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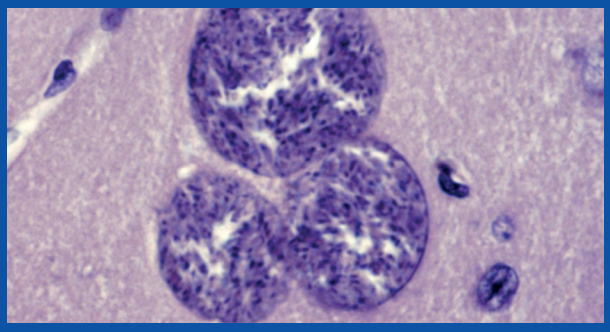


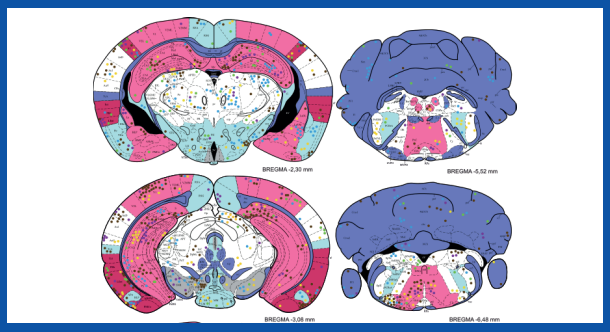

Special Issue

Effects of Latent Toxoplasmosis: Three Decades of Studies

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Special Issue on Toxoplasmosis
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Nuts and bolts of the behavioural manipulation by *Toxoplasma gondii*

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Abstract: In this review, I take the first-person perspective of a neuroscientist interested in *Toxoplasma gondii* (Nicolle et Manceaux, 1908). I reflect on the value of behavioural manipulation as a perturbation tool to understand the organisation of behaviour within the brain. *Toxoplasma gondii* infection reduces the aversion of rats to the olfactory cues of cat presence. This change in behaviour is one of the often-discussed exemplars of host-parasite coevolution, culminating in the manipulation of the host behaviour for the benefit of the parasite. Such coevolution also means that we can use host-parasite systems as tools to derive fundamental insights about the host brain itself.

Keywords: Amygdala, Fear, Life-history, Parasite

Bridge from neuroscience to parasitism

I have not really been trained in the mystical art of formal parasitology, not in my entire academic life. I was a doctoral student in mid-2002, struggling to find words to finish writing my thesis every breakfast and failing to find those by dinner. My chief scientific concern in those days was the neurobiology of behavioural flexibility. It seemed that I could convince rats to become very fearful if I stressed them for ten days. Nothing in the life circumstances of these rats had changed except one hour of stress each day for ten days. They had the same genes, the same tasteless food pallets, and the same sour-looking animal technician. But the intervening stress somehow carved something permanent in their brain, making them fearful for weeks and weeks afterward. I was interested in how that carving in the brain looked, in the literal and physical sense, pondering over the structure of stained neurons under the microscope (Vyas et al. 2002).

It was good fun looking at neurons, measuring them, and arguing about the importance of changes brought about by stress. And yet, while struggling for words for my thesis, I became aware of a conceptual conundrum. I, like other neuroscientists, observed animals making behaviours. This rat likes to spend time in protected dark corners of my maze. That rat is more open to leaning over raised lighted platforms to see what lies underneath. Then, I gave these behaviours names: this rat is more fearful than that one over there. However, I was not at all confident that the names I used for the behaviour were rooted in biological realities rather than the convenience of human language. Was there a cognitively bound state of fear, an entity as real as ears and eyes, descending with modifications through the primal past? As it happens, these issues were already subject to intense discussion by neuroscientists. Just that my uneducated self was unaware of these discussions.

I was weighing these issues one day, eating tasteless toast in the canteen, not making much progress with either the thought or the toast. Then a cell biologist friend joined me and struck up a conversation that seemed peripheral to my interests. The previous week, he read a short article in Science magazine about a parasite that was in the habit of infecting rats but really wanted to be in cats. This parasite allegedly made rats fearless so that they could end up in cats, giving the parasite a free ride to the cat's intestine. That was my first introduction to *Toxoplasma gondii* (Nicolle et Manceaux, 1908), through a commentary written by Carl Zimmer about a now-famous research paper by Joanne Webster and colleagues (Berdoy et al. 2000, Zimmer 2000).

The Zimmer commentary was suffused with a feeling of the strangeness of it all, awe at what natural selection can achieve. Yet, for me, the strangeness appeared somewhere else. If *T. gondii* could remove fear, it logically follows that fear exists as something physical in the brain, something that can be visible to selection rather than a linguistic device. If I kick a ladder and a person comes crashing down, then it follows that there was indeed a person on top of the ladder to start with!

Thus, I ignored my thesis writing for the next week and drank multiple cups of coffee in the company of those two papers. I asked myself if it was possible to use *T. gondii* infection as a perturbation system to study the organisation of fear in the brain in the same manner a pharmacologist might use brain lesions, or a geneticist might use mutations. I really had no interest in the parasite yet. I merely saw the parasite as a useful tool to study the brain.

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A tractable laboratory model

While I slowly became obsessed with *T. gondii*, there was still the practical matter of securing a postdoctoral position. My graduate fellowship was imminently ending and my parents were starting to wonder if their kid was employable at all. So, I started pitching the value of the *T. gondii* system to potential neurobiologists in my familiar circles. I cornered several leading fear neuroscientists in conferences to regale them with this solid scientific opportunity full of promise and aesthetics. To the surprise of my inexperienced self, almost all of them smiled with encouragement and then went right back to the snacks bar without coming back.

My spouse had a postdoctoral offer at Stanford University by that time. In desperation to find a job near her, I wrote to Robert Sapolsky, whom I knew to be a reputed stress neurobiologist. I wrote long paragraphs telling him how impressed I was by his neuroscience, but then I was not actually interested in doing his neuroscience. I told him he should let me work on this parasite, *T. gondii*. No, I did not have parasite experience, but he should pay me for this work and a visa sponsorship. He agreed to do just that! And so it happened in mid-2004 that I found myself in the company of Robert, nervously sitting in the office of John Boothroyd, a well-known *Toxoplasma* biologist at Stanford. I also finally peered down a microscope in the Boothroyd lab to look at tachyzoites of *T. gondii* growing in beautiful rosettes in human foreskin fibroblasts.

Both Robert and I were steeped in the tradition of structure-function relationships, the idea that a change in behaviour is coded by anatomical alterations in specific and well-localised brain regions. A quick succession of studies was already defining brain circuits involved in rodent fear of cat odours; a chain of regions starting from the olfactory bulb, flowing through the medial amygdala and down the medial hypothalamic circuits (Dielenberg et al. 2001, Canteras 2002). These studies were mainly concerned with innate fear, the behaviour that does not require prior exposure to the odour. On the other hand, another set of influential literature defined brain regions centred around basolateral amygdala for conditioned fear and defensive responses that did require prior experience (Blair et al. 2001). And then there was a general push for defining specificities in these circuits. This part of the amygdala is only involved in light-enhanced anxiety and that one over there is important for consolidating prior memories of fear. So, the initial order of the business seemed to be a simple two-step experimental approach.

First, I thought we needed to find which part of the fear response is compromised by the infection. That information should give us a prediction about which brain regions are functionally involved. Second, we needed to see if cysts of *T. gondii* congregated in the same brain regions that we predicted based on the behavioural observations. Before doing any of these, I needed to learn how to handle the parasite and establish a chronic infection that spared normal physiology and behaviour. We managed this after a few trials thanks to Seon-Kyeong Kim, a talented postdoc from the Boothroyd lab who was also very tolerant of rookie neurobiologists pretending to be parasitologists.

We showed that the effects of *T. gondii* behaviour on behaviour were rather specific (Vyas et al. 2007). Rats could learn to attach new emotional meanings to smells and be taught to fear novel sounds. They continued showing healthy anxiety to stuff that should cause anxiety. All the while, they had a deficient aversion to the smell of cat odours. This specificity appeared to be problematic from the perspective of the structure-function relationship in the brain. I could not envision any one brain region that I could remove and hope to recapitulate a similar effect. The change in behaviour was so idiosyncratic that I struggled to place it anywhere in the brain circuits.

When we looked at the localisation of cysts of *T. gondii* in the brain, the same problem prevailed. Cysts were everywhere. They were in olfactory bulbs; we had no odour processing deficit. Cysts were present in the basolateral amygdala which is bits of the brain involved in learned fear, but we did not see any deficit in associative learning of new fears. In other words, we did not have our ducks all in one row, from cyst localisation to brain circuits to eventual behavioural change. We did see some mild tropism in the amygdala, but I was not very hopeful of its importance, given the lack of congruence between the remarkable specificity of the infection effects and the role of the amygdala in a broad range of fear-related behaviour. All of this seemed like a wild goose chase.

Fear and sex

Amidst all the intellectual confusion, I attended the annual conference of the International Behavioural Neuroscience Society. I had a poster there summarising the behavioural and anatomical findings. As a visual aid, I also inserted a schematic of the medial hypothalamic zone, a brain circuit for innate fear. It was in that poster session that Newton Canteras pointed out something that was obvious to his trained eyes and obscure to me. Newton was instrumental in defining neural pathways involved in aversion to predator odours (Canteras 2002). And now he gently pointed out that many parts of the *aversion* circuit were parallel to brain regions that processed *attraction* to sexual pheromones. There was a medial amygdala in my schematic: if you lesion its posteroventral part, you lose aversion, but lesion its posterodorsal part, and there goes attraction to females with it. A similar sort of dichotomy plays out in the ventromedial hypothalamus downstream and accessory olfactory bub upstream. *Aversion* and *attraction* did sit uncomfortably close in these brain circuits. As far as brain circuits were concerned, the opposite of fear was not apathy but attraction.

We approached the localisation problem from a different starting point after I returned from that conference. We started using markers of recent neuronal activity to map which bits of the brain were active in the infected rats smelling cat odour. This technique allowed us to count the number of neurons active in a particular brain region after exposure to cat odour and then compare that number between control and infected groups. We focused on brain

regions where aversion and attraction coexisted. For the moment, we plainly ignored the question of whether the physical location of *T. gondii* had any informational value.

We observed something interesting happening within the medial amygdala when we broke the code for the experiment (House et al. 2011). The medial amygdala receives inputs from the olfactory system for both sexual pheromones and predation-related kairomones. These inputs are anatomically segregated, with posterodorsal and posteroventral parts receiving information about sex and predation, respectively. We observed that the posteroventral medial amygdala of both the control and infected groups remained robustly engaged after exposure to the cat odour. There was no deficiency in the processing of cat odour. Control animals did not light up the posterodorsal part, as would be expected, due to the sexual nature of this region. And yet, lots of activity could be seen in this sexual part of the medial amygdala in the infected animals. Atypical brain regions were added to the processing of cat odour by the infection rather than the removal of typical brain regions from the functional circuit. For a moment, it appeared that infected animals were perceiving cat odours as both *aversive* predator odour and *attractive* sexual cues. The location of the parasite cysts could not explain this strange reorganisation because both the subregions were so nearby and because it was unlikely that the parasite could preferentially find itself in one side of the medial amygdala but not the other.

Around this time, I applied for a faculty position at Nanyang Technological University in Singapore (NTU). Nothing came of it for several months, and then, to my surprise, I was called for an interview and offered a job within a few weeks. It was the only job I had managed to apply for, so I accepted the offer and moved to the warm environs of Singapore. I bought my suitcases of clothes and my confusion about *T. gondii* with me. I patiently arranged both in my new home and office, respectively.

Looking at the body from the other end

From this point onwards, my scientific journey was less dependent on me and more on brilliant PhD students who joined me in forming the Ethoneuro team at NTU. I quickly found that these students had better ideas and were more agile than me. Together, we started to consume copious amounts of caffeine and argue about the best way forward. We made series after series of brain samples from control and infected animals, hoping to compare the recruitment of immune cells, or density of glial cells, or the development of new dendritic branches on neurons and such. We did not finish the analysis of any of these varied experiments because something else was afoot, something much more exciting.

We had been trying hard to perfect our fluorescent staining technique to visualise *T. gondii* cysts in the brain. As part of this, we needed a control tissue, a boring part of the body where we expected to see only the background in the staining preparation. We used testes and bits of epididymis for this purpose. We reasoned that the testis, just like the brain, is an immune-privileged site. Hence, we would be able to continue using it for all sorts of negative staining controls, including in experiments we were planning to visualise immune cells. Hence, we were very surprised when our choice of boring tissue was not so boring after all. Our negative control tissue lit up with fluorescent tags attached to the *Toxoplasma* cyst wall. We saw *T. gondii* in the testes, in the collection of developing sperms ready to be ejaculated in the epididymis, in the vagina of uninfected female rats after mating, and in the brains of developing foetuses resulting from such mating. It appeared that *T. gondii* was a sexually transmitted infection in rats (Dass et al. 2011).

So, for a period, we lost our focus on the brain and quickly educated ourselves about the innards of the male reproductive system. An intern passing through the lab showed that the infection increased the expression of enzymes that convert cholesterol to testosterone in the testes (Lim et al. 2013). A graduate student showed that the infected rats indeed had more testosterone circulating in the blood. Another graduate student, Anand Vasudevan, demonstrated that the rise in testosterone was metabolically meaningful. The liver and urine of infected males had more proteinaceous pheromones used in sexual communication. Urine from infected animals smelled much more attractive to uninfected females (Dass et al. 2011).

This last bit was a mighty surprise to us because it had been drilled into my head during reading behavioural ecology that female mate choice has evolved as a proxy to better immune functioning in males and, hence, lower parasitic burdens. There were hundreds of papers in the field showing that females did not like males infected with all sorts of parasites and pathogens. All of this was new and mighty exciting. The promise of the *Toxoplasma* paradigm was fully visible in these experiments. Using this cool perturbation system, we identified the identity of pheromones used for sexual signaling in rats, a family of α-globulins called major urinary proteins (Vasudevan et al. 2015). Another brilliant PhD student, Donna Tan, used the paradigm to show that all the testosterone in the infected animals made them impulsive decision-makers (Tan et al. 2015). Infected rats were too impatient to wait for delayed gratification and willing to gamble prematurely in tasks that required patience, something analogous to another paper published during that time with stockbrokers. And all these enjoyable detours took us farther and farther from the original question: How does *T. gondii* reduce aversion to cat odours?

Flexibility through epigenetics

A couple of years before I moved to NTU, James Goodson had written an email to Robert. James was interested in the sociality of birds, mainly around what makes birds form social groups. He had discovered that vasotocin, a small neuropeptide, was an important player (Kelly and Goodson 2013). This was especially true in male birds and when a change in behaviour had a sexual connotation. James wondered if we had read these papers about vasotocin because the site of action for this peptide seems to be centred in the medial amygdala and associated regions, parts of the brain

where we had earlier seen atypical neuronal activity in the infected rats. I was intrigued by this, and the conversation remained lodged somewhere in the periphery of my mind while we were being concerned about nether regions of the body far away from the brain.

Then Shantala, a wise graduate student, read a recently published paper that described how testosterone could alter the epigenetic status of the promotor of arginine vasopressin, or AVP, in the brain (Auger et al. 2011). Rodents do not have vasotocin, and AVP is the analogous molecule. Moreover, there is a lot of AVP in the medial amygdala in rats. And where are these AVP-positive cells located in the medial amygdala? In its posterodorsal aspect, the same place that processes sexual pheromones, the same place where we saw atypical recruitment of neuronal activity when infected rats smell cat urine. Scientists have castrated rats and mice, and AVP synthesis in the posterodorsal medial amygdala will go away. They placed a silicone tube full of testosterone in the castrated rats, and AVP-positive neurons came right back. We decided it was all too much for a coincidence.

Shantala soon devised a way to visualise AVP-positive neurons and recent neuronal activity within a single staining paradigm. We used it to observe that *Toxoplasma* infection increased AVP-positive neurons in the medial amygdala, something expected from our earlier observations that the parasite made male rats make more testosterone. Even more exciting was the observation that the infection made these AVP-positive neurons, this guidepost for sexual motivation in the brain, become engaged in the processing of cat odours instead (Hari Dass and Vyas 2014). Just as shown earlier in animals with more testosterone, the infected rats had DNA hypomethylation in AVP promoter, the epigenetic mark of greater transcription. Shantala managed to mimic parasite-induced epigenetic changes and show that she could reduce fear of rats to cat odours, recapitulating the effect of *T. gondii*. After several gain-and-loss-of-function experiments, we managed to convince ourselves that it really did not matter where cysts of *T. gondii* ended up in the brain. Their tropism and location were meaningless. The specificity came not from the localisation but from invading testes, increasing testosterone, and engaging a pre-existing sexual module inside the brain (Abdulai-Saiku et al. 2021). We did not answer our original question of localisation, but we dissolved the question by showing that ocalisation was really not that important for the parasitic manipulation of the behaviour.

Bridge back from parasitism to neuroscience

I became interested in *T. gondii* due to the potential of this paradigm in understanding fear. And the paradigm has indeed brought the neural workings of fear into greater relief. *Toxoplasma gondii* shows us that innate fear is not a monolithic entity etched in non-varying details within the brain. An animal constantly trades off its need for defence from predators with the need to engage in reproduction. These circuits remain closely engaged with each other in the brain because they represent the same organising principle about animal life histories. The brain, like the rest of the animal body, is a reproductive organ subject to vicissitudes of natural selection. Defence from predators is similarly a reproductive behaviour, optimising survival to maximise the probability of eventual reproduction. *Toxoplasma gondii* lays bare the nuts and bolts of how such a tradeoff is negotiated by the interplay between the brain and the hormones.

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