_{C17a}$ | 17β HSD | Aromatase |
|---|---|---|---|---|---|---|---|---|
| Control (mean ± SEM) | 22.1 ± 0.69 | 38.8 ± 0.64 | 33.7 ± 0.60 | 27.9 ± 0.58 | 29.7 ± 0.60 | 30.3 ± 0.58 | 34.1 ± 1.1 | 27.9 ± 0.66 |
| Infected (mean ± SEM) | 20.8 ± 0.63 | 34.0 ± 0.31 | 31.0 ± 0.25 | 25.9 ± 0.41 | 28.2 ± 0.77 | 25.5 ± 0.53 | 28.4 ± 0.58 | 26.4 ± 0.79 |
| $P$ | 0.232 | 0.001 | 0.001 | 0.029 | 0.152 | <0.001 | 0.003 | 0.152 |

$P$-values are derived from exact Mann–Whitney test.

**Table 3** Correlation between mRNA abundance of Luteinizing hormone receptor and enzymes involved in testosterone synthesis

| | StAR | $P450_{scc}$ | 3β HSD | $P450_{C17a}$ | 17β HSD |
|---|---|---|---|---|---|
| Pearson Correlation | 0.879 | 0.800 | 0.382 | 0.707 | 0.754 |
| *P* | 0.00002 | 0.0003 | 0.160 | 0.003 | 0.001 |

$n$ = 15 animals, including seven control and eight infected animals.

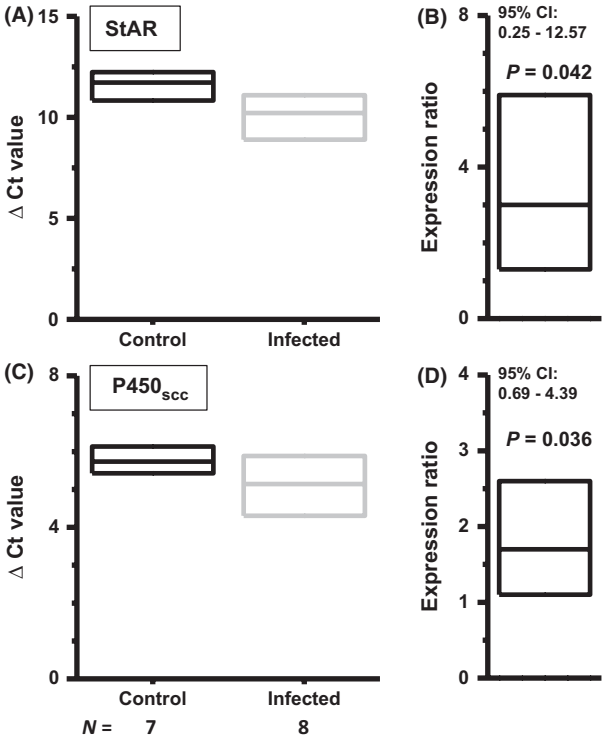


**Fig. 3** *Toxoplasma gondii* infection increased mRNA abundance of enzymes involved in synthesis of precursor pregnenolone. mRNA isolated from testes of infected animals required lower number of PCR amplification cycles for StAR and $P450_{scc}$ to reach a predetermined threshold (panel A and C). These enzymes are involved in transport of cholesterol to the mitochondria and its conversion to pregnenolone, a precursor of testosterone. Testicular mRNA from infected animals contained greater abundance of products of these genes, compared to controls (panel B and panel D).

median $C_t$ = 5.5), demonstrating an 8.6- and 15.9-fold greater mRNA abundance (Fig. 4B, $P$ = 0.042 and Fig. 4D, $P$ = 0.036), respectively. Similar to the LHR, the 75th percentiles of $\Delta C_t$ values for $P450_{C17a}$ and 17β-HSD in infected animals were placed well below the 25th percentile of control animals (Fig. 4A,C). The expression

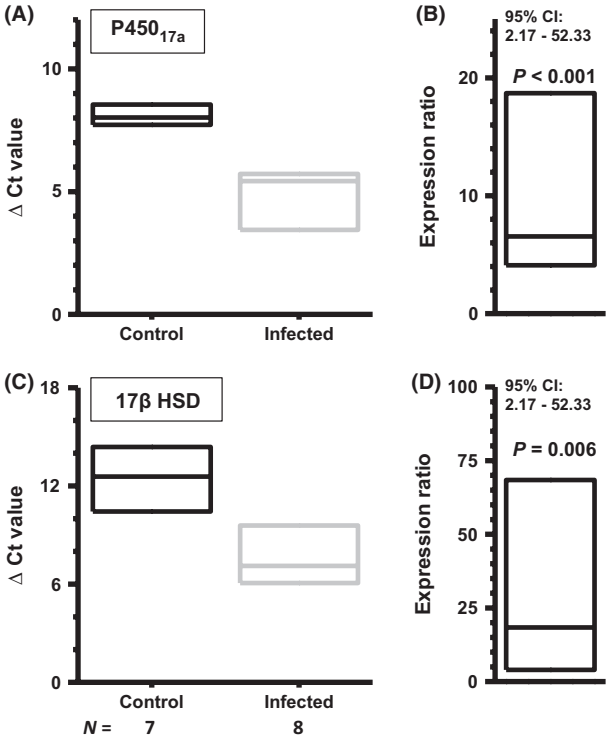


**Fig. 4** *Toxoplasma gondii* infection increased mRNA abundance of steroidogenic enzymes of smooth endoplasmic reticulum. $P450_{C17a}$ (panel A and B) and 17β-HSD (panel C and D) were significantly up-regulated.

of aromatase was not different between control and infected testes ($P$ = 0.724, Table 2).

### *Infection enhanced testicular testosterone concentration*

To validate the physiological effect of greater mRNA abundance of LHR and steroidogenic enzymes, we quantified the amount of testicular testosterone in control and infected animals, both basally and after a stimulus that increases testosterone concentrations (25 min post-exposure 2 mL bobcat urine). Our use of bobcat odour to elicit testosterone secretion was based on pilot data showing a clear increase in plasma testosterone 25 min after exposure to bobcat odour (six experimentally naïve control Long-Evans male rats; $P < 0.05$, paired sample $t$-test). It should be noted that prior studies show a decrease in testosterone in response to predator odour (Kavaliers *et al.* 2001), instead of increased testosterone we consistently observe in rats after bobcat urine exposure.

A two-way analysis of variance (ANOVA) demonstrated a significant interaction between infection and exposure to bobcat odour (Fig. 5A; $F_{1,41}$ = 3.9, $P$ = 0.05). Main effects of infection or induction did not reach statistical significance ($F_{1,41} < 2$, $P > 0.15$).

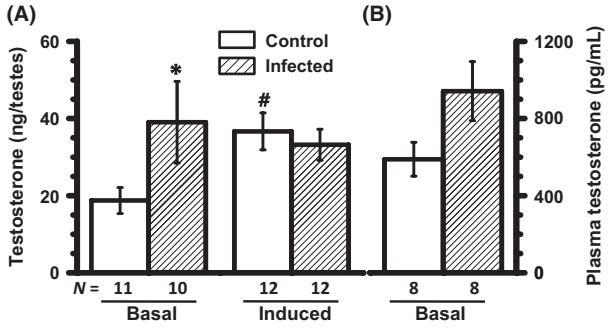


**Fig. 5** *Toxoplasma gondii* infection enhanced testicular testosterone (panel A) under basal condition. Bars depict mean ± SEM. *$P < 0.05$ for comparison between control basal and infected basal; #$P < 0.05$ for comparison between control basal and control induced. Increase in the testosterone concentration in circulating plasma did not reach statistical significance (panel B, $P < 0.057$).

Basally, testes of infected males contained more than twice the testosterone found in controls (Fig. 5A; $P < 0.05$, LSD post hoc test), a substantial increase in the magnitude seen in control rats only after exposure to cat odour ($P < 0.05$, for basal vs. induced testosterone in control animals; $P > 0.5$, for basal vs. induced testosterone in infected animals). Interestingly, all but one of the infected animals had basal testosterone values greater than median of control group. We also quantified amount of the testosterone circulating in blood plasma. The infection produced an increase in the testosterone levels that failed to reach statistical significance (Fig. 5B; 60% increase; $P = 0.057$, student's *t*-test).

We did not measure testes weight in this study. In an earlier pilot experiment, we did not observe significant difference in testes weights between control and infected animals (Long-Evans rats, $n = 11$ control and 18 infected animals; $P > 0.9$).

### *Removal of testes attenuated behavioural effect of infection*

To test the necessity of testosterone in bringing about effects of infection, we quantified innate fear to bobcat urine in control and infected animals, both in castrated and sham-operated males. Castration drastically reduced the amount of circulating testosterone (2.2 ± 0.8 pg/ml plasma, 6 weeks postcastration). A two-way ANOVA demonstrated a significant interaction between infection and surgical removal of testes ($F_{1,19} = 5.42$, $P = 0.03$). Main effects of infection or castration did not reach statistical significance ($F_{1,19} < 3$, $P > 0.1$).

In agreement with prior reports, *Toxoplasma gondii* infection attenuated innate fear of infected animals towards bobcat urine (Fig. 6; $P = 0.02$, LSD post hoc test; 62% increase in occupancy near bobcat urine). In contrast, infection did not result in loss of fear when castrated animals were employed (Fig. 6; $P = 0.49$).

## Discussion

In this study, we demonstrate that *Toxoplasma gondii* infection in male rats enhances synthesis of testicular testosterone.

Testosterone can act directly as ligand of androgen receptors found in a variety of target tissues. The testosterone is also metabolized to alternative ligands like dihydrotestosterone or oestrogen in the target tissue, acting through androgen receptors or oestrogen receptors, respectively. Data presented in this report do not inform whether control and infected animals differ in terms of testosterone conversion to alternative ligands. Our results do not confirm a statistically significant increase in blood testosterone. The testosterone is required to enter bloodstream in order for it to exert any effect in the target tissue. In support of this, we have recently reported that the infection alters an androgen-responsive brain phenotype (House *et al.* 2011). Physiological mechanisms responsible for augmented steroidogenic machinery in infected animals are currently unclear. Potential speculations include greater secretion of luteinizing hormone from the anterior pituitary and/or paracrine influence from macrophages and Sertoli cells.

Effects of *Toxoplasma gondii* on testosterone have been previously studied in mice and humans. In mice, infection reduces blood testosterone levels, an effect that is contrary to the present observations (Kankova *et al.* 2011). Course of *Toxoplasma gondii* infection in mice is

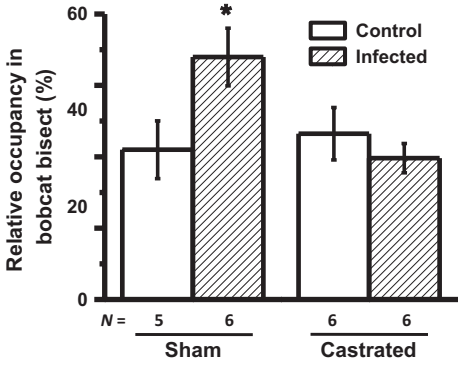


**Fig. 6** Behavioural effects of infection on innate fear were dependant on intact testes. Infection attenuated innate fear in intact, but not in castrated, males. The ordinate depicts relative occupancy in the bobcat bisect relative to total occupancy in the bobcat plus rabbit bisect. *$P < 0.05$ for comparison between control intact and infected intact.

significantly more virulent often leading to sickness and mortality (Hrda *et al.* 2000; Hodkova *et al.* 2007). It is plausible that reduced testosterone levels in mice reflect suppression of gonadal function due to stress and sickness during acute course of infection (Sapolsky 1985). Rats provide an ideal model to study endocrine effects of *Toxoplasma gondii* infection because chronic course of infection in rats seldom results in sickness and mortality. Infected human males exhibit a statistically nonsignificant increase in salivary testosterone levels (Flegr *et al.* 2008). In the present report, we extend these observations by showing greater testosterone synthesis in testes of infected male rats, an organ responsible for most of testosterone. Moreover, we demonstrate a coordinated physiological change in metabolic nodes that regulate testosterone biosynthesis. This is important because a coordinated change requires an organizing principal that is less likely to arise because of accidental by-product of the infection (Poulin 1995).

Immune challenge generally reduces testosterone levels. It is likely that when faced with such challenge, animals partition their resources towards an immune-enabled regime and reduce levels of immune-suppressive testosterone. Several exceptions to this general pattern have been reported, including the present report. For example, male rats infected with Seoul virus contain greater testosterone. This virus is transmitted by wounding during aggressive encounters between males (Easterbrook *et al.* 2007), thus testosterone-mediated increase in aggression is thought to increase the parasite transmission. Similarly, mice infested with tick-borne encephalitis virus synthesize greater testosterone and exhibit greater sexual attractiveness (Moshkin *et al.* 2002). Greater attractiveness of infected individuals in this case is also postulated to increase pathogen transmission. Interestingly, testosterone and attractiveness did not change in nonsusceptible individuals who were inoculated with the pathogen but were able to ward off systemic invasion (Moshkin *et al.* 2002). In both these studies, it is unclear whether pre-existing differences in the testosterone predisposed high-testosterone males to infections through immunosuppressive effects of the steroid.

*Toxoplasma gondii* transmits through both trophic and sexual routes in rats (Dass *et al.* 2011). Two distinct behavioural changes postulated to increase the parasite transmission have been reported in infected host, that is, decrease in aversion to cat odours and increase in sexual attractiveness to females (Berdoy *et al.* 2000; Dass *et al.* 2011). We propose that these two behavioural changes are not distinct but are rather part of a single pattern built around testosterone-mediated interplay between mate choice and predation. Enhanced testosterone levels might explain reduction in fear and increase in attractiveness of infected male rats. Testosterone is believed to reduce fear in a variety of species and experimental paradigms. Administration of exogenous testosterone reduces fear in humans and rodents (Kavaliers *et al.* 2001; Aikey *et al.* 2002; Hermans *et al.* 2006), while castration potentiates fear to predator odours in rodents (King *et al.* 2005). Testosterone also imparts attractiveness to males in birds, goats, rats and humans (Taylor *et al.* 1982; Bottoni *et al.* 1993; Roney *et al.* 2006; Ardia *et al.* 2010; Bonisoli-Alquati *et al.* 2011; Longpre & Katz 2011). It should be noted here that female rats do not produce much testosterone. A testosterone-based explanation does not address if infected female rats also show behavioural change and if disparate mechanisms are involved in two genders. Moreover, it is unclear whether exogenously reinstating testosterone levels in castrated animals to those observed in intact control and infected male will be sufficient to reinstate infection-induced behavioural change.

Testosterone could indicate male genetic quality such as better defence against parasites/diseases, ability to produce heavier muscle mass to fight competitors or ability to combat oxidative stress. These 'good gene' models assume that male phenotypic display is 'honest' proxy of genetic legacy. This honesty is thought to arrive because resources used for sexual signalling produce a handicap in survival or viability of the individual (Folstad & Karter 1992; Wingfield *et al.* 1997). Testosterone is well suited to represent this trade-off because it is required for sexual signalling but imparts costs in terms of metabolism, immune suppression and conflicts with other life history choices (Folstad & Karter 1992; Wingfield *et al.* 1997). We speculate here that in some cases, parasites may manipulate this supposedly honest signalling by manipulating mate choice through enhanced testosterone.

Enhanced testosterone synthesis in infected males results from a coordinated modulation of LHR and steroidogenic enzymes, affecting the same metabolic nodes that are known to normally regulate testosterone synthesis (Parker & Schimmer 1995; Payne & Hales 2004; Payne 2007; Chen *et al.* 2009). We suggest that it is consistent with an adaptive parasitic manipulation. A definitive interpretation of parasitic manipulation in this case will require a demonstration that the behavioural change results in a significant increase in parasite fitness. For example, this demonstration could take a form of greater predation rates of infected animals by cats in field conditions. Similarly, this interpretation will be supported by any demonstration that similar behavioural changes occur in several unrelated lineages through the process of convergent evolution (Poulin 1995).

## Acknowledgements

This research was funded by Nanyang Technological University and Ministry of Education, Singapore. We thank Dr Kevin Lafferty for comments on the manuscript.

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AL and VK performed quantitative PCR, SD performed testosterone and behaviour measurement and AV wrote first draft. VK and SD contributed to revisions. All authors participated in analysis.

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## Data accessibility

Data from quantification of mRNA abundance, testosterone levels and measurement of innate fear are archived on Dryad (doi: 10.5061/dryad.3f2s5).

**Category:** **Original Research**

# Copulation or sensory cues from the female augment Fos expression in arginine vasopressin neurons of the posterodorsal medial amygdala of male rats

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## Abstract

Background:

Posterodorsal part of the medial amygdala is essential for processing reproductively salient sensory information in rodents. This is the initial brain structure where information from olfactory system and male hormones intersect. The neurochemical identity of the neurons participating in the sensory processing in medial amygdala remains presently undetermined. Many neurons in this brain structure express arginine vasopressin in a testosterone-dependent manner, suggesting that this neuropeptide is maintained by the androgenic milieu.

Method:

Here we use Fos, a protein expressed by recently active neurons, to quantify activation of arginine vasopressin neurons after exposure to odor from physically inaccessible female. We compare it to mating with accessible female and to reproductively innocuous odor.

Results:

We show that inaccessible female activate arginine vasopressin neurons in the male posterodorsal medial amygdala. The magnitude of activation is not further enhanced when physical access with resultant mating is granted, even though it remains undetermined if same population of AVP neurons is activated by both inaccessible female and copulation. We also show that arginine vasopressin activation cannot be fully accounted for by mere increase in the number of Fos and AVP neurons.

Conclusion:

These observations posit a role for the medial amygdala arginine vasopressin in reproductive behaviors, suggesting that these neurons serve as integrative node between the hormonal status of the animal and the availability of reproductive opportunities.


Keywords: Affiliation; Mating; Neuropeptide; Nonapeptide; Pheromone; Sexual behavior; Social behavior; Testosterone; Vasotocin.

## Introduction

Medial amygdala (MeA) plays an important role during male reproductive behavior in the rodents [1]. Lesions of the MeA reduce reproductive behavior in hamsters [2], rats [3] and gerbils [4]. In hamsters, anterior MeA is involved in discrimination of conspecific odor from same-sex versus opposite-sex donors, while posterodorsal MeA is selectively activated by opposite-sex conspecifics [5]. Fiber-sparing lesions of anterior MeA in this species reduce number of Fos immunoreactive cells in the posterodorsal MeA and efferent forebrain regions [6], suggesting a unidirectional flow of chemosensory information through anterior MeA to its downstream targets. In male rats, MeA lesions drastically reduce penile erections in response to an inaccessible estrous female[7]. Interestingly, such lesion does not affect reflexive erections in response to penile sheath retraction. These observations suggest that MeA involvement in male reproductive behavior is restricted to motivation and not to the downstream initiation of mating in this species.

MeA is a sexually dimorphic structure (reviewed in[8]), characterized by more neurons and larger neuronal soma in males compared to females [9, 10]. Important from the perspective of this report, male MeA also contains sunstantial number of extra-hypothalamic parvocellular population of arginine vasopressin (AVP) neurons [11]. Testosterone is required for sexual dimorphism of MeA neurons [8] and also for AVP expression in the MeA [12, 13]. The essential nature of testosterone is further supported by the observations that antagonism of androgen receptors in the MeA inhibits penile erection in male rats in presence of estrus females [14]; an effect reversed by testosterone implants within the MeA of castrates [15, 16].

Further evidence suggests that the contribution of the MeA to reproductive behavior is anchored in its ability to integrate pheromonal information with hormonal milieu. Soiled bedding from females increases MeA-Fos in male mice with or without aromatase [17] and in testosterone-primed gonadectomized rats [18, 19]. Since Fos is regarded as proxy for recent neuronal activity [20], this observation suggests that pheromones enhance the activity of MeA neurons. Similarly, female vaginal fluid increases Fos expression in MeA of the mandarin voles [21]. These observations provide correlational support for activation of MeA in pheromonal processing. Experiments in Syrian hamster further strengthen this support. Mating in this species requires intact ability to smell female pheromones and presence of testicular testosterone; absence of either countermands copulation. Interestingly, implantation of testosterone in the MeA of castrates can reinitiate copulatory behavior [22]. Yet, the ability

of the testosterone to rescue effects of the castration is dependent on olfactory inputs, such that surgical removal of olfactory bulbs renders testosterone implants ineffective [22, 23]. Since both olfactory inputs afferent to MeA and testosterone within MeA are required for the rescue, it suggests that MeA integrates information from sensory environment and internal androgenic milieu.

Despite the role of the MeA in processing of reproductively salient sensory cues, the neurochemical identity of the pertinent cell groups is yet undetermined. As mentioned before, many MeA neurons also express AVP in a testosterone dependent manner. This is important because the AVP mediates several of social and sexual behaviors, e.g. monogamy in voles [24] and social recognition of juveniles in male rats [25]. Moreover, AVP neurons are activated during copulation in bed nucleus of stria terminalis [26], a brain region with significant neuro-architectural similarity to the MeA.

In view of androgen-dependent expression of the MeA-AVP and the role of the testosterone in the pheromonal processing, we hypothesized that sensory cues from females selectively activate AVP producing neurons in the MeA. We tested this hypothesis by quantifying co-labeling of AVP and Fos, an immediate early gene product that marks recently activated neurons, post-exposure to rabbit odor or inaccessible estrus female or copulation. Posteroventral and posterodorsal sub-nuclei of MeA were quantified separately in view of their disparate neurochemistry and differential involvement in processing of olfactory signals [27]

## Results and discussion

Figure 1 depicts a representative image acquired after histological staining for arginine vasopressin immunoreactive (AVP-ir) and Fos immunoreactive (Fos-ir) neurons in the MePD. Total number of DAPI cells imaged did not significantly differ between the brain regions or the experimental treatments (ANOVA: $p > 0.29$).

***Reproductive stimuli suppressed number of Fos-ir neurons in the MePV, but increased it in the MePD.***

We utilized repeated measure ANOVA to compare number of Fos-ir neurons in MePV and MePD across experimental treatments. Main effect of the experimental treatments reached statistical significance ($F_{(2,14)} = 4.44$; $p = 0.032$). ANOVA revealed significant differences for the main effect of the sub-nuclei (MePD > MePV, 59.2% change in marginal means; $F_{(1,14)} = 35.46$; $p < 0.001$) and the interaction ($F_{(2,14)} = 29.21$; $p < 0.001$).

Specifically in the MePV, animals exposed to an estrus female or copulation contained a significantly reduced number of Fos-ir neurons compared to a reproductively neutral stimuli (Figure 2A, > 65% reduction; post-hoc LSD test: $p \leq 0.001$). It is not obvious whether this represents a reduction in Fos-ir due to reproductive salient stimuli or if rabbit urine increased Fos-ir above the unquantified quiescent baseline. No statistical difference was evident between the males exposed to estrous females with or without the opportunity of mating (Figure 2A; $p = 0.181$).

In contrast to the MePV, the number of Fos-ir neurons in the MePD exhibited significant increase after copulation, compared to rabbit odor (Figure 2B, 57% increase; $p < 0.05$). Exposure to the estrous female did not result in significant increase ($p = 0.259$). The number of Fos-ir neurons was greater in MePD compared to MePV when animals were treated with reproductive stimuli (post-hoc paired t-test: $p < 0.05$) and not statistically different during control stimulus ($|t_5| = 2.35$, $p = 0.066$).

***MePD contained greater number of AVP-ir neurons.***

ANOVA revealed a significant main effect of the brain regions ($F_{(1,14)} = 177.3$; $p < 0.001$) . Main effect of the experimental treatment did not reach statistical significance ($F_{(2,14)} = 2.13$; $p < 0.156$). Interaction between treatment and sub-nuclei revealed significant differences ($F_{(2,14)} = 4.58$; $p < 0.030$). Marginal mean of AVP-ir number for MePD was substantially greater than that for MePV (181% more, MePD relative to MePV; $p < 0.001$). Similarly for

all experimental treatments, AVP-ir in MePD surpassed observations in MePV (Figure 3; post-hoc paired Student's t-test: $p \leq 0.001$). In view of significant interaction, we further investigated AVP-ir values between experimental groups in MePV and MePD (post-hoc LSD test). Significant inter-group differences were not observed in MePV (Figure 3A; $p = 0.16$). In MePD, no significant differences were observed between control and estrous group (Figure 3B; $p = 0.482$). In contrast, males exposed to estrus females with opportunity to mate exhibited significantly greater number of AVP-ir compared to control stimuli (27% increase; $p < 0.05$).

***Reproductive stimuli increased number of colabeled neurons in MePD, but not in MePV.***

ANOVA revealed significant main effects of experimental treatments ($F_{(2,14)} = 27.28$; $p < 0.001$) and of sub-nuclei ($F_{(1,14)} = 373.72$; $p < 0.0001$). Interaction between treatments and sub-nuclei was also highly significant ($F_{(2,14)} = 73.10$; $p < 0.001$).

Consistent with lesser number of AVP-ir neurons in the MePV, this sub-nuclei also contained lower number of colabeled neurons (AVP-ir and Fos-ir; marginal mean: MePV = 8.33 ± 1.04, MePD = 38.88± 5.56). Across all experimental groups, the MePD contained greater number of colabeled neurons than MePV (Figure 4; post-hoc paired Student's t-test: $p < 0.01$). Within MePV, experimental treatments did not significantly change number of colabeled neurons (Figure 4A; $p > 0.3$).

In the MePD, exposure to estrus females robustly increased number of colabeled neurons compared to the control stimuli (Figure 4B, 233% increase; post-hoc LSD test: $p < 0.00001$). Similarly, mating with females also increased number of colabeled neurons in the male MePD (300% increase; $p < 0.00001$).

***Reproductively salient stimuli specifically activated AVP-ir neurons in the MePD.***

In MePD, >45% of Fos-ir neurons activated by either estrous female or mating expressed AVP (Table 1; ≈ 3-fold increase compared to rabbit odor). Similarly, >58% of all imaged MePD AVP-ir neurons also expressed Fos (Table 1) after exposure to female or copulation. Data described above demonstrate an increase in MePD colabeled neurons. To analyze if the number of colabeled neurons were reflective of more Fos-ir and AVP-ir, we compared observed and expected (based on mathematical product of AVP-ir and Fos-ir frequencies) values using a repeated measure ANOVA.

In case of the MePV, ANOVA revealed that the interaction between treatment and observed/ expected values were not significantly different ($F_{(2,14)} = 1.94$; $p < 0.181$). On the other hand, observed values for the MePD were substantially divergent from the expectations (main effect: $F_{1,14)} = 348.93$, $p < 0.0001$; 119% difference, expected < observed), suggesting a selective Fos activation of AVP-ir neurons. Amongst experimental groups, animals exposed to reproductively salient stimuli exhibited greater departure of the observed values from the expectations (Figure 5A; chance is depicted by diagonal gray line). We further recapitulated this departure by calculating a divergence scale that was indifferent to the distance of expected/observed Cartesian points from the origin. For each point in Figure 5A, we calculated divergence by dividing $(x-y)^2$ with $(x+y)^2$, expressed in percentage (Figure 5B). A one-way ANOVA revealed that exposure to reproductively salient stimuli significantly enhanced divergence between expected and observed values ($F_{(2,14)} = 18.45$, $p < 0.001$; Figure 5B; > 350% increase; $p < 0.001$).

Amongst various nuclei of the extended amygdala, MeA is especially important for the appetitive aspects of the reproduction because it is the initial site during pheromonal processing where main and accessory olfactory information intersects [5, 28, 29].It robustly expresses androgen receptors and is sexually dimorphic in the structure [30-32]. The androgen responsiveness suggests that MEA has access to and can plausibly integrate both the internal hormonal milieu and external pheromonal environment. Lesions of this structure ablate penile erections in male rats in response to an inaccessible female [23], while leaving reflexive erections intact [7]. Importance of MeA efferents for mating can be ascertained by the observations that simultaneous unilateral MeA lesion coupled with contralateral medial preoptic lesion ablates mating behavior [33]. The MeA also shares bidirectional innervation with bed nucleus of stria terminalis (BNST). For example, in male hamsters after exposure to a female odor, greater number of MePD neurons colabel with Fos and a retrograde tracer injected in the BNST [34]. This demonstrates that female odors increases activity in BNST-projecting neurons of the MePD, compared to an odor from male conspecifics. The greater communication between MePD and BNST is consistent with the ontological and hodological similarities between these brain regions [35].

Despite the importance of neurons in the MeA in the processing of reproductively salient cues, neurochemical identity of the activated neurons is still undetermined. Data presented in this report shows that the exposure to a physically inaccessible female rat can selectively

activate AVP neurons in the MePD, in contrast to the MePV or in contrast to reproductively innocuous rabbit urine. Moreover, the extent of AVP neurons being active cannot be explained by arithmetic changes in AVP or Fos neurons, thus suggesting that AVP neurons are activated in a non-random manner. While the present report examined two sub-nuclei of the MeA, it is possible that other MeA sub-nuclei or other parts of the social brain network are also activated during exposure to the reproductively salient stimuli.

We demonstrate a rapid increase in the density of Fos neurons in the MePD after exposure to the inaccessible female or post-copulation. This suggests that Fos in the MePD is dynamically regulated by the presence of sensory cues. Yet, this increase in Fos density is not sufficient to explain the full extent of MePD-AVP activation. This is substantiated by the fact that a greater number of active neurons express AVP and a greater fraction of the AVP neurons become active. Thus, AVP neurons are activated during exposure to sexually salient environmental signals. This is consistent with the prior observations in desert finches expressing vasotocin, a neuropeptide homologous to the AVP. This bird is an opportunistic breeder similar to the rat. In this species co-housing with females enhances activation of the pre-existing vasotocin neurons in the male bed nucleus of stria terminalis of the males, compared to the unisexual group of males [36].

The extent of MePD-AVP activation does not seem to be affected by presence or absence of the physical contact or mating itself. In other words, sensory signals of a physically inaccessible female and the actual mating induce equivalent amount of AVP activation. It should be however noted that experiments described in this report cannot differentiate if same or different population of MePD-AVP neurons are activated by inaccessible female and copulation. Localized manipulations in MeA does increase reproductive motivation of the males even when females are inaccessible or even when soiled bedding or vaginal fluids are used instead of the females [30]. Testosterone implants in the MeA rescue the effects of castration on appetitive aspects of the reproductive behavior. It is not clear if testosterone in these cases directly activates androgen receptor and/or is aromatized to estradiol and subsequently binds to estrogen receptors. Mice lacking aromatase gene exhibit MeA-Fos activation comparable to the wild-type individuals when exposed to female odors [17], suggesting direct role for the androgen receptors. These observations and data in present report suggest, but do not prove, that MePD is involved in processing of information with no additional role in mating. It should be noted that, contrary to the suggestion of non-additional

role of the MeA testosterone in consummatory reproductive behavior, androgen receptor occupancy in MeA does facilitate intromission and ejaculation if simultaneous estrogen receptor binding is available throughout the brain and the periphery [37].

AVP neurons mediate social and sexual behaviors in a variety of species and paradigms. Relevant examples include social recognition in rats [38-40] and development of pair bonding in monogamous voles [24]. Homologous neuropeptides are involved in flocking and territoriality in birds [41]; and in mate recognition and mating in nematode *Caenorhabditis elegans* [42]. AVP neurons in medial bed nucleus of stria terminalis are also activated during copulation in mice [26, 43]. While these strands of evidences suggest a pervasive role for the AVP in a variety of social and sexual behaviors, it is currently unknown if the sexual sensory cues themselves activate AVP neurons. In this backdrop, we show that exposure to inaccessible females is able to activate AVP neurons in MePD, the actual act of copulation not being obligatory. This is consistent with the prior observations that testosterone acting within the MEA promotes sexual arousal to the odor of female rats, without any apparent effect on mating behavior itself.

Reproductive investment and testosterone levels in animals is frequently calibrated to incipient metabolic conditions and mating opportunities [44, 45]. It is plausible that AVP within the MePD serves as integrative node between reproductive status of the animal (signaled by testosterone mediated expression of AVP) and availability of reproductive opportunities (signaled by activation of AVP neurons by sexual pheromones). Related to this, social isolation after weaning reduces the volume of MeA and blunts the sexual behavior in rats [46]. Similarly, ageing reduces MeA-AVP and AVP innervation to brain regions efferent to the MeA [47].

## Conclusion

In conclusion, data presented here show that AVP neurons in MePD are activated during the processing of reproductive cues. It is likely that these neurons play critical role in the mediation of pheromone-directed reproductive behaviors. In addition this report provides support to the role of extra-hypothalamic AVP neurons in reproductive and affiliative behaviors.

## Materials and Methods

### Animals

Wistar rats (47-50 days old) were obtained from vivarium at National University of Singapore. Animals (housed 2/cage) were maintained on 12 hour light-dark cycle with *ad libitum* food and water (lights on at 0700 hours). The Nanyang Technological University institutional animal care and use committee reviewed and approved all procedures. These procedures are compliant with the NIH guidelines. All the male and female subjects used in this paper were sexually naïve at the start of the experiment.

### Exposure to reproductive stimuli

Males were habituated for ten successive days (twenty minutes each day, between 1100 and 1400 hours) in a rectangular arena, in which they would eventually receive the stimuli (46 X 9 cm; 15 cm high). On the day of exposure males were shifted into procedure room just before the beginning of the light phase (7 AM). After a four hour rest period they were exposed to either a physically inaccessible estrus female behind translucent perforated plastic partition (N = 5) or allowed to mate with an accessible receptive female (N = 6). Females were allowed to explore the entire arena for two hours before the start of the trial, during which time they placed urine marks in the arena. To control for novelty of the odor, a third group of males was exposed to rabbit urine on an inaccessible towel (N = 6). All animals were sacrificed two hour after the onset of stimulus exposure.

Naturally cycling females were used as the stimulus. Estrus phase was determined using examination of vaginal lavage, obtained by gentle flushing of cells from vaginal lining using 20 µl buffered saline (between 1030 and 1100 hours). Unstained lavages were examined on a glass slide using 20X magnification. Females in estrus were identified by presence of cornified cells and absence of nucleated cells.

### Histological staining

Animals were deeply anaesthetized and transcardially perfused with 4% paraformaldehyde. Free floating brain sections (40 µm thick) were incubated in a cocktail of primary antibodies for 72 hours at $4^{o}$C (guinea pig anti-AVP, 1:500, Bachem; and, rabbit anti-Fos, 1:100, Santa Cruz Biotenchology). This was followed by incubation with secondary antibodies at room temperature for 2 hours (biotinylated anti guinea pig; 1: 200 + anti rabbit-DyLight 549;1:200;

obtained from Vector Laboratories). The biotinylated antibody signal was developed using Vectastain elite ABC kit (Vector Laboratories) and tyramide signal amplification system (Perkin Elmer). Sections were counter stained with DAPI for 1 minute.

Brain sections between Bregma levels -2.76 mm and -3.24 mm (Interaural 7.28 to 7.08) were selected for analysis. Sections were imaged at 40X magnification and 1.2X digital zoom using a confocal microscope (optically sliced at 4 µm, three set of stacks per animal, Carl Zeiss LSM 710). Neurons positive for DAPI, Fos and AVP were counted. Scores were cumulated per animal.

**Calculation of observed and expected frequencies**

We calculated expected probability of encountering colabeled neurons by multiplying individual probabilities of AVP-ir and Fos-ir neurons. Individual probabilities for AVP-ir were calculated by division of number of AVP-ir neurons with total number of DAPI positive neurons counted (i.e. probability that a particular DAPI positive neuron will be also be AVP-ir). Individual probabilities for Fos-ir were also counted in the similar manner. A product of these probabilities defines the baseline expectation of colabeling by mere chance and assuming biological independence between Fos and AVP activation. The observed numbers of the colabeled cells were compared to the expected baseline, with null hypothesis of colabeling being a mere mathematical coincidence (adapted from [48]).

**Statistics**

Repeated measures analysis of variance (ANOVA) was used to quantify statistical significance for main effects and interactions. In case of within-subject comparisons, paired Student's t-test was employed for post-hoc significance testing. In case of between-subject comparisons, LSD test was used. Values reported are mean ± SEM.

Authors' Contribution

SAHD performed all the experiments. AV and SAHD designed the experiments. AV and SAHD analysed the data. AV wrote the paper. All authors read and approved of the final maucript.

5 Aclnowledments

Funded by Nanyang Technological University and Ministry of Education, Singapore.

Competing Interests

The authors declare that they have no competing interests.

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## Figure legends

**Figure 1. Representative image depicting colabeling of Fos and AVP antigens in MePD.** Fos is stained in red color (DyLight549, emission = 568 nm) and AVP is in green (Fluorescein, emission = 517 nm). DAPI is in blue. Inset on top and right portions depict confocal slice along planes marked by red and green lines, respectively. White arrow highlights a colabelled neuron expressing AVP and FOS.

**Figure 2. Fos expression in MePV and MePD.** Ordinate depicts call counts of Fos-ir neurons () in MePV (A) and MePD (B). N is indicated above abscissa (*italics*). *, $p < 0.05$, post-hoc comparison between experimental treatments within a particular sub-nuclei. #, $p < 0.05$, post-hoc paired comparison between sub-nuclei for a particular experimental treatment. Mean ± SEM.

**Figure 3. AVP expression in MePV and MePD.** Cell counts of AVP-ir neurons () in MePV (A) and MePD (B). N is indicated above abscissa (*italics*). *, $p < 0.05$, post-hoc comparison between experimental treatments within a particular sub-nuclei. #, $p < 0.05$, post-hoc paired comparison between sub-nuclei for a particular experimental treatment.

**Figure 4. Colabeling in MePV and MePD.** Cell counts of colabeled neurons () in MePV (A) and MePD (B). *, $p < 0.05$, post-hoc comparison between experimental treatments within a particular sub-nuclei. #, $p < 0.05$, post-hoc paired comparison between sub-nuclei for a particular experimental treatment.

**Figure 5. Departure of observed colabeling from theoretical prediction.** Expected and observed values of colabeled neurons in the MePD (A). Expected probability (abscissa) was calculated as product of individual probabilities for FOS and AVP neurons. The diagonal gray line from the origin depicts chance level (expected probability equals observed probability). Divergence of observed values from the chance (B). Divergence was calculated for each Cartesian point in panel A by dividing $(x-y)^2$ with $(x+y)^2$. Divergence is expressed as percentage on the ordinate. *, $p < 0.05$, post-hoc comparison between experimental treatments.

**Figure 1**

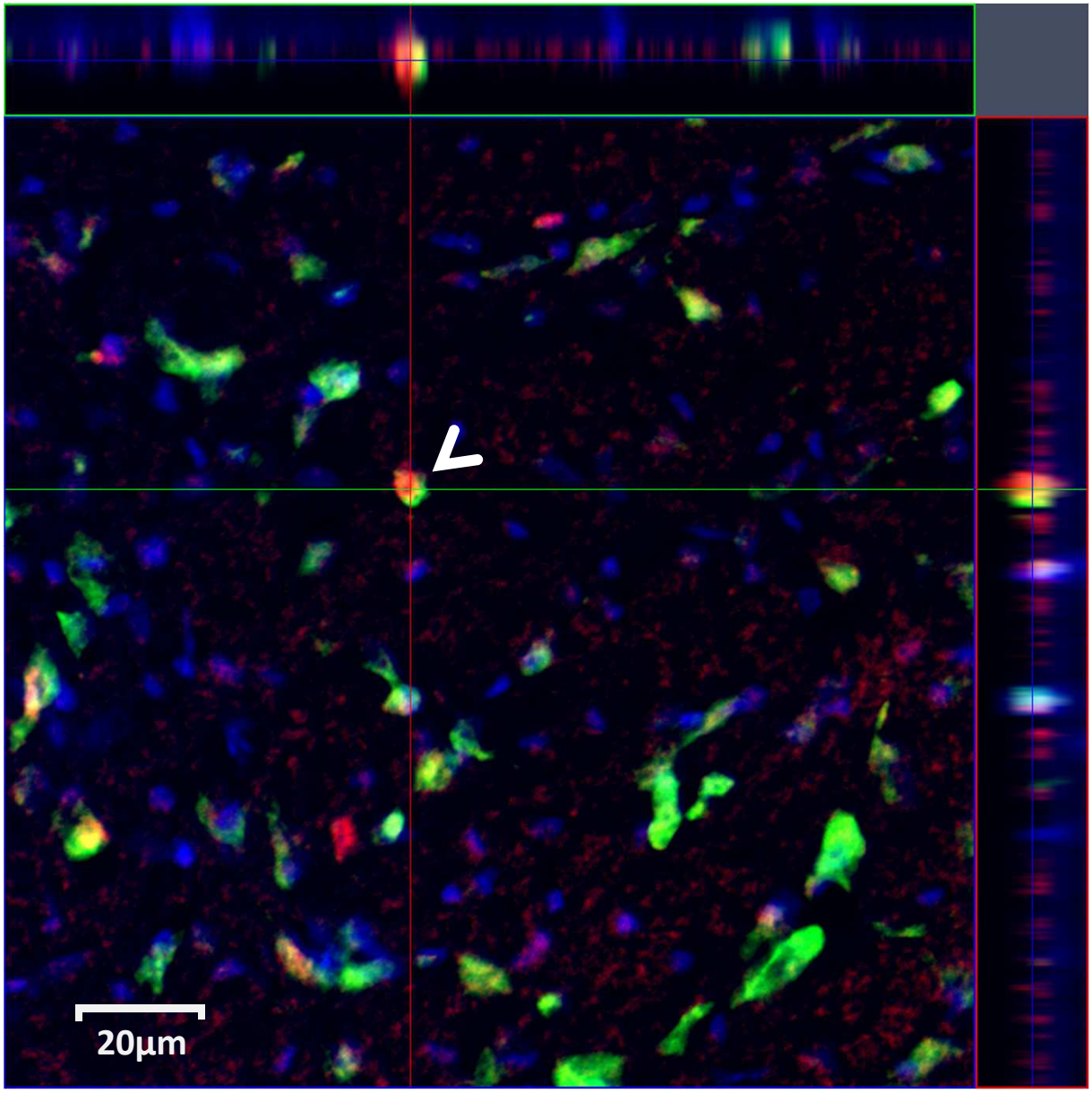
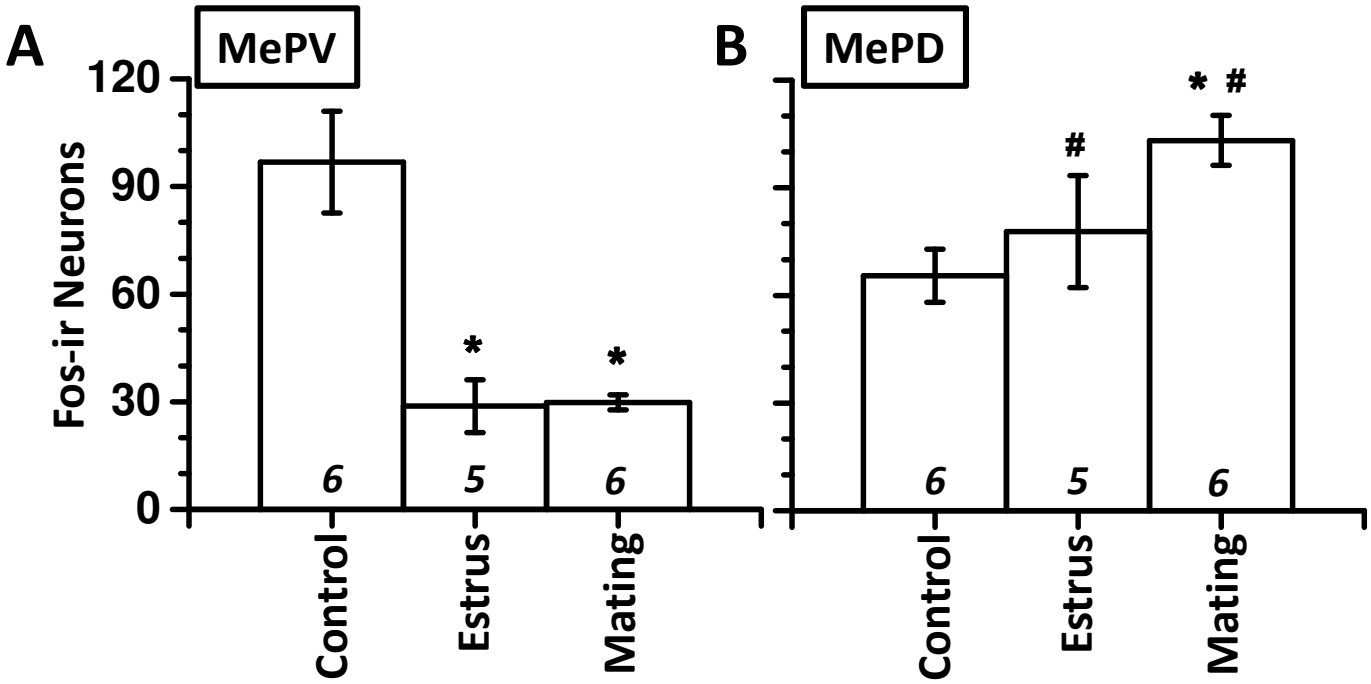


**Figure 2**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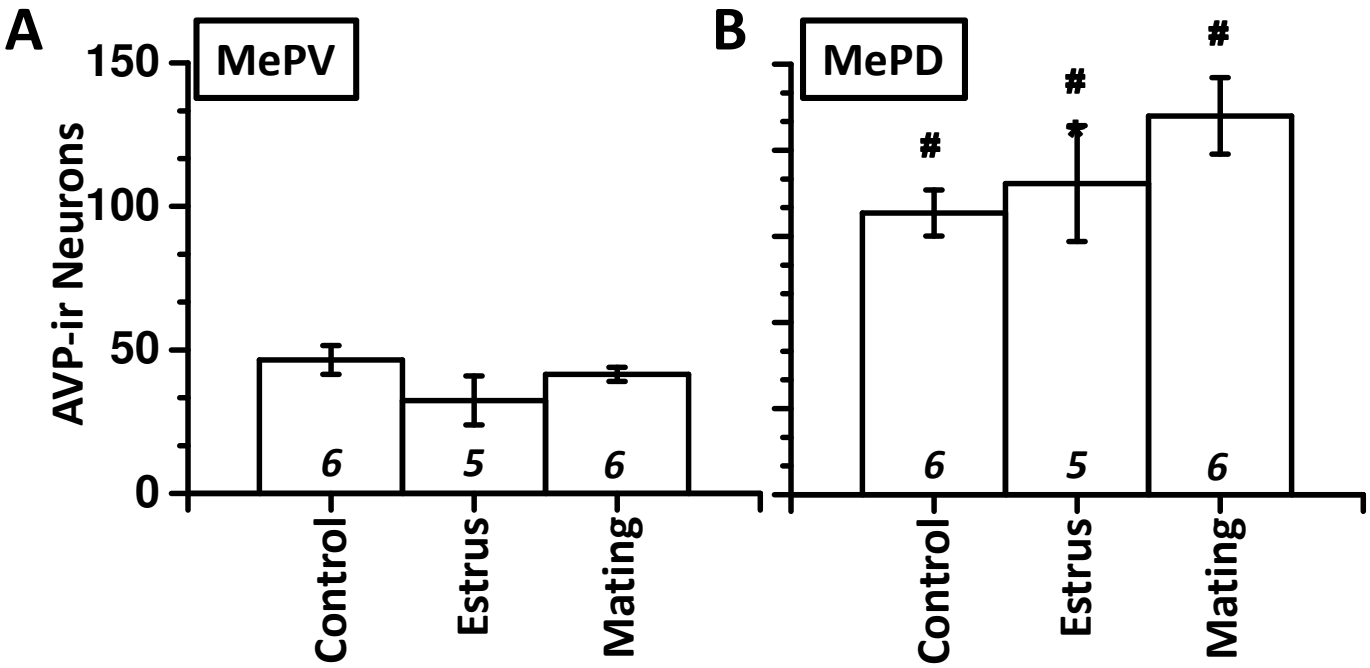
MePV
120
90
60
30
0
Fos-ir Neurons
*
*
6
5
6
Control
Estrus
Mating
B
MePD
# 
* #
6
5
6
Control
Estrus
Mating

**Figure 3**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MePV
AVP-ir Neurons
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100
50
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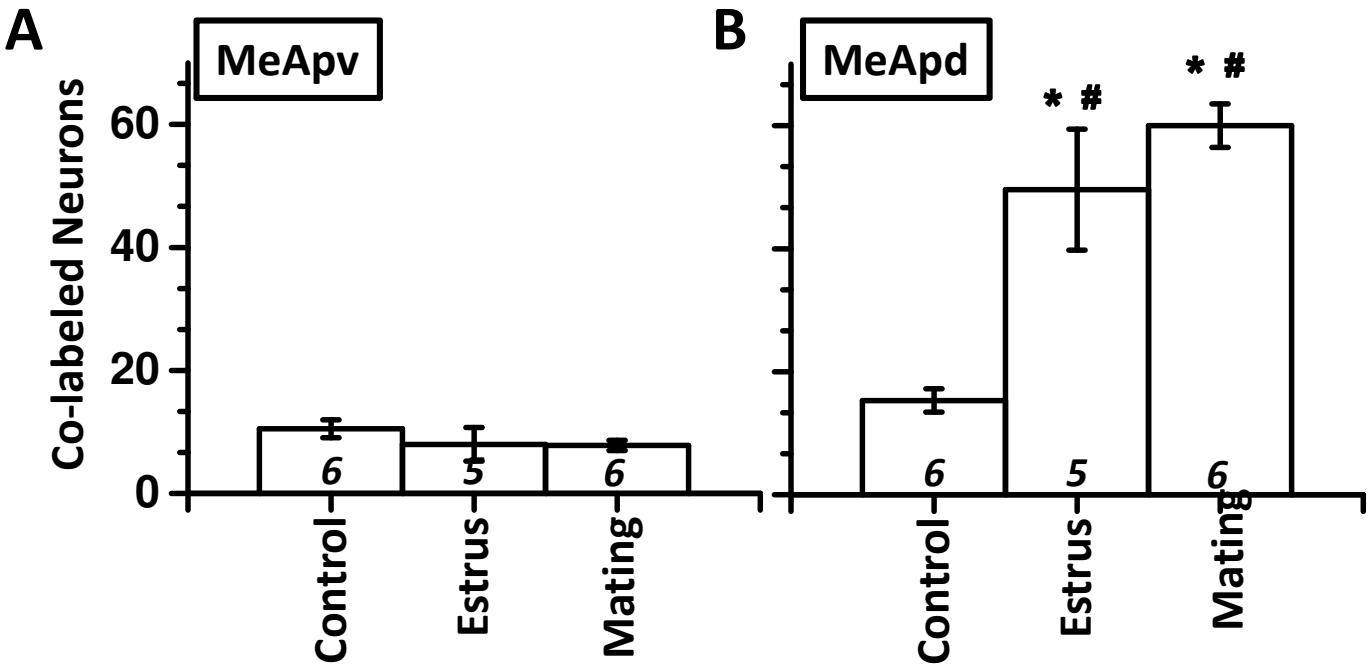
Control
Estrus
Mating
B
MePD
#
#
#
6
5
6
Control
Estrus
Mating

**Figure 4**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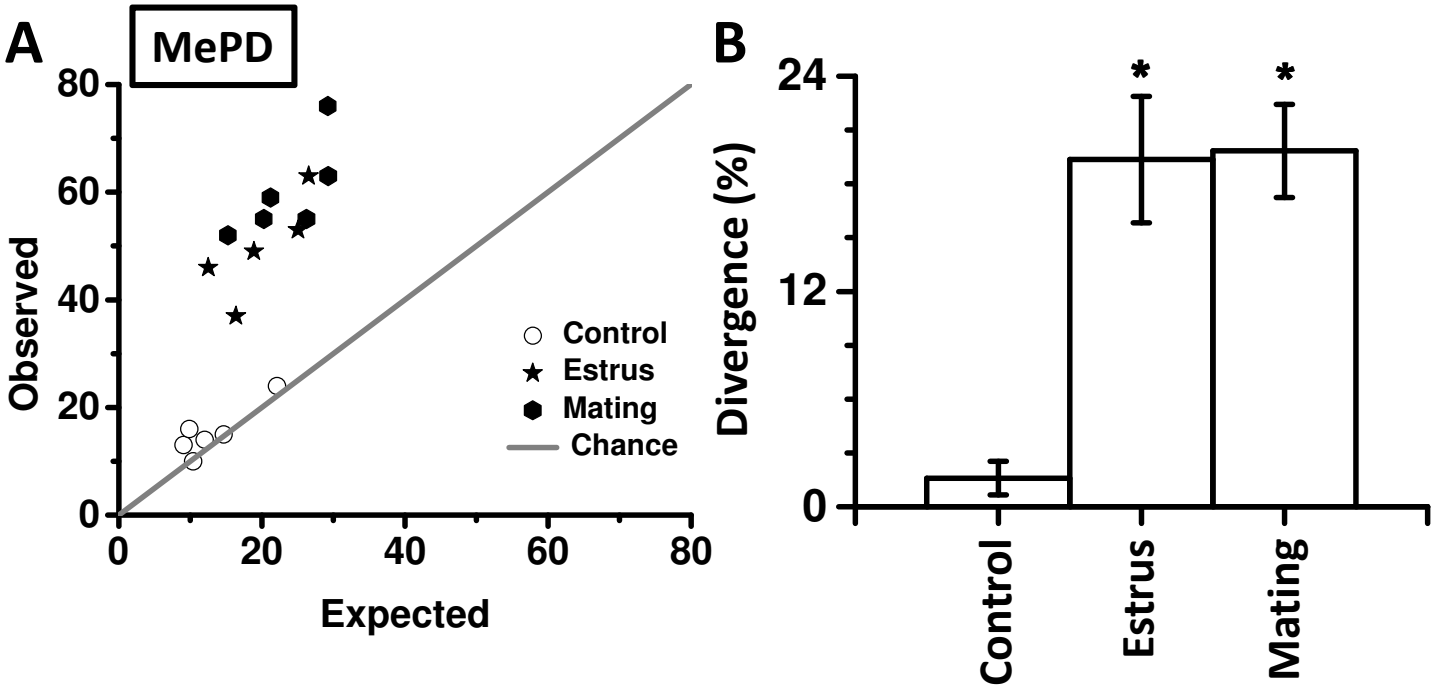
MeApv
Co-labeled Neurons
60
40
20
0
6
5
6
Control
Estrus
Mating
B
MeApd
* #
* #
6
5
6
Control
Estrus
Mating

**Figure 5**


A
MePD
Observed
Expected
0
20
40
60
80
Control
Estrus
Mating
Chance
B
Divergence (%)
24
12
0
*
*
Control
Estrus
Mating

**Additional files provided with this submission:**

Additional file 1: Revised Table 1.docx, 17K
http://www.frontiersinzoology.com/imedia/2058205025128841/supp1.docx