Trypanosoma evansi infection impairs memory, increases anxiety behaviour and alters neurochemical parameters in rats

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SUMMARY

The aim of this study was to investigate neurochemical and enzymatic changes in rats infected with *Trypanosoma evansi*, and their interference in the cognitive parameters. Behavioural assessment (assessment of cognitive performance), evaluation of cerebral L-[^{3}H]glutamate uptake, acetylcholinesterase (AChE) activity and Ca^{+2} and Na^+, K^+-ATPase activity were evaluated at 5 and 30 days post infection (dpi). This study demonstrates a cognitive impairment in rats infected with *T. evansi*. At 5 dpi memory deficit was demonstrated by an inhibitory avoidance test. With the chronicity of the disease (30 dpi) animals showed anxiety symptoms. It is possible the inhibition of cerebral Na^+, K^+-ATPase activity, AChE and synaptosomal glutamate uptake are involved in cognitive impairment in infected rats by *T. evansi*. The understanding of cerebral host–parasite relationship may shed some light on the cryptic symptoms of animals and possibly human infection where patients often present with other central nervous system (CNS) disorders.

Key words: Trypanosomes, Ca^{+2} ATPase, Na^+, K^+ ATPase, cognitive dysfunction, memory, anxiety, glutamate uptake.

INTRODUCTION

Surra is an important disease in a wide geographic region caused by *Trypanosoma evansi*, and infects mainly camels, cattle, buffalos, horses and some wild animals (Brun *et al*. 1998; Al-Qarawi *et al*. 2001; Berlin *et al*. 2009; Habila *et al*. 2012). The parasite is spread by mechanical transmission of infected blood through haematophagous insects such as tabanid flies (Brun *et al*. 1998; Herrera *et al*. 2004). Animals infected with *T. evansi* develop anaemia (Dargie *et al*. 1979; Gutierrez *et al*. 2006; Da Silva *et al*. 2009*a*, *c*; Wolkmer *et al*. 2009; Paim *et al*. 2011*b*; Habila *et al*. 2012) and neurological signs in the final stage of the disease (Tuntasuvan *et al*. 1997, 2000; Berlin *et al*. 2009). In the first report of *T. evansi* infection in humans, sensory deficit, disorientation, agitation and aggression were described (Joshi *et al*. 2005).

Neurological signs might be the result of necrotizing panencephalitis or meningoencephalitis (Berlin *et al*. 2009; Rodrigues *et al*. 2009), but also, due to alteration in the actions of neurotransmitters (Da Silva *et al*. 2011*a*; Paim *et al*. 2011*a*). The mechanism leading to the onset of neurological signs in trypanosomiasis is not completely elucidated, however, in recent studies, we have demonstrated that infection by *T. evansi* affects blood and brain acetylcholinesterase (AChE) activity on hosts (Da Silva *et al*. 2010*b*, 2011*b*; Wolkmer *et al*. 2010). Therefore, the study of the activity of this enzyme in animals infected with *T. evansi* might give us some insight on neurotransmission and could, consequently, associate this enzyme with cognitive dysfunction observed in this disorder.

It is believed that changes in cholinergic system activity have a key role in clinical signs developed by animals infected with the parasite. However, neurotransmission is a dynamic process, supported by a permanent cycle of neurotransmitter release, over a neurotransmitter response to stimulation. Little is known about the effect of infection by *T. evansi* in the neurochemical activity of the hosts.

The glutamate is considered to be the major mediator of excitatory signals in the mammalian central nervous system (CNS) and is probably involved in most aspects of normal brain function including cognition, memory and learning (Fonnum, 1984;

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Headley and Grillner, 1990; Greenamyre and Porter, 1994; Danbolt, 2001). Despite their important role in neurotransmission, the toxicity caused by over-stimulation of glutamate receptors, 'excitotoxicity', has been hypothesized to be a final common pathway of neuronal death in both acute and chronic neurological disease (Choi, 1988; Beal, 1992*a*, *b*; Rothman and Olney, 1995; Abril *et al*. 2004).

The enzymes Na^+, K^+-ATPase and Ca^{+2}-ATPase are embedded in the cell membrane, responsible for the generation of the membrane potential through the active transport of sodium, potassium and calcium ions in the CNS necessary to maintain neuronal excitability (Erecinska and Silver, 1994). These ions are involved in many neuronal signalling processes such as the control of presynaptic neurotransmitter release, regulation of membrane excitability and directly, as a second and third messenger (Smith *et al*. 1983; Gandhi and Ross, 1988). The changes in the neuronal homeostasis in animals infected by *T. evansi* could be involved in disturbances of cognitive functions (Hartmann *et al*. 1994).

Therefore, the present investigation was carried out to determine if infection in rats with *T. evansi* induces neurochemical and enzymatic changes and if these are correlated with neurological signs. These changes can be evaluated by animal performance in various behavioural tests that can identify, for example, the coordination, the state of anxiety and memory of the animals, which are the final manifestations of neural functions (Genn *et al*. 2003; Lalonde *et al*. 2004; Lapiz-Bluhm *et al*. 2008).

MATERIALS AND METHODS

Reagents

[^{3}H]Glutamic acid (1 Ci mL^{-1}) was purchased from Amersham Biosciences. Acetylthiocholine iodide, 5,5′dithiobis-2-nitrobenzoic acid (DTNB), tris (hydroxymethyl)-aminomethane GR, Coomassie brilliant blue G, were obtained from Sigma Chemical Co (St. Louis, MO, USA). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Animals

Male Wistar rats (90–110 days) from the Central Animal House of the Universidade Federal de Santa Maria (UFSM) were used in this experiment. They were housed 5 to a cage on a natural day/night cycle at a temperature of 21 °C with free access to water and standard chow *ad libitum*. This study was approved by the Ethics and Animal Welfare Committee of the Rural Science Center of the Federal University of Santa Maria (CCR/UFSM), No. 017/2012 in accordance with existing legislation and the Ethical Principles published by the Brazilian College of Animal Experiments (COBEA).

Experimental design

Twenty-two rats were used in each treatment group, which consisted of *T. evansi* infected groups (T) and control group (C; non-infected rats). To obtain the total of 22 infected rats, a total of 45 rats were inoculated (a mortality rate of up to 50% can be expected). At 5 (C5, T5) and 30 (C30, T30) dpi 11 animals per group were euthanased with isoflurane in a gas chamber. Behavioural assessments were performed 24 h pre-euthanasia.

Inoculation

The rats were inoculated intraperitoneally with 0·2 mL of blood containing 10^6 parasites. The control rats received 0·2 mL of physiological solution by the same route. The aetiological agent isolate used here is from a naturally infected dog, and maintained in liquid nitrogen at the laboratory of Dr Silvia G. Monteiro (Brazil).

Parasitaemia estimation

The presence and degree of parasitaemia were determined daily for each animal by blood smear examination. The blood films were stained with Romanowsky (Diff-Quick) and visualized under optical microscope (1000×) determining the average number of trypanosomes in 10 homogeneous random fields (considering erythrocytes).

Behavioural assessment – assessment of cognitive performance

During the behavioural study, only one animal was tested at a time. The behavioural experiments were performed at 4 and 29 dpi, and the inhibitory avoidance test was always performed first. Behavioural assessment was performed in triplicate and was evaluated by three different analysers.

Inhibitory avoidance. Animals were subjected to training and test in a step-down inhibitory avoidance apparatus according to Guerra *et al*. (2006). Briefly, the rats were subjected to a single training session in a step-down inhibitory avoidance apparatus, which consisted of a 25 × 25 × 35 cm box with a grid floor whose left portion was covered by a 7 × 25-cm platform, 2·5 cm high. The rat was placed gently on the platform facing the rear left corner, and when the rat stepped down with all four paws on the grid, a 2-s 0·4-mA shock was applied to the grid. Retention test took place in the same apparatus 24 h later. Test step-down latency was taken as a measure of retention, and a cut-off time of 300 s was established.

Open field. Immediately after the inhibitory avoidance test session, the animals were transferred to an open-field that was a 40 × 45 cm arena surrounded by

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50 cm high walls, made of plywood. The floor of the arena was divided into 12 equal squares by black lines. Animals were placed in the rear left corner and left to explore the field freely for 5 min. Line crossings and rearings were counted. This test was carried out to identify motor disabilities, which might influence inhibitory avoidance performance at testing.

Elevated plus maze test. Anxiolytic-like behaviour was evaluated using the task of the elevated plus maze as previously described (Frussa-Filho *et al*. 1999; Rubin *et al*. 2004). The apparatus consists of a wooden structure raised to 50 cm from the floor. This apparatus is composed of 4 arms of the same size, with 2 closed arms (walls 40 cm) and 2 open arms. Initially, the animals were placed on the central platform of the maze in front of an open arm. The animal had 5 min to explore the apparatus, and the time spent and the number of entries in open and closed arms were recorded. The behaviour parameters are expressed in per cent of number of entries and per cent of time spent in the open and closed arms. The apparatus was thoroughly cleaned with 30% ethanol between each session.

Foot-shock sensitivity test. Reaction to shock was evaluated in the same apparatus used for inhibitory avoidance, except that the platform was removed. The modified 'up and down' method (Rubin *et al*. 2004) was used to determine the flinch and jump thresholds in naïve animals. Animals were placed on the grid and allowed a 3 min adaptation period before starting a series of shocks (1 s) delivered at 10 s intervals. Shock intensities ranged from 0·1 to 0·5 mA in 0·1 mA increments. The adjustments in shock intensity were made in accordance to each animal's response. The intensity was raised on one unit when no response occurred and lowered by one unit when a response was made. A flinch response was defined as withdrawal of one paw from the grid floor, and a jump response was defined as withdrawal of three or four paws. Two measurements of each threshold (flinch and/or jump) were made, and the mean of each score was calculated for each animal.

Uptake assay

L-[^{3}H]glutamate uptake assays were carried out in slices of cortex and hippocampus of rats according to the method described by Schweigert *et al*. (2005). Animals (3 rats per group) were decapitated and brains were immediately removed and submerged in Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 0·63 Na_2HPO_4, 4·17 $NaHCO_3$, 5·36 KCl, 0·44 KH_2PO_4, 1·26 $CaCl_2$, 0·41 $MgSO_4$, 0·49 $MgCl_2$ and 1·11 glucose, adjusted to pH 7·2. Cortex and hippocampus were dissected, and coronal slices (0·4 mm) were obtained using a Mcilwain tissue chopper. Slices were transferred to multi-well dishes and washed with 1·0 mL HBSS. After 10 min of pre-incubation, the uptake assay was performed by adding 13·3 μM (hippocampus) and 6·6 μM (cortex) L-[^{3}H]glutamate in 300 μL HBSS at 37 °C. Incubation was terminated after 5 min (hippocampus) or 7 min (cortex) by three ice-cold washes with 1 mL HBSS immediately followed by the addition of 0·5 M NaOH, which was kept overnight. An aliquot of 10 μL was removed to protein determination. Unspecific uptake was measured using the same protocol described above, with differences in temperature (4 °C) and media (choline chloride instead of sodium chloride). Na^+-dependent uptake was considered as the difference between the total uptake and the unspecific uptake. Uptakes were performed in triplicate. Incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1409). Results were expressed as pM of L-[^{3}H]glutamate uptake/mg protein/min.

Biochemical assessment

Biochemical tests were conducted 24 h after the last behavioural test. The animals were anaesthetized and euthanased by decapitation (8 rats per group). The brain structures were removed, separated into cerebral cortex, striatum and hippocampus and placed in a solution of 10 mM Tris–HCl, pH 7·4, on ice. The brain structures were homogenized in a glass Potter homogenizer in Tris–HCl solution and an aliquot was stored for ATPases assay. The homogenate (10%, w/v) was then centrifuged at 1000 ***g*** for 15 min and the supernatant so formed was stored at −80 °C until assessment of acetylcholinesterase activity. Protein was determined by the Coomassie blue method, using bovine serum albumin as standard solution (Bradford, 1976).

Estimation of acetylcholinesterase (AChE) activity. The AchE activity was assessed by the method described by Ellman *et al*. (1961). The reaction mixture (2 mL final volume) contained 100 mM K^+-phosphate buffer, pH 7·5 and 1 mM 5,5′-dithiobisnitrobenzoic acid (DTNB). The method is based on the appearance of a yellow anion, 5,5′-dithio-bis-acid-nitrobenzoic, measured by absorbance at 412 nm during 2-min incubation at 25 °C. The enzyme (40–50 μg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0·8 mM acetylthiocholine iodide (AcSCh). All samples were run in duplicate or triplicate and the enzyme activity was expressed in μM AcSCh h^{-1} mg^{-1} of protein.

Na^+, K^+-ATPase activity measurement. Na^+, K^+-ATPase activity was measured as previously described (Wyse *et al*. 2000) with minor modifications (Carvalho *et al*. 2012). Briefly, the assay medium consisted of (in mM) 30 Tris-HCl buffer (pH 7·4), 0·1 EDTA, 50 NaCl, 5 KCl, 6 $MgCl_2$ and 50 μg of

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protein in the presence or absence of ouabain (1 mM), in a final volume of 350 μL. The reaction was started by adding adenosine triphosphate to a final concentration of 3 mM. After 30 min at 37 °C, the reaction was stopped by adding 70 μL of 50% (w/v) trichloroacetic acid. Saturated substrate concentrations were used, and reaction was linear with protein and time. Appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate (Pi) released was quantified by the colorimetric method, as previously described (Fiske and Subbarow, 1927), using KH_2PO_4 as reference standard. Specific Na^+, K^+-ATPase activity was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain) and expressed in nM of Pi min^{-1} mg^{-1} of protein.

Ca^{+2}-ATPase activity measurement. Ca^{+2}-ATPase activity was measured as previously described (Rohn *et al.* 1993) with minor modifications (Trevisan *et al.* 2009). Briefly, the assay medium consisted of (in mM) 30 Tris-HCl buffer (pH 7·4), 0·1 EGTA, 3 $MgCl_2$ and 100 μg of protein in the presence or absence of 0·4 $CaCl_2$, in a final volume of 200 μL. The reaction was started by adding adenosine triphosphate to a final concentration of 3 mM. After 60 min at 37 °C, the reaction was stopped by adding 70 μL of 50% (w/v) trichloroacetic acid. Saturated substrate concentrations were used, and reaction was linear with protein and time. Appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate (Pi) released was quantified by the colorimetric method, as previously described (Fiske and Subbarow, 1927), using KH_2PO_4 as reference standard. The Ca^{+2}-ATPase activity was determined by subtracting the activity measured in the presence of Ca^{+2} from that determined in the absence of Ca^{+2} (no added Ca^{+2} plus 0·1 mM EGTA) and expressed in nM of Pi min^{-1} mg^{-1} of protein.

Statistical analysis

Statistical analysis of training and test step-down latencies was carried out by the Kruskal–Wallis test (non-parametric two-way ANOVA) and results median ± interquartile. Foot shock sensitivity was analysed by unpaired *t*-test. For the neurochemical analyses, the statistical significance was assessed by analysis of variance (ANOVA) and *post hoc* Duncan's test was carried out when appropriate. A value of $P<0·05$ was considered to be significant.

RESULTS

Parasitaemia

Trypanosoma evansi was detected in peripheral blood of all infected rats between 24 and 48 h after inoculation. Parasitaemia increased progressively in most animals until 5 dpi, when the first peak of parasitaemia was observed (mean of 59 ± 20 trypanosomes/field) and the group T5 was formed. At 6 dpi, a reduction in parasitaemia was observed in the rats from subgroup T30, ranging from 0 to 10 parasites/field until 25 dpi. At 30 dpi the second peak of parasitaemia was detected, with a mean of 46 ± 22 trypanosomes/field. The control animals remained clinically healthy throughout the experimental period.

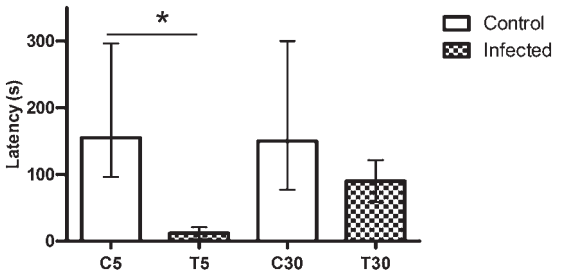

Fig. 1. Inhibitory avoidance: Infection with *Trypanosoma evansi* at 4 dpi (T5) impairs memory in adult rats. Data are the median ± interquartile range for 11 animals in each group. * $P<0·05$ compared with the control group. Performance as three replicas and was evaluated by three different analysers.

Behavioural tests

Memory and learning – Inhibitory avoidance. Figure 1 shows the effect of *T. evansi* infection on step-down latencies. Statistical analysis of testing showed a difference between groups in the step-down latencies during training trials in *T. evansi* infected rats at 5 dpi (T5) ($P<0·05$). Group T5 showed a memory deficit compared with controls (animals not infected – C5). However, despite a reduction in the step-down latencies, there were no significant differences between groups at 30 dpi (T30). Statistical analysis of training showed no significant difference between groups.

Since motivational disparities in the training session may account for differences in inhibitory avoidance at testing, experiments were performed to assess whether trypanosomes affect shock threshold, or locomotor ability of the animals. Statistical analysis of open-field data (one-way ANOVA) revealed that *T. evansi*-infected rats did not change the number of crossing ($P>0·05$) or rearing ($P>0·05$) responses in a subsequent open-field test session, suggesting that the infection does not cause gross motor disabilities at testing. Moreover, infected rats did not change foot shock sensitivity, as demonstrated by the similar flinch (unpaired *t*-test, $P=0·45$) and jump (unpaired *t*-test, $P=0·57$) thresholds exhibited by the animals. These data suggest that *T. evansi* infection does not cause motor disabilities or modify foot shock sensitivity (data not shown).

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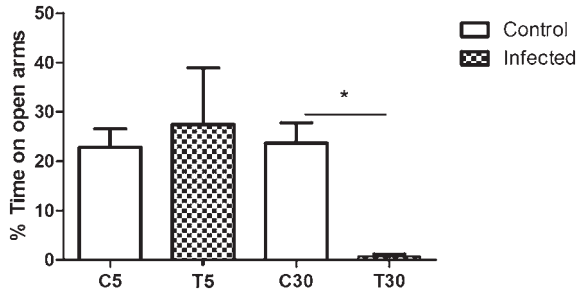

Fig. 2. Elevated plus-maze: Infection with *Trypanosoma evansi* at 29 dpi (T30) decreases the percentage of time spent on the open arms by adult rats tested in the elevated plus-maze. Data are the mean + S.E.M. for 11 animals in each group. * $P<0{\cdot}05$ compared with the control group. Performance as three replicas and was evaluated by three different analysers.

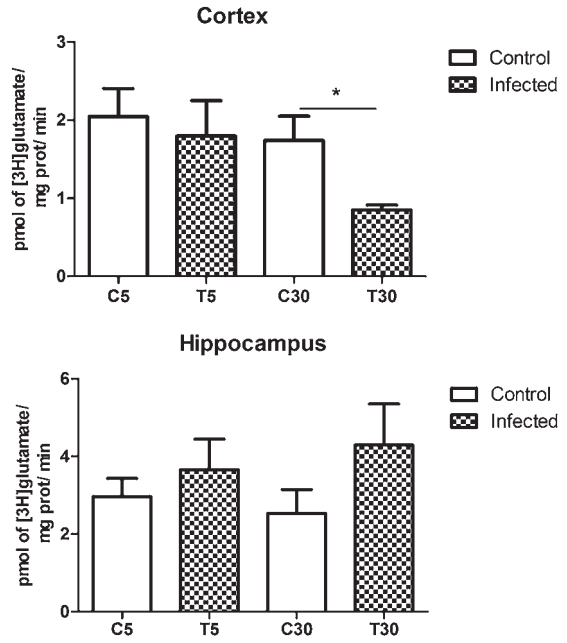

Fig. 3. Evaluation of L-[^{3}H]glutamate uptake assays carried out in slices of cortex and hippocampus rats infected by *Trypanosoma evansi* at 5 (T5) and 30 (T30) dpi. Results are expressed as mean ± S.E.M. * $P<0{\cdot}05$ compared with the control group (n = 8 per group).

Anxiolytic activity – elevated plus-maze test of rats. At 30 dpi, rats infected by *T. evansi* (T30) decreased the percentage of time on open arms ($P<0{\cdot}01$; Fig. 2), and increased the closed arms entries when compared with the control group (C30). There were no significant differences between groups at 5 dpi (T5 *vs.* C5).

L-[^{3}H]Glutamate uptake. L-[^{3}H]Glutamate uptake was decreased in cerebral cortex (Fig. 3) of the T30 group when compared with the control group C30 ($P<0{\cdot}05$). No significant change was observed in L-[^{3}H]glutamate uptake in either cortex or hippocampus in rats at 5 dpi from *T. evansi* group.

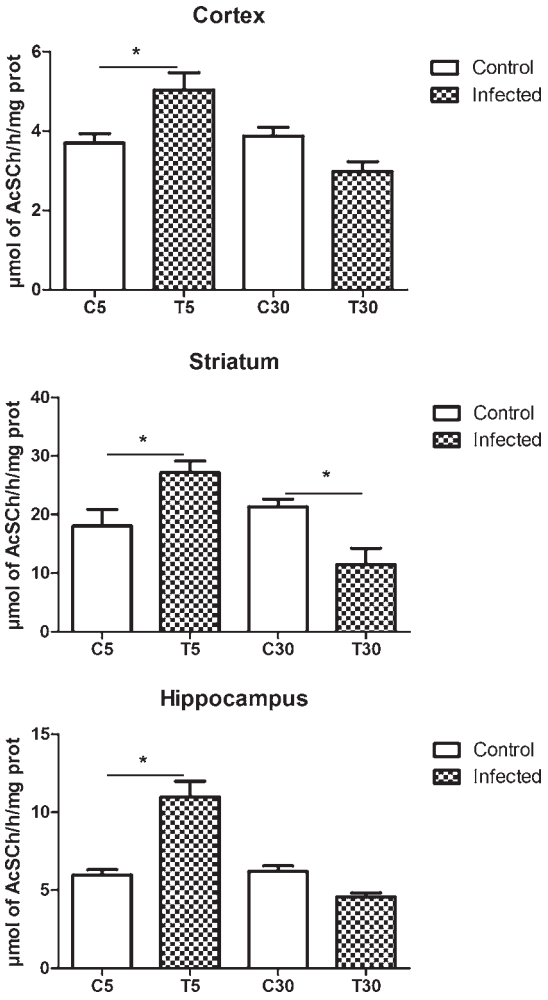

Fig. 4. Evaluation of acetylcholinesterase activity in cortex striatum and hippocampus of rats infected by *Trypanosoma evansi* at 5 (T5) and 30 (T30) dpi. Results are expressed as mean ± S.E.M. * $P<0{\cdot}05$ compared with the control group (n = 8 per group).

Activity of AChE in brain

The results obtained for AChE activity in cerebral cortex, striatum and hippocampus are presented in Fig. 4. As observed, AChE activity was significantly increased in rats infected by *T. evansi* at 5 dpi (T5, $P<0{\cdot}05$) compared with the control group (C5). However, at 30 dpi AChE activity in the T30 group decreased significantly in the striatum ($P<0{\cdot}05$) and showed a tendency to reduction in the cerebral cortex and hippocampus when compared with the C30 group ($P<0{\cdot}058$).

ATPases

Trypanosoma evansi infection decreases the Na^+, K^+-ATPase activity in cerebral cortex, striatum and hippocampus homogenates. Figure 5 shows that infected rats have a significant inhibition of Na^+, K^+-ATPase activity at 5 dpi in cerebral cortex and hippocampus ($P<0{\cdot}05$); and at 30 dpi have a

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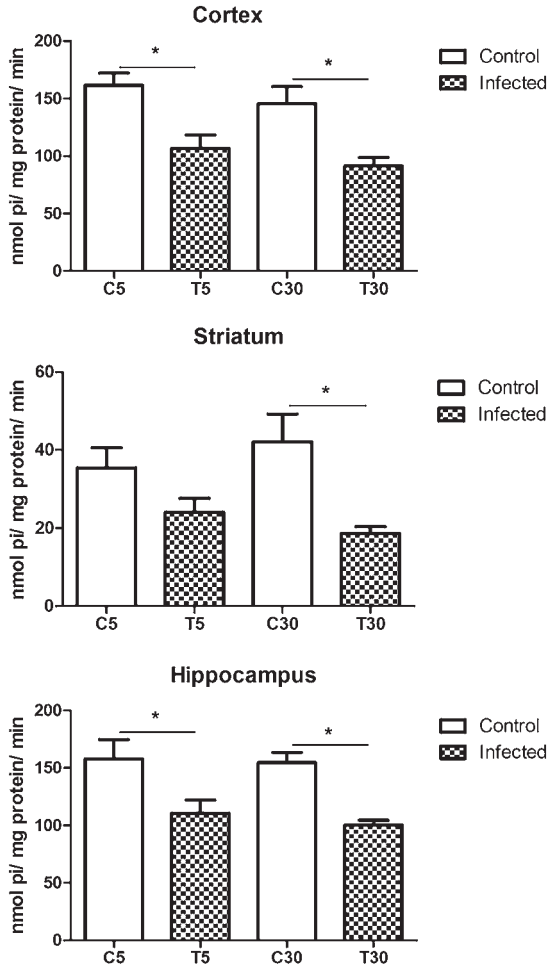

Fig. 5. Evaluation of Na^+, K^+-ATPase activity in cortex, striatum and hippocampus of rats infected by *Trypanosoma evansi* at 5 (T5) and 30 (T30) dpi. Results are expressed as mean ± S.E.M. * $P<0{\cdot}05$ compared with the control group (n = 8 per group).

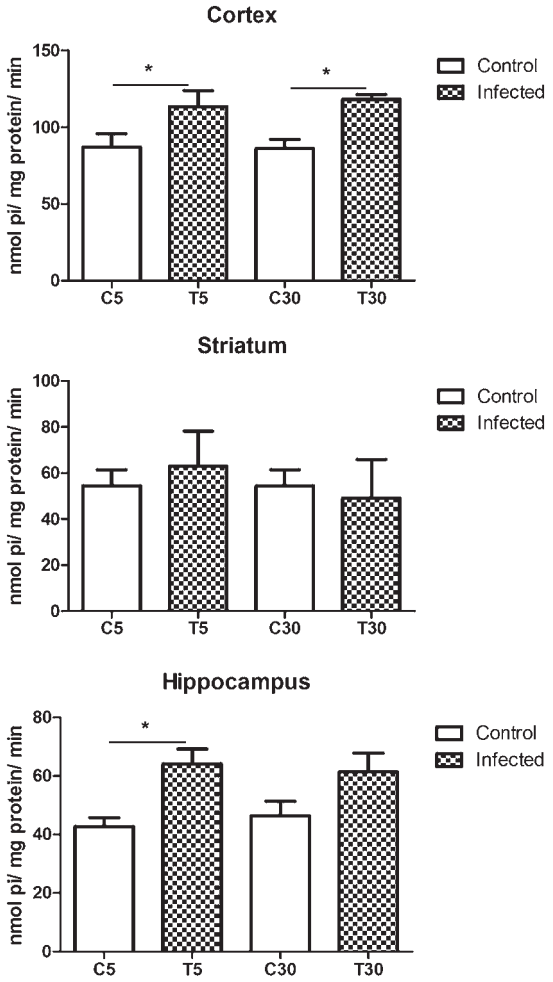

Fig. 6. Evaluation of Ca^{2+}-ATPase activity in cortex, striatum and hippocampus of rats infected by *Trypanosoma evansi* at 5 (T5) and 30 (T30) dpi. Results are expressed as mean ± S.E.M. * $P<0{\cdot}05$ compared with the control group (n = 8 per group).

significant inhibition in all the structures assessed ($P<0{\cdot}05$).

The total activity of the Ca^{+2}-ATPase in the homogenate was higher in the cerebral cortex and hippocampus of rats infected by *T. evansi*. This increase was significant on 5 dpi in cerebral cortex and hippocampus and 30 dpi in the cerebral cortex ($P<0{\cdot}05$; Fig. 6). The striatum showed no variation.

DISCUSSION

This study was conducted to test the contribution of *T. evansi* infection to behaviour changes and its relationship with enzymes and glutamate in CNS. The course of infection has a traditional variation in animals regarding the response to the degree of parasitaemia (Wolkmer *et al*. 2007, 2009, 2012, 2013*b*; Da Silva *et al*. 2009*a*, *b*, 2010*a*, 2011*c*; Franca *et al*. 2011; Paim *et al*. 2011*a*). As expected, *T. evansi* was pathogenic to rats and led to a high parasitaemia at 4–6 dpi. After this period, a reduction in parasitaemia was observed, resembling the presentation of chronic disease at the end of the experimental period.

The inhibitory avoidance test is a classic behavioural test with a strong aversive component and is used to evaluate learning and memory in rats and mice (Cahill *et al*. 1986). In our study, we showed for the first time that *T. evansi* infection decreases step-down latency in the inhibitory avoidance test at 5 dpi in rats (Fig. 1, C5 group), but does not change locomotor activity, suggesting learning and memory impairment of these animals in early infection. Signals of behaviour changes have been reported in animals and human infected by *T. evansi* (Tuntasuvan *et al*. 1997, 2000; Joshi *et al*. 2005; Wolkmer *et al*. 2007; da Silva *et al*. 2011*b*, 2012*a*, *b*).

Regardless of these findings, the involvement of the CNS in infection is widely discussed, because brain lesions are occasionally observed. Some authors have reported the appearance of lesions in the brain and presence of the parasite (Berlin *et al*. 2009; Rodrigues *et al*. 2009). Data based from animal

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models indicate that *Trypanosome brucei* entry into the brain occurs in the first days of infection and a significant level of microvascular inflammation is detectable (Frevert *et al*. 2012). However, we could not see histological abnormalities in CNS sections of rats infected with *T. evansi*, at 5, 15, 30 dpi. Lesions in CNS were only observed after 150 dpi, and, it was determined that posterior member paralysis is a consequence of lesions at the muscular and peripheral nerve systems (Da Silva *et al*. 2012*a*). The abnormalities observed during behavioural tests probably indicate the progression of clinical disease as a result of neurochemical dysfunction.

The importance of the cholinergic system in learning and memory processes is undeniable, and thus changes in AChE activity, as well as in the acetylcholine neurotransmitter levels, are associated with cognitive deficits (Das *et al*. 2005*a*, *b*). In this study, we found increased AChE activity in all cerebral structures of the *T. evansi* group at 5 dpi. This could be the result of a decrease in membrane-bound Na^+, K^+-ATPase concentration, which modifies ion homeostasis and leads to an increase in Ca^{+2} and Na^+ levels within the cell. The concurrent augment of Ca^{+2} and Na^+ concentrations causes a hyperpolarization of neuronal cell membrane and consequently the release of more neurotransmitters such as acetylcholine (ACh). We believe the increased AChE activity might be a compensatory response to these biochemical events. Activation of AChE leads to a rapid degradation of ACh, an important neurotransmitter associated with learning and memory, suggesting that *T. evansi* can promote a dysfunction in the synapse, affecting the modulation of cholinergic neurotransmission.

Studies have shown that Na^+, K^+-ATPase might play a relevant role in neuronal and synaptic plasticity (Brunelli *et al*. 1997; Glushchenko and Izvarina, 1997; Scuri *et al*. 2007) and mediate the modulation of learning and memory (Brunelli *et al*. 1997; Wyse *et al*. 2004; Moseley *et al*. 2007). Our results demonstrated that *T. evansi* infection decreases Na^+, K^+-ATPase activity in cerebral cortex and hippocampus at 5 dpi. The inhibition of Na^+, K^+-ATPase activity and expression has been associated in rats with learning and memory impairment of different behavioural tasks (dos Reis *et al*. 2002; Moseley *et al*. 2007). This evidence corroborates our results that animals infected with *T. evansi* showed memory deficits, possibly due to changes in the Na^+, K^+-ATPase and AChE activities.

Also, an increase in brain Ca^{+2}-ATPase activity in rats infected by *T. evansi* occurs at 5 dpi that may increase the intracellular Ca^{+2}, because the enzyme participates in Ca^{+2} sequestration in cells (Kraus-Friedmann, 1990; Hartmann *et al*. 1994; Hanahisa and Yamaguchi, 1998). The increase of Ca^{+2}-ATPase activity may raise calcium content in brain tissues. This may have a pathophysiological impact in brain function and disturbances of cognitive functions (Magnoni *et al*. 1991; Hartmann *et al*. 1994). *Trypanosoma evansi* infection increases NTPDase activity (ATP and ADP as substrate) in the cerebral cortex (Oliveira *et al*. 2011; Da Silva *et al*. 2012*b*). This study found increased ATP concentration in serum and cerebral cortex. The increase in ATP level was correlated to the inflammatory response and neurotoxicity, since it is an important neurotransmitter (Edwards *et al*. 1992; Agresti *et al*. 2005). In *T. evansi* infection the increased enzymatic activity may be associated with the elevated release of ATP (Oliveira *et al*. 2011; Da Silva *et al*. 2012*b*), which promotes an increase in the levels of intracellular calcium mediated by P2X receptors, and this event could represent significant damage to the cells (Edwards *et al*. 1992). Consequently, the disturbance of brain Ca^{+2} homeostasis may play a pivotal role in brain disease.

On the other hand, different neurochemical parameters and behaviour changes occur with *T. evansi* infected rats at 30 dpi. With the chronicity of the disease, decrease in step-down latency in the inhibitory avoidance test was not significant ($P<0{\cdot}6$), but rats entered the open arm of the elevated maze less frequently and spent less time in it, indicating an anxiogenic-like behaviour in infected animals.

At 30 dpi lower glutamate uptake in the cerebral cortex slices of rats was observed, with normal parameters in the hippocampus slices. Considering glutamate uptake by astrocytes is the main process involved in pathophysiological neuroprotection against glutamatergic excitotoxicity, by reducing the extracellular glutamate concentrations below toxic levels, this inhibitory effect caused by *T. evansi* suggests that infection has excitotoxic properties in cerebral cortex. In addition, it is possible that the reduction in glutamate uptake is mediated by the reduction in Na^+, K^+-ATPase activity caused by *T. evansi*, leading to increased extracellular glutamate concentrations and promoting excitotoxicity. Thus, a reduction in glutamate uptake and Na^+, K^+-ATPase activity may act synergistically and cooperate to induce brain damage in animals infected by this protozoon.

It is important to note that inhibited glutamate uptake (the mechanism that removes glutamate from the extracellular fluid) leads to an increase in extracellular glutamate levels (Danbolt, 2001). Considering glutamate as the major mediator of excitatory signals and that it is probably involved in most aspects of normal brain function including cognition, memory and learning (Fonnum, 1984; Collingridge and Lester, 1989; Headley and Grillner, 1990) the inhibited glutamate uptake could, temporarily, be improving the memory in animals infected by *T. evansi* at 30 dpi.

An increase of glutamate content in the synaptic cleft can activate glutamate receptors, including

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the NMDA receptor. The Ca^{+2} currents of NMDA receptor are responsible for production of nitric oxide (NO) by NO synthase in neurons (Sattler *et al*. 1999; Prast and Philippu, 2001). Our research group has demonstrated that infection by *T. evansi* increases NO in cerebral cortex and hippocampus of rats 20 days after infection (Paim *et al*. 2011*a*). It has been shown that NO can inhibit the activity of the enzyme Na^+, K^+-ATPase, either directly by the action of reactive species, or indirectly through cGMP/PKG pathways signalling (Boldyrev *et al*. 2003, 2004; Carvalho *et al*. 2012). One possible mechanism related to the decrease of Na^+, K^+-ATPase activity in *T. evansi* infected rats is the increase of NO content (Paim *et al*. 2011*a*).

Neurotransmitter systems have an integrated communication system in the brain. *Trypanosoma evansi* infection could affect multiple neurotransmitter systems to influence behaviour. The AChE activity measured in the CNS has been extensively studied not only because it is involved in cholinergic neurotransmission (Soreq and Seidman, 2001; Silman and Sussman, 2005) but also because of the deleterious consequences of its inhibition (Lotti, 1995), and its action as a therapeutic target in neurodegenerative diseases (Rakonczay, 2003). Here and previous studies, we demonstrate that *T. evansi* infection increasing the AChE activity at 30 dpi influences cholinesterases, as indicated by changes in the responses of the cholinergic system. This change could be interfering with cholinergic function and result in disruption of memory and cognitive performance in animals.

In conclusion, the present study establishes a cognitive impairment in rats infected with *T. evansi*. Memory deficit was demonstrated by the performance of these animals in an inhibitory avoidance test. With the chronicity of the disease disease animals showed anxiety symptoms. The inhibition of cerebral Na^+, K^+-ATPase activity, AChE and glutamate uptake could be involved in *T. evansi* cognitive impairment. Additional investigations are necessary to determine the neurochemical mechanisms involved in the effect of *T. evansi* on neurotransmitter systems.

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