Poorer results of mice with latent toxoplasmosis in learning tests: impaired learning processes or the novelty discrimination mechanism?

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(*Received 13 November 2006; revised 7 February 2007; accepted 14 February 2007; first published online 20 April 2007*)

SUMMARY

The heteroxenous protozoan parasite *Toxoplasma gondii* is transmitted from the intermediate host (any warm-blooded animal) to the definitive host (members of the felidae) by carnivory. The infected intermediate hosts develop several specific behavioural changes that are usually considered products of manipulative activity of the parasite aimed to increase the probability of its transmission to the definitive host. Among other changes, the infected rodents were shown to have impaired learning capability. All previous studies were done 2–6 weeks after the infection. Therefore, it was difficult to resolve whether the observed impairment of learning processes was a result of acute or latent toxoplasmosis, i.e. whether it was a side-effect of the disease or a product of manipulation activity. Here we studied the learning capability of *Toxoplasma*-infected mice in the static rod test and 8-arm radial maze test and their spontaneous activity in the wheel running test 10 weeks after the infection. The infected mice achieved worse scores in the learning tests but showed higher spontaneous activity in the wheel running test. However, a detailed study of the obtained results as well as of the data reported by other authors suggested that the differences between infected and control mice were a result of impaired ability to recognize novel stimuli rather than of impaired learning capacity in animals with latent toxoplasmosis.

Key words: *Toxoplasma*, latent toxoplasmosis, novelty seeking, dopamine, manipulation hypothesis, parasite.

INTRODUCTION

Toxoplasma gondii is an intracelluar protozoan heteroxenic parasite with an indirect life-cycle. The asexual part of the life-cycle can take place in any warm-blooded animal including humans while the sexual part of the life-cycle takes place only in members of the *Felidae* (Hutchison and Work, 1969).

In humans, the acute phase of toxoplasmosis can manifest itself as lymphadenopathy, malaise, headache, myalgia and, in immunocompromised individuals, as meningitis (Holliman, 1997). In mice infected with an avirulent strain of *Toxoplasma*, acute toxoplasmosis is characterized by such severe acute symptoms (pneumonia, myocarditis and meningitis) (Kodym *et al*. 2002). After the acute phase, the disease enters the latent stage characterized by the development of tissue cysts in different host organs, preferentially in the brain and muscles. The tissue cysts, which are resistant to anti-parasitic drugs, remain infective until the end of the host life (Gross, 1996). *Toxoplasma* transmitted to the definitive host by carnivory is considered a classical model for studying the parasite manipulation hypothesis assuming that transmission of parasites by carnivorism can be facilitated by manipulation activity of the parasite which results in the induction of behavioural changes in the intermediate hosts that lead to an increased probability of predation (Holmes and Bethel, 1972).

Many studies have shown that *Toxoplasma* has the ability to change the behaviour of its host. Infected mice are hyperactive in the open field (Hay *et al*. 1983, 1984), exhibit increased voluntary wheel running (Hay *et al*. 1985), longer exploration times in the hole board test (Skallová *et al*. 2005), are deficient in motor performance and coordination (Hutchison *et al*. 1980*b*) and have longer reaction times (Hrdá *et al*. 2000).

There are some indices showing that *Toxoplasma* infection also seems to impair the learning capacity, i.e. the ability to acquire new skills, and memory, i.e. the ability to keep acquired skills, in mice. Laboratory mice (strain A) infected with *Toxoplasma gondii* were less responsive to novel stimuli (a novel object) (Hutchison *et al*. 1980*c*). Infected male mice also preferred a known arm of Y-maze to a new arm, spending relatively less time in the new arm than controls. In contrast, *Toxoplasma*-infected rats

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doi:10.1017/S0031182007002673 Printed in the United Kingdom

showed less neophobia (fear of novelty) (Webster *et al*. 1994). Laboratory hybrid rats infected with *Toxoplasma* also reached higher exploration scores for novel objects (Berdoy *et al*. 1995).

Toxoplasma infection also seems to impair the learning capacity and memory of mice. The mice infected for 4 weeks have significantly worse learning performance than controls (Piekarski *et al*. 1978). Witting (1979) has demonstrated a significant learning deficit in the mouse infected 14 days prior to the experiment. However, this deficit was substantially reduced 6 weeks after the infection. Moreover, the previous studies did not discriminate and discuss whether the impairment of the learning performance was caused by the effect on learning capacity or by the effect of other involved processes, such as memory, motivation, ability to discriminate between familiar and novel stimuli, activity etc.

The aim of the present study was to search for another index of impaired learning abilities in *Toxoplasma*-infected mice using the static rod test and 8-arm radial maze test. All previous studies were performed with mice infected with a rather large inoculum and mostly at a relatively early stage of infection (or with congenitally infected animals). In our experiments, we used an avirulent strain of *Toxoplasma* given in a very low inoculum and ran our tests 10 weeks post-infection (p.i.). This experimental setup reduces the risk that the observed behavioural changes would be side-effects of recent acute toxoplasmosis rather than a result of manipulative activity of the parasite at the latent stage of infection. The wheel running test was included into the battery of tests to study the possible role of changes in spontaneous activity in the observed behavioural changes.

MATERIALS AND METHODS

Animals and infection

Sixty-one female F1 crosses between mouse inbred strains BALB/c (females) and C57 BL (males) (AnLab, Czech Republic) were used. The mice were group-housed in plastic cages, 7–8 animals per cage, with wood shaving bedding and maintained on a 12-h light/dark cycle (dark from 2 p.m.). They were fed on mouse pellets (Velaz, Czech Republic) and water *ad libitum*.

Ten-week-old mice were inoculated orally with 0·5 ml of brain suspension in saline containing 10 tissue cysts of the avirulent HIF strain of *Toxoplasma gondii* isolated from the cerebrospinal fluid of a male HIV-positive patient on 22 February1993 in Prague (Kodym *et al*. 2002). Controls received 0·5 ml of saline perorally.

The animals were checked at least once a day for physical signs of discomfort or distress and their weight was recorded once a week.

At the end of experiments (at 13 weeks p.i.) all mice were examined serologically for anti-toxoplasmic antibodies with the complement-fixation test (SEVAPHARMA, Czech Republic). Brains of 12 randomly selected infected mice were homogenized in 1 ml of sterile saline using a glass homogenizer. The number of cysts was counted in ten 15 μl samples of each suspension under $\times 250$ magnification.

Apparatus and procedures

Behavioural testing started 10 weeks after infection. The experiments (the static rod and 8-arm radial maze tests) were carried out during the dark phase between 3 p.m. and 10 p.m. The behaviour of mice was videotaped and the videotapes were analysed using the Observer software (Noldus). The wheel running activity was recorded for 48 h by impulse sensors connected to a computer.

The day of the oestrous cycle was determined daily for each female by taking vaginal smears and assessing the proportions of cell types present in the smear. Smears were taken 2 h prior to behavioural experiments (Hubscher *et al*. 2005).

The 8-arm radial maze. The 8-arm radial maze was made of transparent plastic. The central area had a perimeter of 64 cm and each arm was 32 cm long, 8 cm wide and 23 cm high. Before test day 1 all animals were given no food for 24 h. The animals were fed again immediately after tests on day 1 for 30 min. Each mouse was placed at the end of the Start arm facing the wall and videotaped until it found 3 millet seeds located at the far end of the end arm (always the third arm to the left of the start arm). Each mouse was tested 4 times on day 1 and 4 times on day 2. The first run of day 1 (habituation run) was the same for all mice; each mouse was left in the maze for 3 min. The time needed for finding the millet seeds (searching time) and number of errors (entries in the wrong arms) were recorded. The mice that did not find the millet seeds within 180 s had the worst scores in terms of errors (12 errors) and searching time (180 s). The 8-arm radial maze was lit by 2 red bulbs (24 W). After each mouse, faeces and urine were removed and the maze was cleaned with 96% ethanol.

Static rods. The static rod apparatus consisted of 50 cm long wooden rod with one end fixed to a solid platform 30×30 cm. We used 2 different rods, one was 4·3 cm and the other 2·5 cm wide. The apparatus was placed 50 cm above a surface. In the test, the mice were placed at the free end of the rod, facing away from the platform. For every mouse we recorded the time necessary to turn 180° to face the platform (orientation time) and the time necessary to reach the platform (travel time). If the mouse did not

reach the platform within 90 s, the maximum travel time (90 s) was recorded. The mice were tested on 6 consecutive days (1 trial per day). For the first 3 days the 4·3 cm diameter static rod was used while for the next 3 days the 2·5 cm diameter static rod was employed in the test. After each mouse, faeces and urine were removed, and the rod was cleaned with 96% ethanol.

Running wheel. The test apparatus was a transparent plastic cage, 36 cm × 20 cm × 25 cm, equipped with a metal running wheel (14 cm in diameter) in the centre. It had a metal lid with a magnetic field sensor connected to a computer which recorded impulses from the moving running wheel with 4 embedded magnets. Each mouse was placed alone in the test cage and its wheel running activity was recorded for 48 h. All experiments began at the same time (at 10:00 a.m.). For each mouse tested, the wheel running activity data, i.e. average velocity, distance and duration, were recorded. Mouse pellets and water were available *ad libitum*. The running wheel test was done immediately after the end of learning tests, i.e. 12 weeks after the infection.

Data analysis

Statistica ® v.6.0 software was used to analyse the data. The simple models, i.e. influence of a single factor, toxoplasmosis, on particular behavioural variables (that failed in the tests of normality) was tested with the non-parametric Kendall test. It is usually used for testing the relation of a continous variable with an ordinal factor; however, it performs equally well in testing the relation with any binary factor. All multivariate models including repeated measure models were tested with General Linear Models (GLM) which are relatively robust with respect to normality assumptions. These tests were used to study the effects of toxoplasmosis and experience (number of previous trials) on each of the behavioural variables. The phase of the oestrous cycle (fertile/nonfertile) was available only for the maze test and the information was included in particular models as an independent categorical (binary) variable. All tests yielded approximately the same results when the weight of animals was included into the models as a continuous independent factor. The results were considered statistically significant when $P<0{\cdot}05$.

RESULTS

Clinical appearance

No typical symptoms of acute toxoplasmosis, i.e. lethargy, ruffled fur, and hunched posture, were observed in infected mice. However, the infected animals had significantly lower weight then controls on days 11, 12, 13 and 14 p.i. ($P<0{\cdot}0007$), the maximum difference was 1·9 g on day 11. At the beginning of behavioural tests, the difference in weight of infected and control mice was not significant. The serological examination at the end of experiments showed that all mice inoculated with brain suspension were *Toxoplasma* positive while all controls remained *Toxoplasma* negative. After 13 weeks of infection the brains of 15 mice were examined microscopically and the cyst count ranged from 40 to 280 cysts/brain (geometrical mean of 94 cysts/brain).

The 8-arm radial maze test

The data were analysed again using both non-parametric and parametric tests and analyses of the corresponding models gave qualitatively the same results. On test day 1, there were no significant differences in time spent in the maze or the number of errors between *Toxoplasma*-infected mice and controls in most simple models and more complex models (repeated measure model and model with 2 independent variables, i.e. *Toxoplasma* infection and oestrous cycle phase). On day 2 of the experiments (trials 4–7) the *Toxoplasma*-infected mice performed worse than controls (Table 1). The effect of the infection was highly significant, especially in complex models of GLM repeated measures tests with 3 independent factors, i.e. toxoplasmosis (binary), oestrus cycle phase (binary), and within-subject factor trial (4 levels). Here the P value for the difference in total time spent in the maze and in the number of errors between infected and control mice was 0·010 and 0·003, respectively. The effect of trial was also highly significant; however, the tests revealed no interaction between trial and toxoplasmosis or trial, toxoplasmosis and oestrus cycle phase (Fig. 1).

Static rod test

As the data failed tests of normality, we studied the effects of toxoplasmosis using first the non-parametric Kendall test (simple models) and then more complicated models with relatively robust GLM tests. When the same simple model was analysed by both the non-parametric and GLM tests, the results obtained were qualitatively the same (Table 2). All differences between control and infected mice in the time required to turn 180° were non-significant. In the first trial of the first day (wider rod) the infected mice needed (non-significantly) less time to reach the platform. In the other trials, the infected mice needed more time to reach the platform. The difference was significant for trials 3, 5 and 6. For analysis of differences in learning abilities between infected and control mice, we used a GLM

Table 1. The 8-arm radial maze test

(The table shows the results of the ethological test, i.e. mean times (in sec) and number of errors and standard deviations (S.D.), and results of the statistical testing of differences between 30 control and 31 infected mice in time necessary to reach the food in the maze and number of errors (entries into a wrong arm) during the trials. Trials 1–3: day 1, trials 4–7: day 2. Columns 5–7: results of the non-parametric Kendall test, columns 8–9: GLM test results.)

	Controls	S.D.	Infected	S.D.	Kendall Tau	Z	*P*-level	F	*P*-level
Trial 1	56·5	35·4	66·5	42·4	0·065	0·738	0·461	0·639	0·427
Mistakes 1	3·4	2·5	3·7	2·7	0·035	0·401	0·688	0·079	0·780
Trial 2	56·7	42·1	50·4	43·3	−0·085	−0·970	0·332	0·217	0·643
Mistakes 2	3·3	2·7	2·4	2·8	−0·245	−2·794	0·005	1·002	0·321
Trial 3	50·1	23·6	69·8	40·1	0·048	0·544	0·586	2·345	0·131
Mistakes 3	2·4	1·9	3·5	3·1	−0·009	−0·103	0·918	1·544	0·219
Trial 4	76·3	45·7	85·6	50·8	0·110	1·258	0·208	0·284	0·596
Mistakes 4	4·4	2·7	5·3	2·8	0·125	1·425	0·154	0·616	0·436
Trial 5	49·1	41·8	77·7	43·7	0·221	2·513	0·012	3·747	0·058
Mistakes 5	3·0	1·8	5·2	2·6	0·226	2·573	0·010	4·217	0·044
Trial 6	53·3	37·6	92·2	49·8	0·236	2·687	0·007	6·438	0·014
Mistakes 6	2·4	1·8	5·9	3·2	0·273	3·108	0·002	10·657	0·002
Trial 7	34·2	22·7	62·0	43·2	0·126	1·435	0·151	4·608	0·036
Mistakes 7	1·9	1·2	3·5	2·5	0·099	1·128	0·259	3·269	0·076

repeated measures test with time as a dependent variable in trials 1–6, infection as an independent factor and 2 independent within-subject factors, i.e. day of experiment (2 levels) and trial (3 levels). The results are shown in Table 3 and Fig. 2. The significant two-way interaction Toxo-trial ($P=0{\cdot}004$) suggests that infected mice have lower learning abilities than non-infected controls.

Wheel running test

In the wheel running tests, the *Toxoplasma*-infected mice ran faster and covered larger distances on both test days than controls. No significant effect of infection on time spent running was observed (Table 4).

DISCUSSION

The *Toxoplasma*-infected mice performed worse in most tests including a learning process. The results of the static rod tests as well as those of the maze test suggested that infected mice had impaired ability to learn. They had similar or even better results in the first trial of both tests; however, the time required to finish the task remained unchanged or decreased only slowly in the next trials of the same tests. Infected and control mice exhibited a similar decrease in time required to finish the task between days 1 and 2 for the same experiment, suggesting no effect of the infection on memory functions. In agreement with the previous reports (Hay *et al*. 1985) the infected mice showed higher activity in the wheel running tests, ran faster and covered larger distances.

At face value, toxoplasmosis seems to impair the learning ability of infected mice as the infected animals exhibited higher activity in the wheel running test but achieved worse results (longer times and more errors) in learning tests. The results of trial 4 on day 2 in the 8-arm radial maze test and those of the wheel running test, however, suggested another possibility for interpretation of our results. In fact, the efficiency of learning (relative increase in performance in consecutive trials on the same day) during all 4 trials was the same in infected and control mice and the absolute performance of mice in the fertile oestrus cycle phase was nearly the same in infected and control animals. It may indicate that the infected mice had the same learning capacity as controls but differed from the latter in motivation or perception of their environment. There are several lines of independent indices suggesting that *Toxoplasma*-infected mice could have impaired ability to recognize novel stimuli which resulted in poorer performance in learning tests. (1) The infected mice spent less time exploring a new environment during the first run of the maze test and more time in the following runs. Possibly, the control mice are able to recognize more quickly that the maze is not a new environment and to cut down their exploration activity. (2) The same mechanism, i.e. the lower ability of discrimination between novel and familiar activity, could explain the higher wheel running activity observed in our infected mice. Tarr *et al*. (2004) suggested that higher wheel running activity could reflect lower sensitivity to dopaminergic activity in the striatum, a trait associated, for example, with locally increased dopamine concentration. The mice with lower sensitivity to dopaminergic activity in the striatum, for example our *Toxoplasma*-infected mice, have lower ability to respond to novel stimuli and probably need more intensive wheel running activity to achieve the

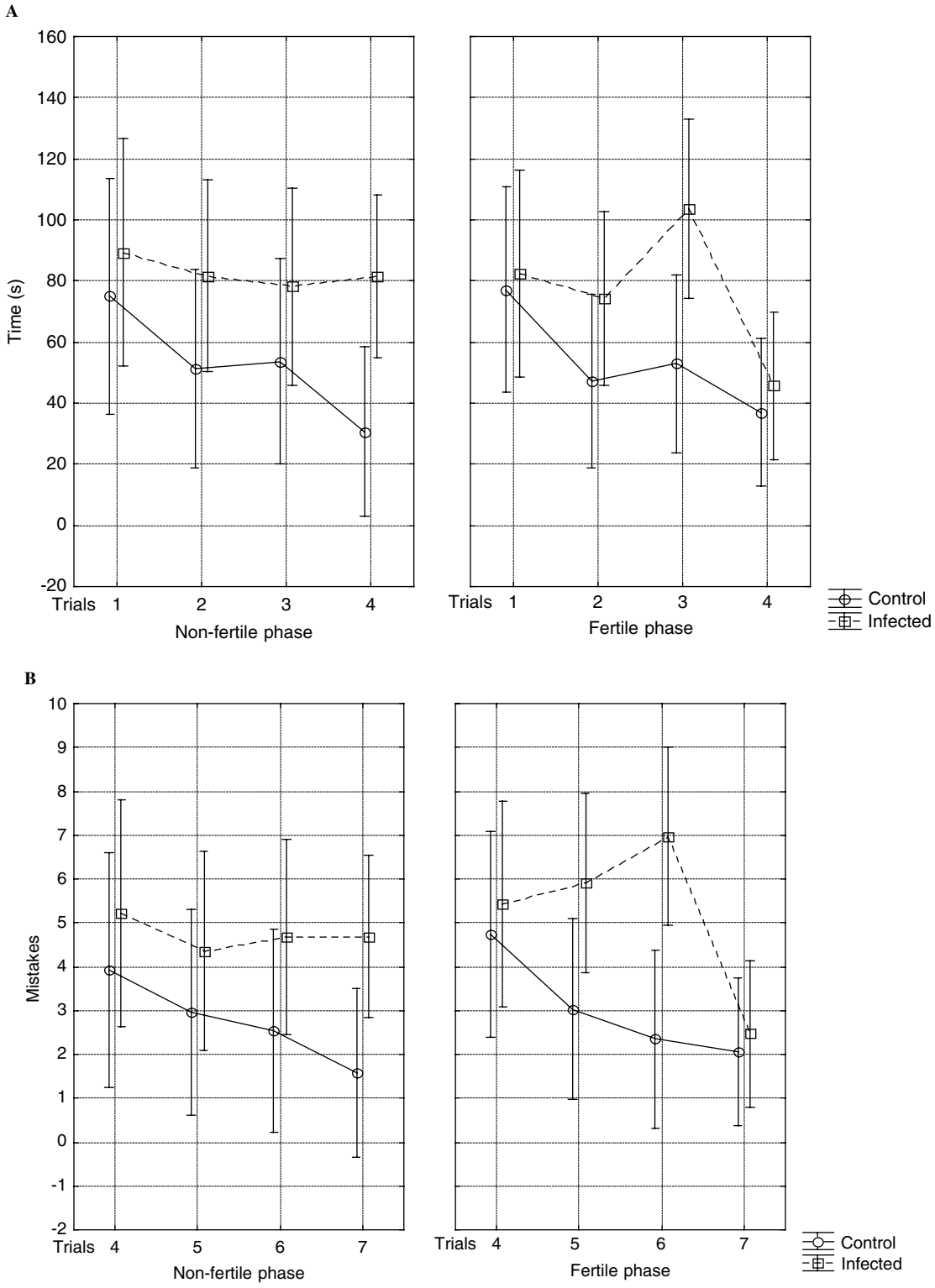

Fig. 1. Performance of infected and control mice in the 8-arm radial maze test on day 2. (A) Time necessary to reach the food in the maze; (B) number of errors (entries into a wrong arm) during the trial.

same level of endorphine reward than controls do. (3) The worse performance of infected animals in the static rod tests could be explained again by lower ability of infected animals to recognize a novel, this time uncomfortable, environment and consequently by lower motivation of animals to leave the adverse environment. The same mechanism could also play a role in the phenomenon described by Berdoy *et al*. (1995), i.e. in the lack of fear of infected mice for the smell of cat urine. Here, however, another more specific phenomenon probably plays a role, as the infected mice exhibited affinity to the smell of cat urine rather than the lack of fear of it. (4) The representatives of neophilic species, mice and humans,

Table 2. Static rod test

(The table shows the results of the ethological test, i.e. mean times (in sec) and standard deviations (S.D.), and results of the statistical testing of differences between 30 control and 31 infected mice in time necessary to turn 180° and total time necessary to reach the platform. Trials 1–3: day 1, rod 4·3 cm in diameter, trials 4–6: day 2, rod 2·5 cm in diameter. Columns 5–7: results of the non-parametric Kendall test, columns 8–9: GLM test results.)

	Controls	S.D.	Infected	S.D.	Kendall Tau	Z	*P*-level	F	*P*-level
Trial 1 turn	10·6	2·9	11·5	7·3	−0·074	−0·848	0·397	0·054	0·817
Trial 1 total	71·6	24·9	59·6	33·4	−0·129	−1·463	0·143	3·418	0·069
Trial 2 turn	9·9	5·9	6·1	4·8	−0·11	−1·258	0·208	3·174	0·08
Trial 2 total	28·1	30·6	32·2	33·9	−0·063	−0·721	0·471	0·007	0·932
Trial 3 turn	6·0	6·1	11·9	7·5	0·115	1·311	0·19	2·389	0·128
Trial 3 total	26·6	32·6	45·7	36·4	0·251	2·854	0·004	5·797	0·019
Trial 4 turn	5·2	4·6	7·9	13·0	0·058	0·656	0·512	1·212	0·275
Trial 4 total	39·9	33·8	39·1	35·4	−0·014	−0·163	0·871	0·017	0·897
Trial 5 turn	9·2	6·8	9·3	6·6	−0·035	−0·393	0·694	0·002	0·967
Trial 5 total	39·6	34·1	61·1	34·4	0·182	2·069	0·039	4·327	0·042
Trial 6 turn	5·2	4·9	5·0	6·7	0·05	0·568	0·57	0·018	0·893
Trial 6 total	31·8	30·2	48·4	33·8	0·178	2·032	0·042	3·391	0·071

Table 3. Effects of *Toxoplasma* infection on learning abilities of mice in the static rod test

(Model: time as a dependent variable in trials 1–6, infection as an independent factor and test day and trial no. as 2 within-subject independent factors.)

	SS	D.F.	MS	F	P
Intercept	843877·8	1	843877·8	629·986	0·000
Toxo	4157·9	1	4157·9	3·104	0·083
Error	79031·6	59	1339·5		
DAY	143·5	1	143·5	0·151	0·699
DAY*toxo	1492·9	1	1492·9	1·567	0·216
Error	56216·0	59	952·8		
TRIAL	14266·3	2	7133·1	9·933	0·000
TRIAL*toxo	8197·4	2	4098·7	5·707	0·004
Error	84739·5	118	718·1		
DAY*TRIAL	30378·8	2	15189·4	15·350	0·000
DAY*TRIAL*toxo	2248·6	2	1124·3	1·136	0·325
Error	116763·9	118	989·5		

show lower neophily after the *Toxoplasma* infection, while the opposite is true of neophobic rats (Hutchison *et al*. 1980*a*, *c*; Hay *et al*. 1984; Webster *et al*. 1994; Berdoy *et al*. 1995; Flegr *et al*. 2003; Skallová *et al*. 2005). The most parsimonious explanation of such an opposite effect of toxoplasmosis in two phylogenetically related rodent species is reduced ability of infected animals to recognize (or to react to) novel stimuli. In the neophilic species (e.g. mice and human), this effect may manifest itself in reduced neophily while in the neophobic species (e.g. rats) in reduced neophoby.

The proximal mechanism responsible for the observed effect of latent toxoplasmosis on learning processes can be only speculated upon. Generally speaking, the neurological mechanism of behavioural effects of toxoplasmosis is unknown; however, two molecules, testosterone and dopamine, are suspected to be implicated in these effects. The following indices of increased testosterone level were observed in humans: higher body height, lower 2nd to 4th digit length ratio and increased secondary sex ratio (higher probability of the birth of a boy) (Flegr *et al*. 2005; Kaňková *et al*. 2007). The increased dopamine concentration in the brains of infected mice (Stibbs, 1985), nature of behavioural changes in rats and mice (Jones *et al*. 1996; Kabbaj and Akil, 2001; Nieoullon, 2002; Viggiano *et al*. 2003) and that of psychological changes in infected humans (decrease of the psychobiological factor novelty seeking) (Flegr *et al*. 2003, Flegr, 2007) and results of a pharmacoethological study with a selective dopamine uptake inhibitor, GBR 12909 (inhibited hole board exploration in infected mice and an opposite effect, i.e. enhanced hole board exploration, in controls) (Skallová *et al*. 2006) suggest that an imbalance in the dopamine level in particular parts of the brain could play a role in the observed effects. This notion is also supported by the observed association between toxoplasmosis and schizophrenia (Minto and Roberts,

Table 4. Wheel running test

(The table shows the results of the ethological test, i.e. the average velocity (in meters per sec), total distance (in meters) and active length (in min), and standard deviations (S.D.), and results of the statistical testing of differences between 25 control and 25 infected mice in average velocity, total distance and activity length during the test (first and second day). Columns 5–7: results of the non-parametric Kendall test, columns 8–9: GLM test results.)

	Controls	S.D.	Infected	S.D.	Kendall Tau	Z	*P*-level	F	*P*-level
Velocity 1 day (m/s)	0·12	0·06	0·16	0·04	0·17	1·79	0·073	5·77	0·020
Velocity 2 day (m/s)	0·08	0·04	0·11	0·03	0·25	2·56	0·01	6·98	0·011
Distance 1 day (m)	6198	3923·9	8411	2849·6	0·209	2·14	0·032	5·2	0·027
Distance 2 day (m)	3030	1756·8	4010	1493·1	0·177	1·81	0·069	4·51	0·038
Active time 1 day (min)	791·2	326·7	850·6	84·3	−0·1	−1·031	0·30	0·72	0·399
Active time 2 day (min)	525·9	236·2	601·3	143·2	0·028	0·29	0·76	2·25	0·14

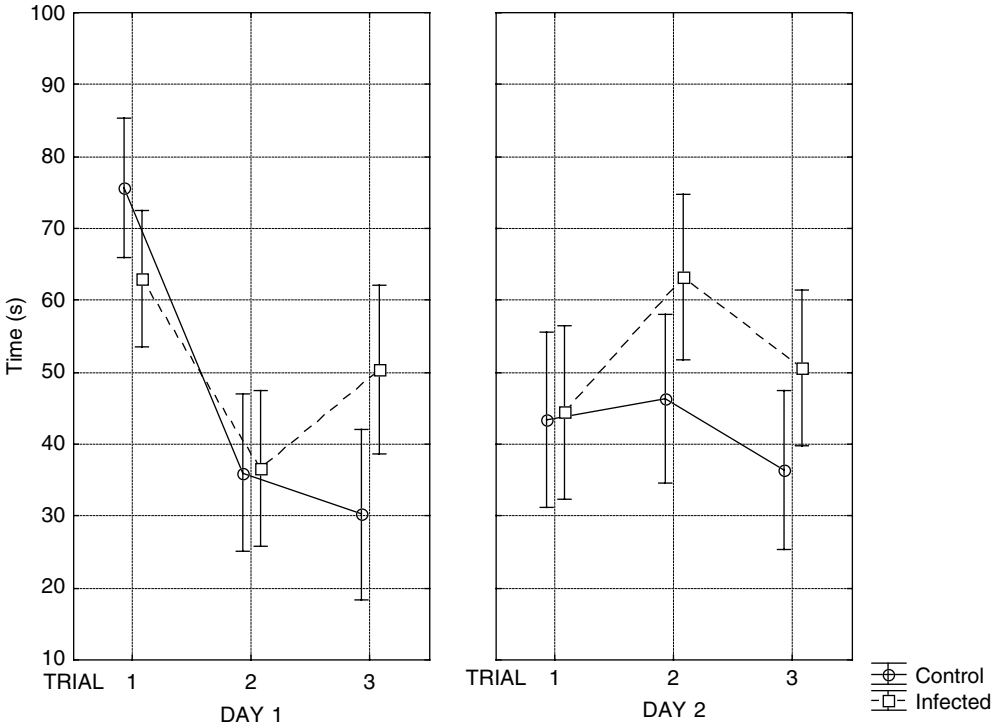

Fig. 2. Static rod test. Time necessary to reach the platform in infected and control mice.

1959; Kramer, 1966; Ladee *et al*. 1966; Torrey and Yolken, 2003; Bachmann *et al*. 2005), a mental illness reportedly caused by higher dopamine levels in the brain mesolimbic regions, or rather dopamine imbalance between the mesolimbic and mesocortical regions in the brain (Creese *et al*. 1976; Sawa and Snyder, 2002). The higher level of dopamine in particular regions of the brain could be a side-effect of increased levels of lymphokines as a result of local inflammation in the infected brain tissue (Alonso *et al*. 1993; Petitto *et al*. 1997; Novotná *et al*. 2005; Skallová *et al*. 2005) and could result, among other things, in an observed increase of testosterone. In the context of our results, it is important to stress that the association between dopaminergic activity and intensity of response to novel stimuli was clearly demonstrated in rodents (Dellu *et al*. 1996; Kabbaj and Akil, 2001).

Based on the results of the present study, we can conclude that latent (not only acute or congenital) toxoplasmosis has a negative effect on learning processes of infected mice. At the present state of knowledge, it cannot be decided whether latent toxoplasmosis impairs the learning ability or the ability to recognize novel stimuli. However, the ability to recognize novel stimuli reportedly plays a role in most learning processes. Therefore, latent toxoplasmosis is highly likely to have either a primary or a secondary effect on the learning abilities of infected hosts.

We would like to thank Anna Skallová for her advice. This work was supported by the Czech Ministry of Education (Grant 0021620828).

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