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Histopathological characterization of *Toxocara canis*- and *T. cati*-induced neurotoxocarosis in the mouse model

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Abstract

Infective larvae of *Toxocara canis* and *T. cati*, the common roundworms of dogs and cats, may invade the central nervous system of paratenic hosts, including humans, causing neurotoxocarosis (NT). Previous studies on NT in the model organism "mouse" have indicated distinct differences between *T. canis* and *T. cati* regarding larval migration patterns as well as the severity of clinical symptoms and behavioural alterations. The objective of the present study was to provide an extensive characterization of the underlying histopathological alterations, comparing *T. canis*- and *T. cati*-induced changes in different brain areas over the course of murine infection. Four histological sections of five brains each of *T. canis*- and *T. cati*-infected as well as uninfected C57Bl/6 mice were investigated 7, 14, 28, 42, 70 and 98 days post infection (dpi), while brains of *T. cati*-infected and control mice were also available 120 and 150 dpi. In addition to haematoxylin-eosin and luxol fast blue-cresyl violet staining, immunohistochemistry was employed to study microglia/macrophage cell morphology and to detect accumulation of β-amyloid precursor protein (β-APP) as an indicator of axonal damage. Haemorrhages, eosinophilic vasculitis and activated microglia/macrophages were detected in both infection groups starting 7 dpi, followed by eosinophilic meningitis in cerebra as from 14 dpi. Overall, little differences in the proportion of animals affected by these alterations were found between the two infection groups. In contrast, the proportion of animals displaying β-APP accumulation was significantly higher in the *T. canis* than *T. cati* group as from 28 dpi regarding the cerebrum as well as at 98 dpi regarding the cerebellum. In *T. canis*-infected mice, myelinophagic microglia/macrophages ("gitter cells") appeared as from 14 dpi, whereas these were first observed at 70 dpi in *T. cati*-infected animals. The proportion of animals displaying demyelination and/or gitter cells in the cerebrum was significantly higher in the *T. canis* than *T. cati* group as from 28 dpi, and at 28 and 42 dpi regarding the cerebellum. Earlier and more severe neurodegeneration during *T. canis*- than *T. cati*-induced NT, especially in the cerebrum, may explain the differences in behavioural alterations observed in previous studies. In addition to differences in larval migration preferences, immunological processes may contribute to these patterns, which warrant further investigation.

Keywords Neurotoxocarosis · Demyelination · Neurodegeneration · Zoonosis · Roundworms

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Introduction

Zoonotic helminths of the genus *Toxocara*, the common roundworms of dogs and cats, may cause several forms of disease in humans. These parasites are highly prevalent worldwide, with serological evidence of infection in humans ranging from 2.4% in Denmark (Stensvold et al. 2009) to more than 90% in tropical countries (Magnaval et al. 1994). Humans become infected by ingesting embryonated *Toxocara canis* or *Toxocara cati* eggs via contaminated soil, food or water, or infective larvae contained in raw or undercooked meat of paratenic hosts. After ingestion, the third-stage larvae (L3) hatch, penetrate the intestinal wall and are transported to different tissues via the bloodstream,

where they can remain viable for several years (Strube et al. 2013). As a consequence, the clinical pictures of *larva migrans visceralis* (LMV), *larva migrans ocularis* (OLM; ocular toxocarosis [OT]), covert toxocarosis (CT) or—upon invasion of the central nervous system (CNS)—neurotoxocarosis (NT) may develop (Strube et al. 2013). In humans, *Toxocara*-neuroinfection may cause eosinophilic meningitis, encephalitis and myelitis, accompanied by a wide spectrum of neurological symptoms such as headache, paresis, seizures and behavioural disorders (Fan et al. 2015). However, asymptomatic CNS infection is probably more common (Sánchez et al. 2018). Nevertheless, an association of *Toxocara*-seropositivity with reduced cognitive function in children and young adults has been described (Walsh and Haseeb 2012; Erickson et al. 2015) and a possible silent progression to neurodegenerative disorders like Idiopathic Parkinson's disease and Alzheimer's disease is being discussed (Fan et al. 2015).

Most cases of human NT have been attributed to infection with *T. canis* based on larval migration behaviour. Indeed, *T. canis* larvae show a higher affinity towards the CNS than *T. cati* larvae in the mouse model (Janecek et al. 2014). However, human NT due to *T. cati* infection has recently been described (Fukae et al. 2012). Since a large proportion of *Toxocara* eggs found in public places may originate from *T. cati* (Shimizu 1993; Otero et al. 2018), the role of *T. cati* as a zoonotic infectious agent may currently be underestimated (Holland 2015).

In mice, which are considered a suitable model of human NT, distinct differences between *T. canis*- and *T. cati*-induced NT have been described. In addition to their overall stronger neuroaffinity, *T. canis* larvae display a stronger preference for the cerebrum than *T. cati* larvae, which mainly migrate to the cerebellum upon neuroinvasion (Janecek et al. 2014). Behavioural alterations such as reduced exploration behaviour and impaired memory function as well as learning ability due to *T. canis* infection have been observed in several studies (Dolinsky et al. 1985; Holland and Cox 2001; Hamilton et al. 2006). When directly comparing *T. canis*- and *T. cati*-induced NT in C57Bl/6 mice, Janecek et al. (2017) observed an earlier onset and more severe behavioural alterations, such as sensorimotoric impairment, during *T. canis*- than *T. cati*-induced NT. Stereotypical circling was only observed in *T. canis*-infected mice, indicating severe neurological involvement, whereas *T. cati*-infected mice rather displayed reduced excitability and flight behaviour. Indeed, previous studies indicate that *T. canis* infection causes more severe structural damage to the brain than *T. cati* infection (Heuer et al. 2015). Nevertheless, impaired memory function was observed in both infection groups, although it occurred later during *T. cati*-induced NT (Janecek et al. 2017). Apart from more severe structural brain damage in *T. canis*- than *T. cati*-infected mice, observed behavioural differences may be due to differential involvement of certain brain areas in *T. canis*- vs. *T. cati*-induced NT, and a detailed comparative analysis of the induced histopathological changes has not been available to date.

Therefore, the objective of this study was to provide an extensive histopathological characterization of NT in the mouse model, comparing *T. canis*- and *T. cati*-induced changes in different brain areas over the course of infection. In addition to haematoxylin and eosin (H&E) and luxol fast blue-cresyl violet (LFB) staining, immunohistochemistry was carried out to study microglia/macrophage cell morphology by staining the Iba1-protein and to detect accumulation of β-amyloid precursor protein (β-APP) as an indicator of axonal damage.

Materials and methods

Animals and experimental procedure

Brains analysed in this study originated from C57Bl/6JRccHsd mice (Envigo, Huntingdon, UK) used in previously published *Toxocara*-infection experiments (Heuer et al. 2015; Janecek et al. 2015; Janecek et al. 2017), which were permitted by the ethics commission of the Institutional Animal Care and Use Committee (IACUC) of the German Lower Saxony State Office for Consumer Protection and Food Safety (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit) under reference numbers 33.14-42502-04-12/0790, 33.9-42502-04-13/1080 and 33.12-42502-04-15/1869. *T. canis* and *T. cati* eggs for the infection experiments in mice were obtained from faeces of experimentally infected dogs and cats (dog/cat infection permitted under reference number 33.9-42502-05-01A038) and purified by a combined sedimentation-flotation technique. After egg embryonation at 25 °C for 4–5 weeks, mice were infected orally with 2000 *T. canis* or *T. cati* eggs, respectively, in a total volume of 0.5 ml tap water. The control group received the vehicle (tap water) only. Maintenance of mice included a 12/12 h dark/light cycle and a standard rodent diet ad libitum. Mice were sacrificed by cervical dislocation at days 7, 14, 28, 42, 70 and 98 post infection (pi). Five brains per group and time point were analysed in the present study. In addition, five brains each of the control group and the *T. cati*-infected group were available from day 120 and 150 pi. The difference in duration of trials was based on severe progression of clinical symptoms and resulting ethical concerns in *T. canis*-infected mice, but delayed symptoms in *T. cati*-infected mice (Heuer et al. 2015).

Histology

Brains were cut coronally at the bregma zero mark, bregma -3 mm and bregma -7 mm. The four resulting segments (cranial and caudal cerebrum, cranial and caudal cerebellum) of each

brain were fixed in 10% phosphate-buffered formalin (Roti®-Histofix; Carl Roth, Karlsruhe, Germany) and embedded in paraffin wax. Histological sections were stained with H&E and LFB.

Immunohistochemistry (IHC) for Iba-1 and β-APP was carried out using a modified avidin-biotin-peroxidase (ABC) method (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) in combination with the chromogen contained in the Dako EnVision+ System-HRP (DAB) kit (Agilent Technologies, Santa Clara, CA, USA). Antigen retrieval was performed by immersing the sections in citrate buffer (pH 6.0) at 70 °C overnight. Inactivated goat serum was used for blocking at a 1:5 dilution in phosphate-buffered saline (PBS). Primary antibodies were used as follows: mouse anti-β-APP monoclonal antibody (Merck Millipore, Darmstadt, Germany) at a 1:2000 dilution in PBS supplemented with 1% bovine serum albumin (PBS-BSA) and rabbit anti-Iba1 polyclonal antibody (Wako Chemicals GmbH, Neuss, Germany) in a 1:500 dilution in PBS-BSA. Secondary antibodies were biotinylated goat anti-mouse and goat anti-rabbit antibodies (Vector Laboratories, Burlingame, CA, USA) diluted 1:200 in PBS. Negative control sections were incubated with naïve mouse (diluted 1:1000 in PBS-BSA) or rabbit (diluted 1:3000 in PBS-BSA) serum, respectively, instead of the primary antibody.

Microgliosis and microglia/macrophage activation was evaluated by examining density and morphology of Iba-1-stained cells, as described e.g. in Boche et al. (2013).

Statistical analyses

For each time point pi, the proportion of animals displaying haemorrhages/haemosiderophages, eosinophilic inflammatory infiltrates, microglia/macrophage activation, demyelination/presence of gitter cells and β-APP-positive axons in the cerebrum and in the cerebellum, respectively, was compared between the *T. canis* and *T. cati* group using Fisher's exact tests in R v. 3.3.1 (R Core Team 2018). Results were considered significant at $P \leq 0.05$.

Results

Numbers of mice and detailed histopathological changes observed in different brain areas in the *Toxocara*-infection groups compared with the uninfected control group over the course of infection are displayed in Table 1 (cerebellum) and Table 2 (cerebrum). Control mice displayed no histopathological abnormalities, with the exception of one individual showing unilateral, focal eosinophilic meningitis between the thalamus and hippocampus, unilateral eosinophilic perivascular cuffing in the hippocampus and minor, unilateral activation of microglia/macrophages as well as focal accumulation of a few gitter cells in the anterior commissure, but no abnormalities with regard to the cerebellum and no evidence of *Toxocara* larvae.

In *T. canis*-infected animals, larvae were visible in histological sections as from day 7 pi (Fig. 1a), while they were noted in the *T. cati* infection group from day 14 pi. In addition, cerebra and cerebella of both infection groups showed haemorrhages (Fig. 1b) from day 7 pi and haemosiderophages (Fig. 1c) from day 14 pi. At day 42 pi, significantly more *T. canis*-infected mice displayed evidence of haemorrhages in the cerebrum than *T. cati*-infected mice (Fisher's Exact test, $P = 0.048$, Fig. 2a), whereas no statistically significant differences were found for the other time points pi or with regard to the cerebellum.

Perivascular lymphocytic cuffs involving neutrophilic and eosinophilic granulocytes (Fig. 1d) were observed in the cerebrum as well as the cerebellum of a single *T. cati*-infected animal already on day 7 pi (Fig. 2c, d; Tables 1 and 2). From day 14 pi on until the end of the experiment, perivascular cuffs as well as eosinophilic meningitis (Fig. 1e) were noted in the majority of animals in both infection groups (Tables 1 and 2). Furthermore, microglia/macrophage activation (Fig. 1f, g) was also evident in both infection groups from day 7 pi (Fig. 2e, f). No statistically significant differences were found between both infection groups regarding the proportion of animals displaying granulocytic inflammatory changes and activation of microglia/macrophages, neither regarding the cerebrum nor the cerebellum (Fisher's exact test, $P > 0.05$, Fig. 2c–f). Haemorrhages, inflammatory infiltrates and perivascular cuffs containing eosinophilic granulocytes as well as activation of microglia/macrophages were observed in all studied brain areas of *Toxocara*-infected mice (Tables 1 and 2).

Degenerative changes were mainly observed in the cerebellar white matter (*arbor vitae*/cerebellar nuclei) and cerebral fibre tracts (*corpus callosum*, fornix, corticospinal tract and anterior commissure). Axonal damage, indicated by β-APP accumulation (Fig. 3a), occurred from day 7 pi in *T. canis*-infected mice. This was noted in the cerebrum as well as in the cerebellum throughout the course of infection, affecting the majority of *T. canis*-infected animals from day 28 pi (Tables 1 and 2). In the cerebrum of *T. cati*-infected mice, β-APP-positive axons were only found in three animals in a focal pattern in conjunction with haemorrhages on day 7 pi, but not at any later time point during the course of infection. In contrast, in the cerebellum of *T. cati*-infected mice, β-APP-positive axons were noted sporadically over the entire course of infection. The proportion of animals displaying β-APP accumulation was significantly higher in *T. canis*- than *T. cati*-infected mice on days 28, 42, 70 (Fisher's Exact test, $P = 0.008$ each) and 98 pi ($P = 0.048$) regarding the cerebrum as well as on day 98 pi regarding the cerebellum ($P = 0.008$, Fig. 2g, h).

Table 1 Number of animals displaying histopathological changes in different regions of the cerebrum in *Toxocara canis*- and *Toxocara cati*-infection groups compared with the uninfected control group. Shading indicates the number of affected animals in each group. Italic written numbers indicate that sections from < 5 individuals could be analysed

Brain area and histopathology	Control group (dpi)								*T. canis*-infection (dpi)						*T. cati*-infection (dpi)							
	7	14	28	42	70	98	120	150	7	14	28	42	70	98	7	14	28	42	70	98	120	150
Eosinophilic meningitis	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	3/5	5/5	3/5	3/5	1/5	0/5	4/5	4/5	4/5	4/5	5/5	5/5	5/5
Cerebral cortex																						
Presence of larvae	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	3/5	3/5	1/5	1/5	2/5	3/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Haemorrhages/ haemosiderophages	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	3/5	2/5	3/5	3/5	5/5	3/5	1/5	2/5	2/5	0/5	2/5	1/5	3/5	5/5
Eosinophilic perivascular cuffs	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	3/5	3/5	3/5	4/5	1/5	0/5	1/5	0/5	1/5	1/5	0/5	0/5	0/5
Inflammatory infiltrates	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	3/5	2/5	1/5	1/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5
Vacuolisation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	1/5	2/5	0/5	0/5	0/5	1/5	0/5	0/5	1/5	0/5	0/5
Demyelination	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Presence of „gitter cells“	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	1/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Activated microglia	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	4/5	5/5	5/5	5/5	4/5	5/5	3/5	3/5	1/5	3/5	0/5	0/5	0/5	1/5
APP-positive axons	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	1/5	0/5	0/5	1/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Cerebral fiber tracts[a]																						
Presence of larvae	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	3/5	3/5	4/5	5/5	2/5	0/5	0/5	0/5	0/5	0/5	1/5	1/5	0/5
Haemorrhages/ haemosiderophages	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	0/5	0/5	1/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5
Eosinophilic perivascular cuffs	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	2/5	2/5	3/5	3/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	1/5
Inflammatory infiltrates	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	3/5	2/5	1/5	1/5	3/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Vacuolisation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	3/5	3/5	3/5	4/5	3/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Demyelination	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	1/5	4/5	4/5	5/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Presence of „gitter cells“	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	1/5	4/5	4/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Activated microglia	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	4/5	5/5	5/5	5/5	5/5	5/5	1/5	2/5	2/5	2/5	1/5	4/5	5/5	3/5
APP-positive axons	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	2/5	5/5	5/5	5/5	4/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Hippocampus																						
Presence of larvae	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	2/5	0/5	0/5	0/5	1/5	*0/3*	*0/4*	*0/4*	0/5	0/5	1/5	0/5	0/5
Haemorrhages/ haemosiderophages	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	1/5	1/5	0/5	*0/3*	*0/4*	*0/4*	0/5	0/5	1/5	0/5	1/5
Eosinophilic perivascular cuffs	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	2/5	3/5	4/5	0/5	1/5	*0/3*	*0/4*	*0/4*	2/5	1/5	2/5	3/5	2/5
Inflammatory infiltrates	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	1/5	1/5	0/5	*0/3*	*0/4*	*0/4*	0/5	0/5	1/5	1/5	0/5
Vacuolisation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	*0/3*	*0/4*	*0/4*	0/5	0/5	0/5	0/5	0/5
Demyelination	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	*0/4*	0/5	0/5	0/5	*0/3*	*0/4*	*0/4*	0/5	0/5	0/5	0/5	0/5
Presence of „gitter cells“	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	1/5	*0/3*	*0/4*	*0/4*	0/5	0/5	0/5	0/5	0/5
Activated microglia	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	4/5	*4/4*	*4/4*	4/5	3/5	3/5	*0/3*	*2/3*	*3/3*	*2/4*	3/5	*2/4*	5/5	5/5
APP-positive axons	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	*0/4*	0/5	0/5	0/5	*0/4*	*0/4*	*0/4*	0/5	0/5	0/5	0/5	0/5
Thalamus/hypothalamus																						
Presence of larvae	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	1/5	0/5	1/5	1/5	2/5	0/5	0/5	1/5	0/5	0/5	0/5	1/5	0/5
Haemorrhages/ haemosiderophages	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Eosinophilic perivascular cuffs	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	3/5	4/5	2/5	3/5	0/5	0/5	0/5	2/5	2/5	3/5	1/5	2/5
Inflammatory infiltrates	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	1/5	0/5	2/5	0/5	0/5	0/5	0/5	0/5	1/5	1/5	1/5
Vacuolisation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	3/5	0/5	4/5	3/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5
Demyelination	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	1/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Presence of „gitter cells“	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	1/5	4/5	3/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5
Activated microglia	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	4/5	4/5	5/5	4/5	5/5	5/5	2/5	3/5	3/5	2/5	4/5	5/5	4/5	3/5
APP-positive axons	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	3/5	2/5	4/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Midbrain																						
Presence of larvae	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	0/5	1/5	1/5	3/5	3/5	*0/3*	*0/4*	*0/4*	0/5	0/5	1/5	1/5	0/5
Haemorrhages/ haemosiderophages	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	4/5	0/5	1/5	0/5	2/5	1/5	*0/3*	*0/4*	*1/4*	0/5	0/5	0/5	0/5	0/5
Eosinophilic perivascular cuffs	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	3/5	3/5	2/5	1/5	1/5	*1/3*	*0/4*	*0/4*	1/5	0/5	2/5	1/5	0/5
Inflammatory infiltrates	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	*0/3*	*0/4*	*1/4*	0/5	0/5	2/5	0/5	0/5
Vacuolisation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	3/5	3/5	4/5	3/5	*0/3*	*0/4*	*1/4*	0/5	0/5	2/5	1/5	0/5
Demyelination	0/5	0/5	0/5	*0/4*	0/5	0/5	0/5	0/5	*0/4*	0/5	*1/4*	1/5	2/5	0/5	*0/3*	*0/4*	*0/4*	0/5	0/5	0/5	*0/4*	0/5
Presence of „gitter cells“	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	2/5	5/5	2/5	*0/3*	*0/4*	*0/4*	0/5	0/5	0/5	0/5	0/5
Activated microglia	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	*3/4*	*4/4*	5/5	5/5	4/5	*1/3*	*1/3*	*3/3*	*2/4*	3/5	*2/4*	5/5	3/5
APP-positive axons	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	*2/4*	1/5	4/5	2/5	*0/4*	*0/4*	*0/4*	0/5	0/5	0/5	0/5	0/5
Cerebral nuclei																						
Presence of larvae	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	2/5	3/5	2/5	3/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Haemorrhages/ haemosiderophages	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	1/5	1/5	1/5	1/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5
Eosinophilic perivascular cuffs	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	1/5	1/5	3/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	0/5
Inflammatory infiltrates	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Vacuolisation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	1/5	0/5	3/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5
Demyelination	0/5	0/5	0/5	*0/4*	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	*0/3*	0/5
Presence of „gitter cells“	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	3/5	3/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Activated microglia	0/5	0/5	0/5	*0/4*	*0/4*	0/5	0/5	0/5	2/5	3/5	4/5	4/5	5/5	3/5	2/5	0/5	3/5	0/5	0/5	0/5	*1/3*	0/5
APP-positive axons	0/5	0/5	0/5	0/5	*0/4*	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	0/5	2/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5

[a]Cerebral fiber tracts: *Corpus callosum*, corticospinal tract, fornix, anterior commissure

Table 2 Number of animals displaying histopathological changes in different regions of the cerebellum in *Toxocara canis*- and *Toxocara cati*-infection groups compared with the uninfected control group. Shading indicates the number of affected animals in each group. Italic written numbers indicate that sections from < 5 individuals could be analysed

Brain area and histopathology	Control group (dpi)								*T. canis*-infection (dpi)						*T. cati*-infection (dpi)							
	7	14	28	42	70	98	120	150	7	14	28	42	70	98	7	14	28	42	70	98	120	150
Eosinophilic meningitis	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	2/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	2/5	4/5
Cerebellar cortex																						
Presence of larvae	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	3/5	3/5	3/5	3/5	2/5	2/5	0/5	1/5	2/5	1/5	0/5	1/5	1/5	0/5
Haemorrhages/ haemosiderophages	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	4/5	2/5	2/5	3/5	4/5	2/5	1/5	2/5	2/5	2/5	3/5	3/5	4/5	3/5
Perivascular cuffs	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	3/5	2/5	1/5	0/5	0/5	2/5	2/5	0/5	0/5	0/5	0/5	0/5
Inflammatory infiltrates	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	3/5	1/5	1/5	2/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	1/5
Vacuolisation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	1/5	0/5	3/5	3/5	2/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
Demyelination	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	*0/4*
Presence of „gitter cells“	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	4/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Activated microglia	0/5	*0/4*	0/5	0/5	0/5	0/5	*0/4*	0/5	3/5	5/5	4/5	4/5	4/5	5/5	3/5	3/5	3/5	3/5	2/5	3/5	3/5	3/5
APP-positive axons	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Cerebellar nuclei/arbor vitae																						
Presence of larvae	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	3/5	4/5	4/5	4/5	3/5	4/5	0/5	1/5	2/5	0/5	2/5	1/5	2/5	2/5
Haemorrhages/ haemosiderophages	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	1/5	1/5	0/5	0/5	0/5	0/5	0/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5
Perivascular cuffs	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	0/5	5/5	5/5	4/5	3/5	4/5	0/5	2/5	1/5	4/5	4/5	4/5	4/5	4/5
Inflammatory infiltrates	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	0/5	2/5	3/5	3/5	3/5	2/5	1/5	0/5	0/5	0/5	2/5	2/5	0/5	1/5
Vacuolisation	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	0/5	5/5	4/5	5/5	5/5	4/5	0/5	1/5	2/5	0/5	4/5	5/5	2/5	4/5
Demyelination	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	0/5	3/5	4/5	3/5	5/5	5/5	0/5	0/5	0/5	0/5	2/5	0/5	*0/4*	2/5
Presence of „gitter cells“	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	0/5	2/5	5/5	5/5	5/5	5/5	0/5	0/5	0/5	0/5	2/5	2/5	0/5	4/5
Activated microglia	*0/4*	*0/4*	0/5	0/5	0/5	0/5	*0/3*	*0/4*	3/5	5/5	5/5	5/5	5/5	5/5	3/5	4/5	5/5	4/5	*4/4*	5/5	*3/3*	*4/4*
APP-positive axons	*0/4*	0/5	0/5	0/5	*0/4*	0/5	*0/4*	*0/4*	3/5	1/5	*3/4*	4/5	5/5	5/5	0/5	0/5	*1/4*	0/5	*1/4*	0/5	*0/4*	1/5
Medulla																						
Presence of larvae	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	2/5	2/5	4/5	4/5	5/5	3/5	0/5	0/5	0/5	0/5	0/5	0/5	4/5	2/5
Haemorrhages/ haemosiderophages	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	3/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	1/5	0/5	1/5
Perivascular cuffs	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	0/5	3/5	4/5	3/5	0/5	2/5	0/5	2/5	0/5	0/5	1/5	2/5	2/5	2/5
Inflammatory infiltrates	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	0/5	0/5	1/5	1/5	0/5	2/5	0/5	0/5	1/5	0/5	0/5	0/5	1/5	2/5
Vacuolisation	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	0/5	1/5	3/5	2/5	5/5	3/5	0/5	0/5	1/5	0/5	0/5	2/5	2/5	4/5
Demyelination	0/5	0/5	0/5	*0/4*	0/5	0/5	*0/4*	0/5	*0/4*	0/5	1/5	1/5	2/5	2/5	0/5	0/5	0/5	0/5	*0/4*	0/5	*0/4*	1/5
Presence of „gitter cells“	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	0/5	1/5	2/5	3/5	5/5	3/5	0/5	0/5	0/5	0/5	0/5	3/5	0/5	4/5
Activated microglia	0/5	0/5	0/5	0/5	0/5	0/5	*0/3*	0/5	3/5	5/5	4/5	5/5	4/5	5/5	3/5	2/5	4/5	4/5	5/5	4/5	4/5	5/5
APP-positive axons	0/5	0/5	0/5	0/5	0/5	0/5	*0/4*	0/5	0/5	1/5	3/5	2/5	5/5	2/5	0/5	1/5	1/5	2/5	1/5	0/5	0/5	0/5

In the cerebrum as well as the cerebellum of *T. canis*-infected mice, microglia/macrophages displaying myelinophagia (“gitter cells”; Fig. 3b) appeared from day 14 pi, whereas these were first noted at day 70 pi in cerebella of *T. cati*-infected animals. In the cerebrum of *T. cati*-infected mice, gitter cells were only noted in one animal at day 98 pi. The proportion of animals displaying demyelination in LFB-stained histological sections (Fig. 3c) and/or presence of gitter cells was significantly higher in *T. canis*- than *T. cati*-infected mice on day 28, 42 (Fisher’s Exact test, $P = 0.048$ each), 70 and 98 pi ($P = 0.008$ each) with regard to the cerebrum, and on day 28 and 42 pi concerning the cerebellum ($P = 0.008$ each, Fig. 2i, j).

In addition to described pathology, cholesterol crystals were observed in the cerebellar nuclei/*arbor vitae* of two *T. canis*-infected mice at day 98 pi and one *T. cati*-infected mouse at day 150 pi.

Discussion

In humans, invasion of *Toxocara* larvae into the CNS may cause meningitis, meningoencephalitis and myelitis, provoking symptoms such as epileptic seizures, sensory and/or motor impairments and loss of cognitive functions (e.g. Kazek et al. 2006; Moiyadi et al. 2007; Scheid et al. 2008). Similar to human NT, histopathological findings in *Toxocara*-infected C57/Bl6 mice in the present study included haemorrhages, eosinophilic inflammation and neurodegenerative processes, confirming previous observations (Summers et al. 1983; Janecek et al. 2014; Heuer et al. 2015). In addition, the present study demonstrated profound differences between *T. canis*- and *T. cati*-infected mice on the histopathological level, in line with differences between both pathogens regarding larval migration patterns, host clinical symptoms and brain transcriptional changes (Janecek et al. 2014; Heuer et al. 2015; Janecek et al. 2015; Janecek et al. 2017).

Evidence of inflammation, e.g. perivascular cuffs involving eosinophilic and neutrophilic granulocytes and activation of microglia/macrophages, was observed as from day 7 pi in *Toxocara*-infected C57Bl/6 mice. In contrast, neither Liao et al. (2008), Othman et al. (2010) nor Eid et al. (2015) observed leucocytic infiltrations in *T. canis*-infected brains of outbred mouse strains (ICR and Swiss albino mice, respectively) examined at several time points up to a maximum of

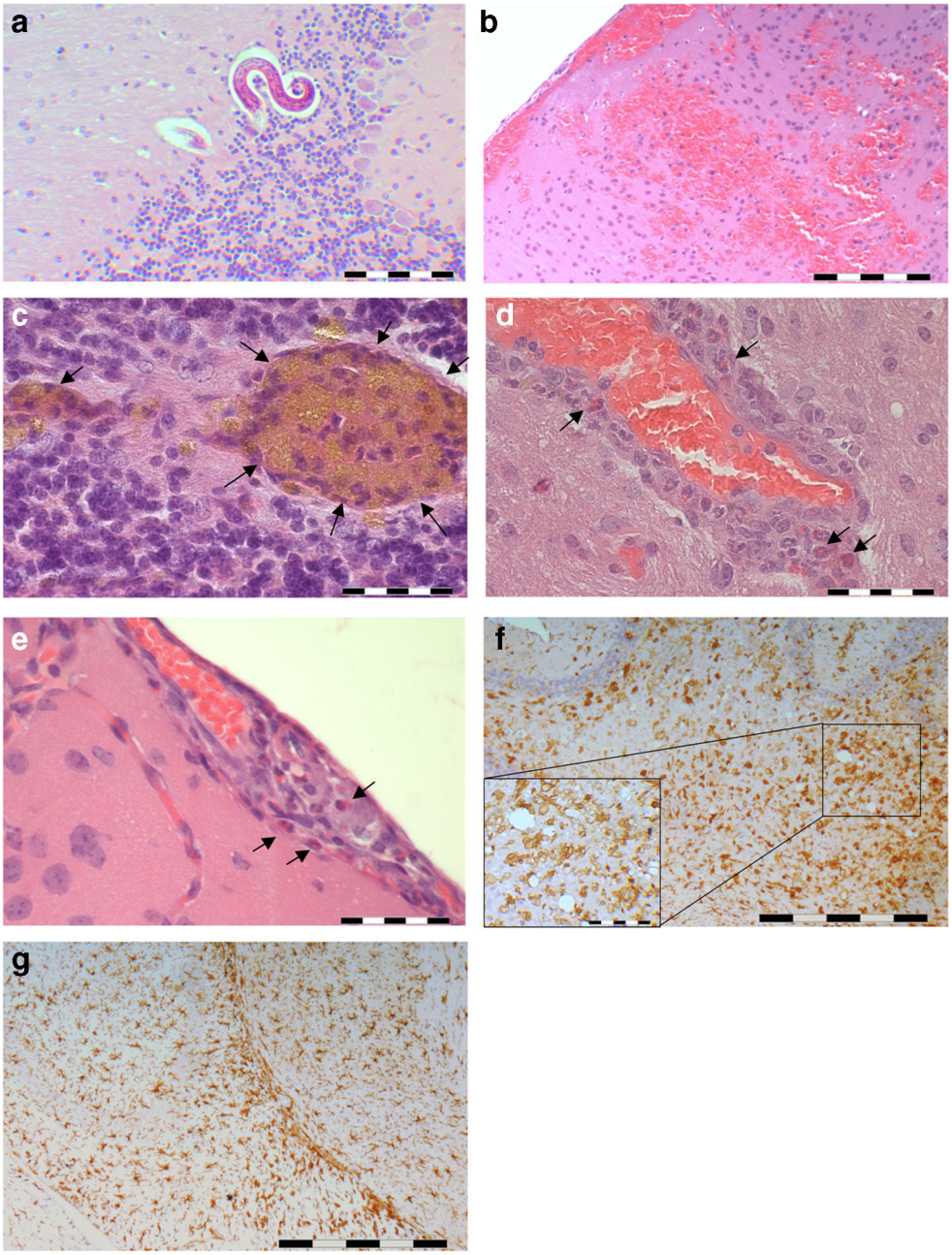

Fig. 1 Larvae, haemorrhages and inflammatory changes observed in brains of *T. canis*- (left) and *T. cati* (right)-infected mice. **a** *T. canis* larva, cerebellum, day 7 p.i., bar = 100 μm. **b** Haemorrhages, cerebral cortex, *T. canis* infection, day 7 pi, bar = 200 μm. **c** Golden-pigmented haemosiderophages (arrows), cerebellar cortex, *T. cati* infection, day 28 pi, bar = 50 μm. **d** Perivascular cuffing involving eosinophilic granulocytes (arrows), cerebellum, *T. canis* infection, day 14 pi, bar = 50 μm. **e** Meningitis involving eosinophilic granulocytes (arrows), cerebrum, *T. cati* infection, day 14 pi, bar = 50 μm. **f** Microgliosis and microglia/macrophage activation, cerebellum, *T. canis* infection, 70 dpi, ABC method, bar = 500 μm. Inset: amoeboid morphology of microglia, bar = 50 μm. **g** Microgliosis and microglia/macrophage activation, cerebrum (hippocampus/thalamus), *T. cati* infection, 70 dpi, ABC method, bar = 500 μm

16 weeks post infection. This might be due to differences between outbred mice and the inbred strain used in the present study, as larval migration behaviour and pathological changes differ between mouse strains, probably due to immunological differences (Epe et al. 1994). Here, C57Bl/6 mice were used because previous studies comparing *T. canis*- and *T. cati*-induced NT have been conducted with this strain (Janecek et al. 2014; Heuer et al. 2015; Janecek et al. 2015; Janecek et al. 2017). It should be kept in mind, however, that the pathological and behavioural changes observed in C57Bl/6 mice may be more severe than in outbred or wild mice. Nevertheless, Liao et al. (2008), Othman et al. (2010) and Eid et al. (2015) observed reactive astrocytes indicating brain injury in infected outbred mouse strains, while no staining to visualize microglia/macrophages was performed in their studies. Furthermore, Liao et al. (2008) reported increased levels of β-APP in *T. canis*-infected mouse brains, similar to observations made via immunohistochemistry in the present study.

Although no significant differences were found between *T. canis* and *T. cati* regarding the proportion of animals affected by meningitis, perivascular cuffs and microglia/macrophage activation, neurodegenerative processes showed an earlier onset and affected a larger proportion of *T. canis*- than *T. cati*-infected mice, especially with regard to the

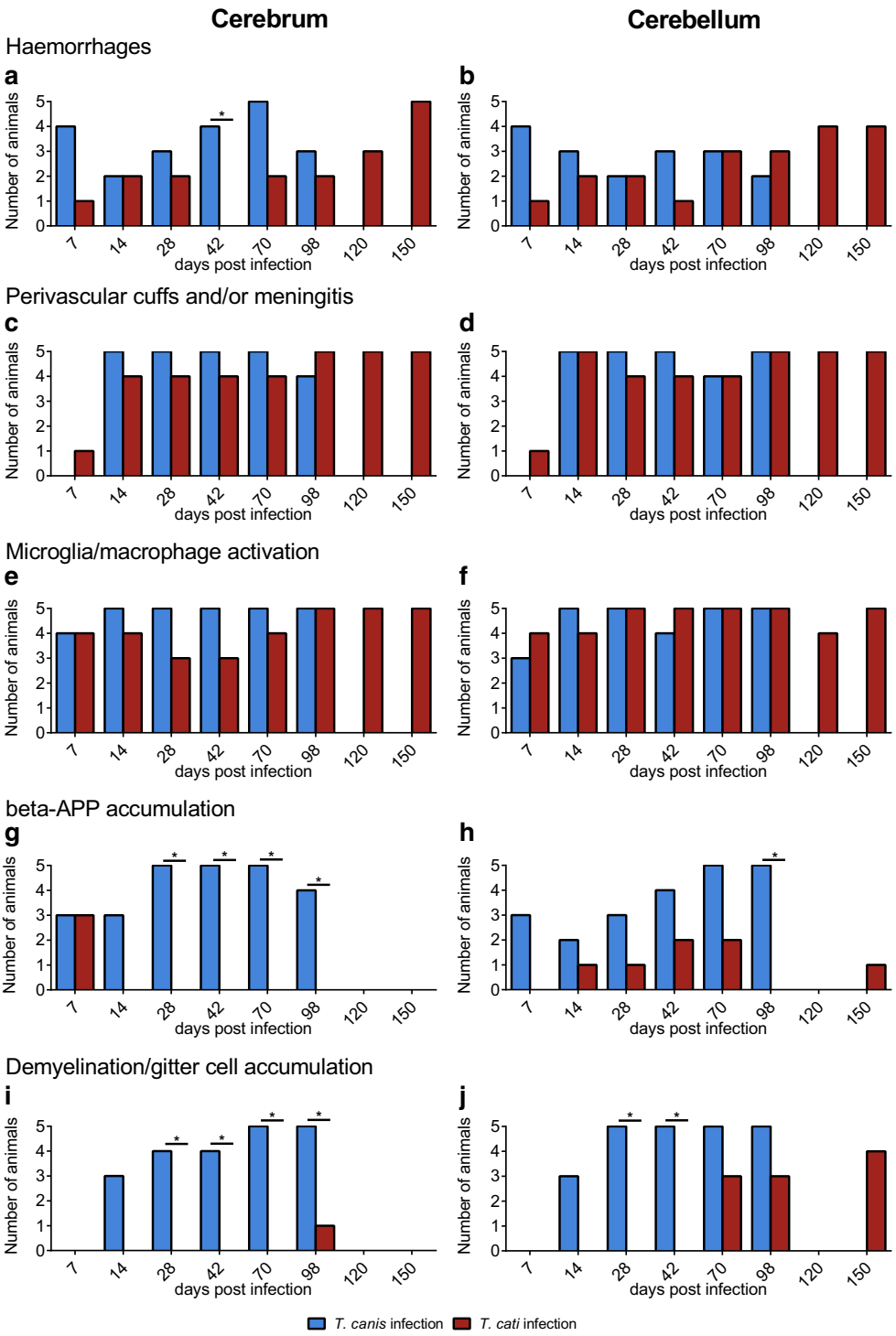

Fig. 2 Proportion of *T. canis*- and *T. cati*-infected animals showing **a**, **b** haemorrhages, **c**, **d** perivascular cuffs and/or meningitis, **e**, **f** microglia/macrophage activation, **g**, **h** β-APP accumulation and **i**, **j** demyelination/gitter cell accumulation in the cerebrum and cerebellum

cerebrum. Specifically, β-APP accumulation in the cerebrum, indicating axonal damage, was only found in the acute stage of infection on day 7 pi in three *T. cati*-infected mice, associated with focal haemorrhages. In contrast, the majority of *T. canis*-infected mice showed β-APP accumulation in the cerebrum throughout the whole course of infection until the end of the study period on day 98 pi. Similarly, accumulation of gitter cells was noted in the cerebral fibre tracts of the majority of *T. canis*-infected mice over the entire course of infection, but was found in the cerebrum of a single *T. cati*-infected mouse only. Even on day 120 and 150 pi, *T. cati*-infected mice did not show β-APP accumulation or gitter cells in the cerebrum, indicating that the difference between both infection groups is not merely due to a delayed onset.

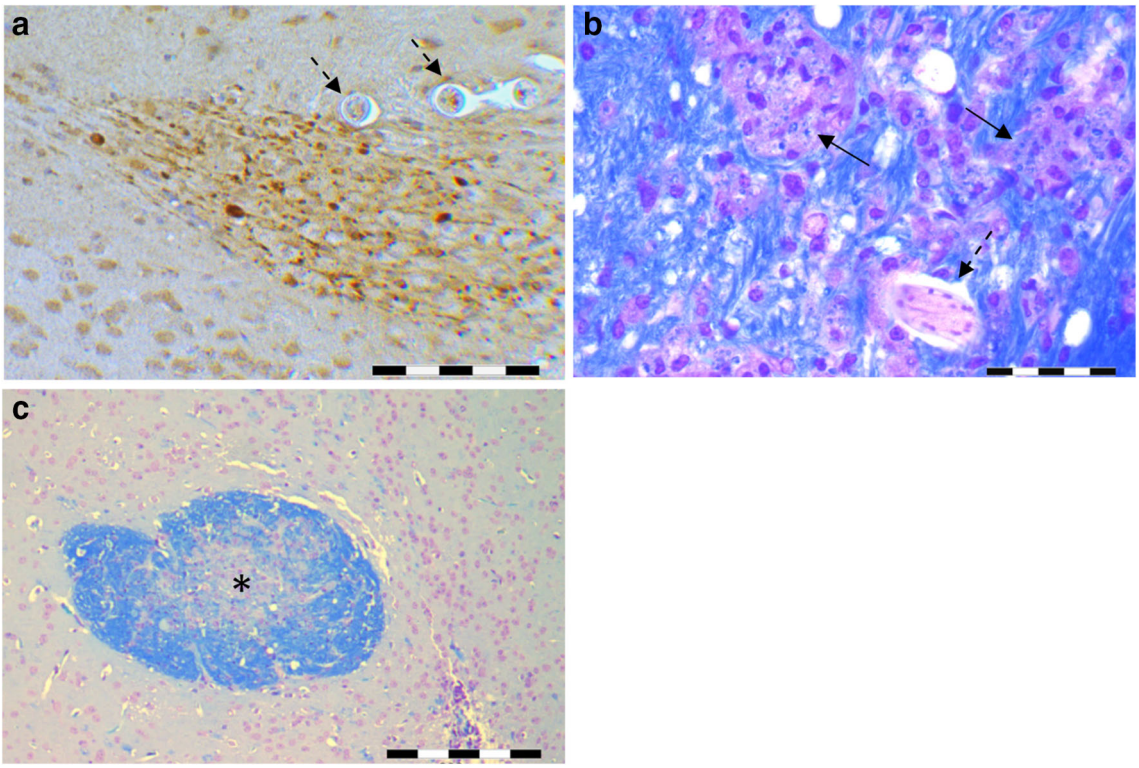

Fig. 3 Degenerative changes observed in brains of *T. canis*-infected mice. **a** β-APP-positive axons, cerebrum (anterior commissure) and *T. canis* larvae (dashed arrows), day 98 pi, ABC method, bar = 100 μm. **b** Gitter cells displaying myelinophagia (solid arrows) and *T. canis* larva (dashed arrow), cerebellum (*arbor vitae*), day 42 pi, LFB stain, bar = 50 μm. **c** Demyelinated area (indicated by asterisk), cerebrum (anterior commissure), day 98 pi, LFB stain, bar = 200 μm

The larger proportion of *T. canis*- than *T. cati*-infected mice displaying neurodegenerative changes in the cerebrum is in line with the observed preference of *T. canis* larvae for this brain area (Janecek et al. 2014). In contrast, enhanced expression of several myelin genes has been noted in both brain areas of *T. canis*- and *T. cati*-infected mice, suggesting that demyelination and remyelination processes in the cerebrum might also be ongoing during *T. cati*-induced NT, despite not being visible in histological slides (Heuer et al. 2015). Alternatively, however, this enhanced myelin gene expression in *T. cati*-infected mice might be due to microglia activation or ischemic processes, since inflammatory cells might stimulate myelin gene expression, as shown in peri-infarct areas during focal cerebral ischemia (Gregersen et al. 2001).

The histologically observed differences in cerebral lesions between *T. canis*- and *T. cati*-infected mice provide a plausible explanation for differences regarding spatial memory tests between both infection groups (Janecek et al. 2017). In cerebra of *T. canis*-infected mice, *Toxocara* larvae and β-APP and gitter cell accumulation were mainly localized in the *corpus callosum*, fornix and anterior commissure. It has been suggested that *Toxocara* larvae preferentially migrate within the white matter because of a nutritional advantage offered by myelin, or simply because this may be the pathway of least resistance through the brain (Summers et al. 1983). Especially the fornix, the major fibre tract linking the medial temporal lobe, including the hippocampus, to the medial diencephalon, plays an important role regarding memory function. In humans, damage to the fornix has been associated with impairment of recall memory (Park et al. 2000; Tsivilis et al. 2008; Vann et al. 2008). In rats as well as in monkeys, experimental transection of the fornix leads to performance deficits in spatial memory tasks (Aggleton et al. 1992; Buckley et al. 2004). Thus, progressive damage to the fornix provides a plausible explanation for the worse performance of *T. canis*-infected mice in a classical maze test as compared to *T. cati*-infected mice (Janecek et al. 2017). This memory impairment was observed from day 20 pi on in *T. canis* infection, which is in line with β-APP accumulation and demyelination in cerebral fibre tracts observed from day 28 pi in the majority of *T. canis*-infected mice in the present study. In humans, parasite-induced damage to cerebral fibre tracts could explain the association between *Toxocara*-seropositivity and deficits in cognitive tests (Walsh and Haseeb 2012; Erickson et al. 2015).

In the cerebellum, which is vital for motor control, neurodegenerative lesions affected fewer animals and occurred later during the course of *T. cati* than *T. canis* infection. Gitter cells in the cerebellar white matter were detected only as from day 70 pi in *T. cati*-infected mice, but already at 14 dpi during *T. canis* infection. In line with these histopathological

observations, Janecek et al. (2017) noted statistically significant motoric impairments much earlier in *T. canis*- than *T. cati*-infected mice (day 41 pi vs. day 83 pi).

Overall, *T. cati* seems to be better adapted to mice than *T. canis*, causing less pathology but reduced flight behaviour (Janecek et al. 2017) and thus facilitating transmission of larvae to the definitive host via predation without the risk of an early death of the paratenic host. The causative mechanisms underlying the differences in neurodegenerative pathology between both infection groups remain the subject of further study. Stronger neuroaffinity and a relative preference of *T. canis* for the cerebrum (Janecek et al. 2014) certainly result in more mechanical damage as compared to *T. cati* infection. On the other hand, immunological processes may play a role. Surprisingly, animals of both infection groups were similarly affected by granulocytic infiltration, involving eosinophils and neutrophils, as well as microglia/macrophage activation in the cerebrum and the cerebellum from day 7 pi on. Like other peripheral macrophages, microglia may have two different activation states. Upon activation via the classical pathway, they acquire the M1 phenotype, characterized by the production of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-12 and tumour necrosis factor (TNF) α, and reactive oxygen species (ROS) (Subramaniam and Federoff 2017). Excess production of these potentially neurotoxic substances may exacerbate neurodegeneration, a process termed reactive microgliosis (Lull and Block 2010). In contrast, the alternative M2 state is associated with immunoregulation and tissue repair (Subramaniam and Federoff 2017). Hypothetically, these two different activation states could be involved in the different pathological outcomes of *T. canis*- and *T. cati*-induced NT. However, a recent study on patterns of cytokine and chemokine concentrations in brains of *Toxocara*-infected C57Bl/6 mice showed no elevation of M1- or M2-specific cytokines in either infection group, but a reduction of most pro-inflammatory cytokines when compared to uninfected controls (Waindok and Strube 2019). However, this study did show a distinct increase in IL-4 and IL-5 levels in *T. canis*-, but not in *T. cati*-infected animals. IL-5 contributes to the recruitment of eosinophils, and less tissue damage was observed in *T. canis*-infected mice genetically deficient for IL-5 compared to mice with the functional IL-5 gene, at least regarding the lungs (Takamoto et al. 1997). Thus, the IL-5 increase might contribute to the observed extent of neurodegeneration during *T. canis* infection. In addition, a microarray gene expression study has shown that genes associated with "lipid/cholesterol biosynthetic processes" were significantly downregulated in *T. canis*- but not *T. cati*-infected mice at 42 dpi (Janecek et al. 2015). Since cholesterol is a vital component of myelin, and downregulation of these genes may lead to cholesterol concentration changes, the authors concluded that this downregulation may contribute to demyelination during *T. canis*-induced NT.

In uninfected control mice, mentioned inflammatory and neurodegenerative changes were not observed. Only one uninfected individual showed focal evidence of eosinophilic meningitis, perivascular cuffing and activation of microglia/macrophages as well as focal accumulation of a few gitter cells in the cerebrum. Due to the fact that only the cerebrum was affected, neurotoxocarosis as a cause of these lesions seems unlikely. Besides parasitic and fungal infections, possible non-infectious causes of meningeal eosinophilic reactions include allergic diseases or idiopathic eosinophilic meningitis, while eosinophils have also been rarely associated with cerebral ischemia and haemorrhages (Graeff-Teixeira et al. 2009).

Conclusions

In conclusion, the present study demonstrates distinct differences in the pattern of neurodegenerative lesions caused by *T. canis* and *T. cati* in mice, despite similarities regarding the recruitment of leucocytes and the activation of microglia/macrophages. Neurodegeneration of cerebral fibre tracts, especially the fornix, during *T. canis*-induced NT may explain the earlier and more severe memory impairment relative to *T. cati*-infected animals observed previously (Janecek et al. 2017). In addition to differences in larval migration preferences, immunological processes may contribute to these patterns, which warrant further investigation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Abbreviations ABC, avidin-biotin-peroxidase; BSA, bovine serum albumin; β-APP, β-amyloid precursor protein; dpi, day post infection; H&E, haematoxylin-eosin; LFB, luxol fast blue-cresyl violet; PBS, phosphate-buffered saline; pi, post infection; NT, neurotoxocarosis

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