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Correlation between behavioral alterations and dopamine changes in mice experimentally infected with *Toxoplasma gondii*

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ABSTRACT

The mechanism of the parasite to induce alterations in host behavior is suggested due to changes in the dopaminergic system. This study aims to clarify the effects of latent toxoplasmosis on infected mice's behavior and to assess the dopamine neurotransmitter in their brains. Experimental infection with Toxoplasma gondii (T. gondii) and monitoring of behavioral alterations in mice using open field and hole-board tests were carried. Mice were sacrificed, then brains histo-pathologically and neuro-chemically assessed. Open field test revealed a decrease in locomotion in both infected mice genders, whereas the hole-board test displayed an increased level of exploration only by infected female mice. Significant changes in the dopamine concentration in the brain with regard to status of infection were detected. The results suggest an association between T. gondii infection and changes in the behavior. Neuromodulators may represent an ideal mechanism by which T. gondii, at least in part of the expression, can influence the behavior of the infected animal or even human host.

Keywords: Toxoplasmosis; infection; mice; behavioral alterations; dopamine

1. Introduction

Toxoplasma gondii (*T. gondii*) is an intracellular parasite in the phylum Apicomplexan. Its life cycle can be completed only in cats and other members of family Felidae, which are the definitive hosts. However, *T. gondii* also infects a wide variety of intermediate hosts, including humans (Sreekumar et al., 2005). In many mammals, *T. gondii* is known to be an important cause of abortions and stillbirths and to selectively infect muscle and brain tissue (Shaapan, 2016). Humans may become infected by contact with cat feces or by eating the undercooked meat. The importance of these modes of transmission may vary in different populations. Individual response to *Toxoplasma* infection is determined by immune status, the timing of infection, and the genetic composition of the host and the organism (Hide et al., 2009).

Some cases of acute toxoplasmosis in adults are associated with psychiatric symptoms such as delusions and hallucinations. Psychiatric manifestations of *T. gondii* are also prominent in immunocompromised persons with AIDS in whom latent infections have become reactivated (Torrey & Yolken, 2003). Schizophrenia is a neuropsychiatric brain disease of uncertain cause that begins in young adults, typically between the ages of 16 and 30, and is characterized by some combination of auditory hallucinations, delusions, flattened affect, disordered thought patterns, bizarre behavior, and social withdrawal. Schizophrenia affects approximately 1% of the adult population and in most cases is a lifelong disease with remissions and exacerbations. An increased occurrence of schizophrenia in family members of affected persons suggests that genetic factors play a role in its etiology, and some candidate predisposing genes have been identified (Webster et al., 2006).

Extensive research has been carried on infectious agents as one of the possible causes of schizophrenia. Among the infectious agents that appear most promising is *T. gondii*, a protozoan parasite that causes toxoplasmosis and is carried by cats and other felines (Yolken & Torrey, 2008). The interest in dopamine and protozoal tissue infection have been initiated by the 10 years-long study of trypanosomes and sleeping sickness and the discover that this organism increased dopamine level by 34 percent in infected rats (Yolken et al., 2009). Hence, the attention paid to *T. gondii* because of its known ability to alter learning, memory, and behavior in infected mice and rats; the infection of mice with *T. gondii* resulted in normal dopamine levels in acute infection, but the level increased with chronic infection and it was concluded that *T. gondii* causes abnormalities in catecholamine metabolism and that these "may be factors contributing to the psychological and motor changes" seen in experimentally infected rodents (Singh et al., 2010). The potential link between *Toxoplasma* infection and schizophrenia has been detected by several

epidemiological, neuropathological, serological and neurophysiological studies carried out on both schizophrenic and non-schizophrenic populations (Holub et al., 2013).

It has been known that neurotransmitters are involved in the pathogenesis of schizophrenia. An excess of dopamine has been widely suspected, and along with genetics, dopamine-excess has been one of the most thoroughly researched theories. Despite hundreds of research projects, however, relatively few abnormalities in the dopamine system have ever been identified in individuals with schizophrenia (Lewis & Lieberman, 2000). Several important diseases of the nervous system are associated with dysfunctions of the dopamine system, and some of the key medications used to treat them work by altering the effects of dopamine. Dopamine itself is available as a manufactured medication for intravenous injection: although it cannot reach the brain from the bloodstream, its peripheral effects make it useful in the treatment of heart failure or shock, especially in newborn babies (Swanson et al., 2007). Inside the brain, dopamine plays important roles in executive functions, motor control, motivation, arousal, reinforcement, and reward, as well as lower-level functions including lactation, sexual gratification, and nausea. The dopaminergic cell groups and pathways make up the dopamine system which is neuromodulator (Björklund & Dunnett, 2007).

T. gondii has the genes encoding two critical enzymes needed to make dopamine. It has the gene for phenylalanine hydroxylase, which changes phenylalanine to tyrosine, and also the gene for tyrosine hydroxylase, which changes tyrosine to dopa, the precursor of dopamine. These genes were not found in any other closely related parasites except *Neospora caninum*. It was subsequently confirmed that *T. gondii* does actually make dopamine and showed a direct correlation between the number of infected cells and the quantity of dopamine released (Prandovszky et al., 2011). Whereas, the chronic *T. gondii* infection causes high dopamine levels in mice brain, so, experimentally infected mice could be considered as a good representative model for the study of human chronic toxoplasmosis that may lead to schizophrenia (Tedford & McConkey, 2017). The above finding suggests the possibility that the excess dopamine thought to occur in individuals with schizophrenia might be introduced by *T. gondii* rather than made by the affected individuals (McConkey et al., 2013). So, the aim of this study is to investigate of the possible causal relationship between toxoplasmosis and behavioral changes by its monitoring in infected mice and clarify the effects of the activity of the dopamine neurotransmitter in its brain.

2. METHOD

2.1 T. gondii strain

Virulent RH strain of *T. gondii* used for experimental infection was obtained from colony kept in the Department of Zoonotic Diseases, NRC Cairo, Egypt. The strain is maintained at their lab by propagation in mice through intraperitoneal passage every 3-4 days according to the procedures described by Shaapan & Ghazy (2007). All procedures performed in studies involving human and animal participants were by the ethical standards of the Scientific Research Ethical Committee on Ethical Conduct in Human and Animal Research at Suez Canal University, Ismailia, Egypt (No. FWA 00014301).

2.2 Experimental animals

Forty-four, Swiss Webster mice, aged 8 weeks, maintained at five mice per cage with a timed lightning schedule set with 16 hours light and 8 hours dark and fed dry rodent chow and water ad libitum, were used in the study.

2.3. Experimental design and infection

Mice were divided into two groups: Group I (Control group), which included 22 pathogen-free mice (11 males and 11 females) that were not inoculated with any *Toxoplasma* strain and Group II (Chronic infection group), which included 22 mice (11 males and 11 females) that were experimentally infected by the RH *T. gondii* strain, sulfadiazine sodium administrated to group II mice to prevent death from acute toxoplasmosis before chronic stage formation (Shaapan et al., 2015). Serology was performed on mice 8 weeks post-inoculation by Enzyme-linked immunosorbent assay (ELISA) to detect IgG antibodies. Tachyzoites antigens were used in ELISA to detect the *T. gondii* antibodies in the collected mice sera. The optimum antigen, serum, and conjugate concentrations were determined by checkerboard titration (Toaleb et al., 2013).

2.4 Monitoring of behavioral alterations

Behavioral testing started 10 weeks after infection. Males and females from both groups were tested in separate trials. The behavior of the mice was videotaped and all of the videos were analyzed using observer scoring software. Analyses were done blindly.

2.4.1 Open field test of activity (OFT)

It is an experiment used to assay general locomotors activity levels and anxiety in rodents in scientific research and willingness to explore in rodents. The open field test consisted of a 60 x 60 x 40 cm box with a black plastic arena floor, divided into 16 symmetrical squares. The mouse is placed in a corner square, facing the walls, and videotaped for 10 min (Figure 1A). The following parameters were recorded: the total number of squares crossed, the total number of rears, time spent grooming, time spent sitting and the relative number of central square entries (Deacon et al., 2002).

2.4.2 Hole-board test of exploratory behavior (HBT)

This is an experimental method used in scientific research to measure anxiety, stress, neophilia and emotionality in animals. The hole-board consisted of a 60 x 60 x 40 cm box with a black plastic arena false floor that is 2.5 cm high from the true floor. It is divided into 25 squares, each 12.5 x 12.5 cm. There are 16 holes (2 cm diameter) in the floor in a symmetrical pattern in the middle of each square. Each mouse was placed in a corner square, facing the walls, and videotaped for 10 min (Figure 1B). The number and duration of head dip into the holes, time spent sniffing at the holes and the number of squares crossed were recorded. Head dipping was defined as a whole entry up to the ears (Tang & Sanford, 2005).

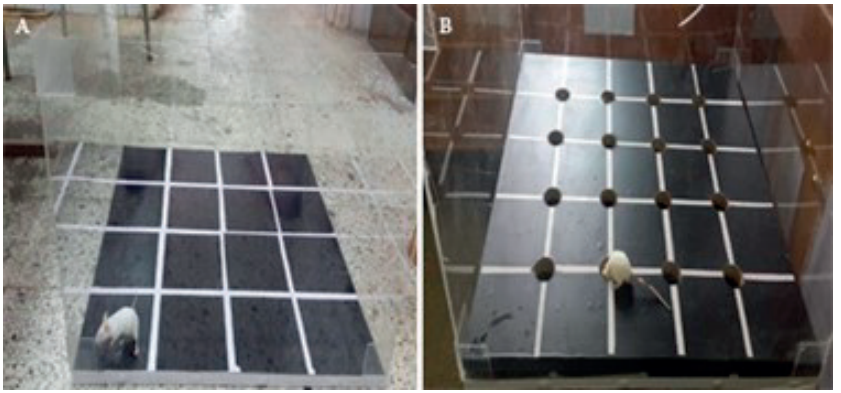

Figure 1. Open field test of activity (A) Hole-board test (B) for exploratory mice behavior

2.5 T. gondii tissue cyst preparation

Mice were anesthetized 12 weeks post-infection and bled out completely by rapid slaughtering before removing their brains. The brains were dissected and removed within 90 seconds of death. A brain imprint smear was taken immediately on a slide and fixed by dipping in methanol twice to be examined later for *T. gondii* cysts. Brains were placed on ice-cooled glass plates and each brain was divided into two halves (El-Nawawi et al., 2008).

2.6 Detection of T. gondii cysts in the infected mice the brain

Pre-alcohol-fixed brain print slides were left in a coupling jar filled with the working Giemsa stain (10 %) for 10 minutes. Slides were washed by dipping twice in buffered distilled water and left in the air to dry, then were examined microscopically with the power X40 and X100 (Garcia et al., 1991). Also, to confirm the presence of *T. gondii* cysts in the brains of mice chosen for neurotransmitters evaluation, histopathological examination of brain samples using hematoxylin and eosin (H&E) staining. The stained tissue sections were examined under the microscope at power magnification of X100 for the identification of *Toxoplasma* cysts (Ross & Pawlina, 2016).

2.7 Neurochemical analysis for the dopamine neurotransmitter

Neurochemical analysis brain sample was carried out using HPLC system, Agilent technologies 1100 series equipped with a quaternary pump (Quat pump, G131A model), with electrochemical detection (Bio-analytical Systems Inc., West Lafayette, IN, USA) together with an ODS-reversed phase column (C18, 25 9 0.46 cm i.d. 5 lm) at the Biochemistry Department, Medical Research Division, NRC, Cairo. The analysis procedures were carried out according to Abdel-Salam et al. (2012), briefly as follows, 5 µl of the sample supernatant was directly injected into the HPLC for analysis. Standard dopamine (from Sigma aldrich®) was used to quantify and identify the peaks on the chromatographs. The retention time for dopamine was approximately 4.231 under the set conditions. The concentration of dopamine was determined by an external standard method using peak areas by running the known concentrations of dopamine standard separately in the HPLC system under the set condition.

2.8 Statistical analysis

SPSS (version 20) statistical program (SPSS Inc., Chicago, IL) was used to carry out a one-way analysis of variance (ANOVA) and Chi-Square (χ^2) on our data.

3. Results

3.1 Clinical features

The clinical signs in mice with established chronic toxoplasmosis i.e., lethargy, ruffled fur, hunched posture, were not observed in infected mice.

3.2 Serology

ELISA test confirmed anti-*Toxoplasma* antibodies in every inoculated mouse. All control mice were *Toxoplasma*-negative. Specific IgG antibody titers for toxoplasmosis were determined by ELISA, optimized for the detection of chronic toxoplasmosis.

3.3 Behavioral assays

3.3.1 Open field test

The results of the open field test displayed the effect of Toxoplasmosis on the ambulatory level of infected mice. The latent toxoplasmosis decreased ambulation (walking or movement) activity of infected mice in the open field test. Separate sex analysis demonstrated that there was a significant interaction between gender and status of infection (Table 1).

3.3.2 Hole-board test

The results of the Hole board test displayed the effect of toxoplasmosis on the exploratory behavior of infected mice. Separate sex analysis demonstrated that there was no significant interaction between gender and status of infection (Table 2).

3.4 Histopathology

The presence of *T. gondii* cysts in the brain was verified by light microscopy of mice brain sections stained with hematoxylin and eosin stain. The *T. gondii* cysts were found to be present in all experimentally chronic infected mice and were not found in all non-infected mice (Figure 2).

Table 1. Open Field Test results

	Measure	Infected (n=20)		Controls (n=22)		P value
		Mean	S.E.M.	Mean	S.E.M.	
Both male and female mice	Squares crossed	179.3	3.9	196.9	3.9	< 0.05 Significant
	Rears	94.8	3.1	105.3	2.4	< 0.05 Significant
	Time spent grooming (sec)	9.3	0.8	8.1	0.6	> 0.05 Non-Significant
	Time spent sitting (sec)	1.5	0.3	1.3	0.3	> 0.05 Non-Significant
	Entries to central square	15.3	0.7	15.0	0.5	> 0.05 Non-Significant
Male mice	Squares crossed	185.3	7.1	191.8	5.9	> 0.05 Non-Significant
	Rears	106.5	5.2	117.9	2.7	< 0.05 Significant
	Time spent grooming (sec)	12.8	1.2	10.1	0.9	> 0.05 Non-Significant
	Time spent sitting (sec)	2.0	0.5	2.5	0.5	> 0.05 Non-Significant
	Entries to central square	20.0	0.8	17.4	0.8	< 0.05 Significant
Female mice	Squares crossed	174.7	4.2	200.9	5.2	> 0.05 Non-Significant
	Rears	85.1	2.6	95.4	2.7	< 0.05 Significant
	Time spent grooming (sec)	6.4	0.8	6.6	0.6	> 0.05 Non-Significant
	Time spent sitting (sec)	1.1	0.4	0.3	0.2	> 0.05 Non-Significant
	Entries to central square	174.7	4.2	200.9	5.2	> 0.05 Non-Significant

Table 2. Hole Board test results

	Measure	Infected (n=20)		Controls (n=22)		P value
		Mean	S.E.M.	Mean	S.E.M.	
Both male and female mice	Head dips	46.3	2.4	42.6	2.4	> 0.05 Non-Significant
	Time spent head dipping (sec)	86.1	5.5	73.2	5.5	> 0.05 Non-Significant
	Time spent sniffing at holes (sec)	92.4	2.5	81.4	2.5	< 0.05 Significant
	Squares crossed	143.2	4.0	145.9	4.0	> 0.05 Non-Significant
Male mice	Head dips	43.2	4.1	45.2	3.4	> 0.05 Non-Significant
	Time spent head dipping (sec)	66.3	8.1	67.8	6.6	> 0.05 Non-Significant
	Time spent sniffing at holes (sec)	78.8	3.2	72.6	3.3	> 0.05 Non-Significant
	Squares crossed	162.6	5.1	163.5	5.6	> 0.05 Non-Significant
Female mice	Head dips	48.8	2.1	40.5	3.4	> 0.05 Non-Significant
	Time spent head dipping (sec)	102.4	6.6	77.5	8.4	< 0.05 Significant
	Time spent sniffing at holes (sec)	103.7	2.7	88.3	3.1	< 0.05 Significant
	Squares crossed	128.5	3.5	131.9	4.2	> 0.05 Non-Significant

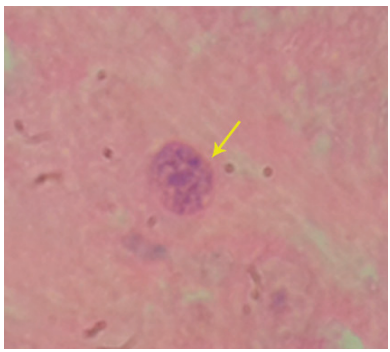

Figure 2. Cut section in the brain of infected chronic mouse (X 400) stained by hematoxylin and eosin stain, showing Toxoplasma gondii cyst (yellow arrow)

3.5 Neurochemical analysis

Chronically infected mice displayed significantly higher dopamine levels in the brain (P <0.05) than non-infected controls. Mean level of dopamine in the brains was 1102 ng/mg for unexposed mice, while it was 1257 ng/mg for chronically infected mice i.e., dopamine was 14% higher in the chronic infected mice group than controls. However, there was not any observed interaction between infection status and mouse sex. Within the male mice group, chronically infected animals displayed significantly higher dopamine concentrations in the brain (P <0.05) than non-infected male controls. The mean level of dopamine in the brain was 1083 ng/mg for non-infected male mice, while it was 1249 ng/mg for chronically infected male mice. Dopamine was 15% higher in the chronic group than controls. Chronically infected female mice displayed significantly higher dopamine concentrations in the brain (P <0.05) than unexposed female controls. The mean level of Dopamine in the brain was 1121 ng/mg for unexposed male mice, while it was 1265 ng/mg for chronically infected male mice. Dopamine was 13% higher in the chronic group than controls (Table 3).

Table 3. Brain dopamine concentration

	Control Group		Chronic infected Group		P value	% of Change
	Mean	S.E.M	Mean	S.E.M		
Both male and female mice	1102	±33	1257	±53	0.01 Significant	+14%
Male mice	1083	±33	1256	±53	<0.01 Significant	+15%
Female mice	1121	±33	1265	±53	<0.01 Significant	+13%

3.6 Correlations between physiological and behavioral data

There is evidence that dopamine levels in chronic infected female mice are linked with behavioral outcomes in the open field test. Dopamine concentrations in females appear to be positively correlated with the number of squares crossed representing ambulatory activity and anxiety. According to the type of obtained data in this study, chi-square test was used to test the difference for significance (chi-sq = 3.65, $p < 0.05$) (Figure 3).

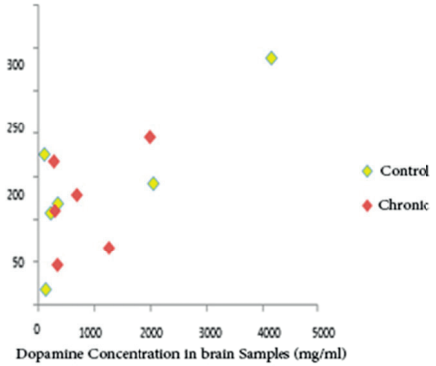

Figure 3. Scatter graph showing proportion of number of squares crossed in the Open field test compared against dopamine concentration in brain of control and infected female mice

4. Discussion

The chronically infected mice toxoplasmosis in the current study revealed some observed clinical signs as lethargy, ruffled fur and hunched posture. The outcome of *T. gondii* infection depends on the virulence of the parasite strain, the size of the challenge dose, the initial port of entry of the parasite, on the host species, and the immunological and genetic status of the host (Skallova et al., 2006). Most of the studies concerning *T. gondii*-induced behavioral alterations in mice were performed with intraperitoneal infected animalls using a relatively high challenge dose (Berenreiterová et al., 2011). However, intraperitoneal inoculation is undoubtedly unnatural and can result in different pathogenicity than per-oral inoculation (Szabo & Finney, 2017). Using subcutaneous inoculation and a low challenge dose, achieve a similar-to-natural and therefore more biologically relevant outcome of *T. gondii* infection, the latent-toxoplasmosis (chronic infection) was obtained and hosts have been found to last throughout their lifetime as positive seroprevalence (Koshy et al., 2020).

In this work, specific IgG antibody titers for toxoplasmosis were determined by ELISA in the chronic infected mice group while control group were *Toxoplasma*-negative. The use of ELISA test as serologically confirmatory

anti-*Toxoplasma* antibodies test for the incidence of chronic toxoplasmosis infection in each experimentally inoculated mouse was advised and recommended several studies (Ghazy et al., 2007; Shaapan et al., 2008) ELISA is a great, sensitive, objective, quantitative, low cost and automatically adopted, test for diagnosis of anti-*Toxoplasma* IgG in infected animals, but it needs refinement in the procedures and standardization of the antigen used (Toaleb et al., 2013).

Concerning behavioral assays in this study, *Toxoplasma*-infected mice (males, as well as females) showed hypo activity (decreased ambulation activity) in a novel environment by both open field and hole-board tests, while in the hole-board test, infected female mice showed more exploration than male and controls. The altered behavior may be a direct effect or an indirect effect of *T. gondii* infection (File, 2001). Infected mice displayed a lower level of ambulatory activity and reared less than control mice in the Open field test. Rearing is considered to reflect exploration, while ambulatory activity mainly reflects general activity, but also a level of anxiety or exploration (Deacon et al., 2002). Therefore, the obtained results suggest that the observed changes in ambulatory activity may be mainly due to alterations in general activity or exploration and only in a minor part of changes in anxiety.

There was a significant sex infection interaction in the case of the relative number of central square entries. Infected males entered the most forbidding central area of the arena more often than controls, while infected females less often than controls. Frequent entries to the central square may reflect exploration, whereas avoiding the center is supposed to be linked to greater anxiety based on the natural aversion of rodents to open spaces (Xiao et al., 2012). However, in this study there was no correlation between movement activity (number of crossed squares) and the relative number of central square entrances by open field test in both infected mice genders, whereas the hole-board test displayed an increased level of exploration only by infected female mice; this suggests that sexual differences observed in this test may be due to the adverse effect of *T. gondii* in female than male mice in this parameter. These results support other studies which indicate that *T. gondii* infection may have opposing or differential effect in males and females, and reiterate that a wide range of behavioral assays must be conducted to further understand this, particularly as rat anxiety behavior tests themselves are strongly influenced by sex (Berdoy et al., 2000).

The *T. gondii* cysts were found in unstained, Giemsa stained brain imprints and H & E stained brain sections of all experimentally infected chronic mice, while there were no *T. gondii* cysts found in all non-infected mice. The microscopical finding of *T. gondii* cysts in the brain tissue of the experimentally infected mice in this study were consistent to the ones

demonstrated by other authors who studied chronic *T. gondii* infection and histopathological finding in animals and man (El-Nawawi et al., 2008; Hassanain et al., 2011).

Although a strong body of evidence suggests a specific *T. gondii* influence on the intermediate host behavior, the underlying mechanism remains unknown (Torrey et al., 2007). One of the main objectives of this study was to evaluate the dopaminergic system activity in brains of *T. gondii* infected mice which revealed that the mean level of dopamine neurotransmitter was 14% higher in the chronic infected mice group than controls. The increased levels of dopamine have been previously reported by many others who infected dopaminergic cells both *in vitro* and *in vivo* (Wang et al., 2015; Hassanain et al., 2011). This finding, therefore, supports the original hypothesis that *T. gondii* infection elevates dopamine levels within the brain (Yolken & Torrey, 2008).

The obtained results from this study revealed statistically significant changes in the dopamine concentration in the brain concerning the status of infection, but it seemed to be there is no apparent difference in neurotransmission activity between males and females, which revealed about 13 and 15% of changes, respectively than non-infected groups. This result is not in agreement with the study that revealed an apparent difference in neurotransmission activity between females and males, also in non-infected control mice (Gatkowska et al., 2013). The positive correlations observed between anxiolytic behavior and dopamine levels suggest that an increase in dopamine levels associated with *T. gondii* infection may also be linked with an increase in anxiety associated behaviors. It would be of interest to analyze the ability of other pathogens associated with schizophrenia, and other neurological disorders, to directly alter dopamine metabolism, thus it is crucial to determine if other pathogens associated with neurological disorders also can directly alter dopamine levels. It is also critical to determine the possible contributions of *T. gondii* infection to other dopamine-related diseases (Tedford & McConkey, 2017). In this study, the positive correlations observed between dopamine levels in infected mice with toxoplasmosis suggest analyzing the ability of other pathogens associated with schizophrenia, and other neurological disorders, to directly alter dopamine metabolism. Despite growing evidence that *T. gondii* infection is linked to a variety of human diseases, there is still a not yet understand full range of neurophysiological changes caused by *T. gondii* infection and it is not fully clear how this host behavior changes.

5. Conclusion

The study concluded that the Toxoplasma-infected mice (males, as well as females) showed hypo activity in a novel environment. Infected female mice showed more exploration than controls in the hole-board test. The nature of the behavioral alterations in Toxoplasma- infected mice, as well as the observed difference in effects of infection on the behavior of infected and control mice, suggests that the proximal causes of alterations in behavior are induced by T. gondii. Significant changes in the dopamine concentration in the brain concerning the status of infection were reported in this study but it seemed that there is no apparent difference in neurotransmission activity between males and females. The mechanism of action by which T. gondii alters rodent behavior is unknown. Histo-pathological, immunological, and/or neuromodulator changes are all potential candidates while neuromodulators may represent an ideal mechanism whereby T. gondii can influence, at least in part, the expression of host behavior.

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