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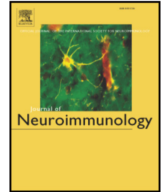

Effect of Chlorovirus ATCV-1 infection on behavior of C57Bl/6 mice

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ABSTRACT

Neuroinflammation induced during immune responses to viral infections in the brain affect behavior. Unexpected evidence that oral gavage of an algal virus in its host algal cells could alter cognition was further examined by directly injecting purified algal virus ATCV-1 intracranially into C57BL/6 mice. After 4 weeks, the ATCV-1 infection impaired delayed location recognition memory, and also reduced and anxiety. Corresponding to these effects, heightened ATCV-1, IL-6, iNOS, IFN-γ, and CD11b expression in brains was observed 3-days and/or 8-weeks post infection compared with control mice. These results imply that ATCV-1 infection damages the hippocampus via induction of inflammatory factors.

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1. Introduction

Behavioral changes after viral infections are not without precedence. However, the observation of a significant relationship between deficits in visual motor speed, delayed memory and attention, and the presence of DNA from Chlorella virus ATCV-1 in oropharyngeal samples from humans was surprising in that algal viruses were not known to infect mammals (Yolken et al., 2014). Recent evidence indicates that ATCV-1 does infect mammalian macrophages (Petro et al., 2015) resulting in inflammatory cytokines, viral persistence, and production of new viruses. In addition to the cognitive effects observed in humans with ATCV-1 DNA, Yolken and associates observed cognitive and behavioral changes in C57BL/6 mice six weeks after being orally gavaged with ATCV-1-infected *Chlorella heliozoae* compared with mice receiving uninfected algae. Mice receiving the ATCV-1 infected algae showed deficits in delayed recognition memory in both object recognition and object location, as well as impaired sensorimotor gating, which is indicative of neurological illnesses (Swerdlow et al., 2001). These data indicate that infection with ATCV-1 affects cognition.

Several cytokines and factors produced during viral infection of macrophages are known to affect cognition. Others and we have shown that during viral infections of the CNS, inflammatory cytokines damage the hippocampus (Howe et al., 2012; Buenz et al., 2006; Poluektova et al., 2005; Moore et al., 2013). Interleukin-6 (IL-6), which is produced normally by pyramidal and granular cells of the hippocampus, impairs learning (Heyser et al., 1997; Sparkman et al., 2006) at the hippocampus (Samuelsson et al., 2006) when it is produced by macrophage lineage cells in excess during neuroinflammation. Likewise, nitric oxide (NO) produced by macrophage lineage cells during neuroinflammation is also associated with memory impairments (Kamat et al., 2012). Therefore, ATCV-1 stimulation of inflammatory macrophages and induction of inflammatory mediators from these cells may be related to certain memory impairments.

The present study aimed to replicate the initial finding and extend further our understanding of the relationship between ATCV-1 infection and cognitive-behavioral changes using a mouse model. As a *proof-of-concept* to evaluate effects of locally produced inflammatory cytokines, ATCV-1 viral particles free of *C. heliozoae* were injected directly into the CNS. Cognitive and behavioral tests were then conducted to examine the effect on specific domains identified in the Research Domain Criteria framework (RDoC; http://www.nimh.nih.gov/research-priorities/rdoc/index.shtml), which was developed to aid in understanding functional relationships between biology and behavior. The results indicate that similar to oral gavage with ATCV-1-infected *C. heliozoae*, intracranial infection with purified ATCV-1 resulted in impairments in delayed location recognition memory and sociability, which

Abbreviations: ATCV-1, *Acanthocystis turfacea* Chlorellavirus-1; iNOS, inducible nitric oxide synthase; i.c., intracranially; RDoC, Research Domain Criteria framework; NOR, novel object recognition; NOL, novel object location; PCNA, proliferating cell nuclear antigen; IFN, interferon; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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occurred in conjunction with expression of ATCV-1 RNA and inflammatory factors.

2. Method

2.1. Animal usage

Three cohorts of C57BL/6 mice (N = 33; Harlan Sprague Dawley) were tested for behavioral and cognitive effects of an algal-infecting Chlorella virus, ATCV-1. In each cohort, eleven female mice (age 10 weeks) were randomly assigned to receive intracranial (i.c.) injections of either 50 μl PBS (n = 5) or 50 μl enriched 5×10^8 ATCV-1 plaque forming units (PFUs) in PBS (n = 6), providing a total of 15 PBS-injected mice and 18 ATCV-1-injected mice. Fifty microliters was injected by punching with a 27 gauge ¼ inch needle 3 mm below the surface to the left approximately 1.0 mm anterior to the bregma and 1.0 mm lateral to the sagittal suture. Following injection, topical lidocaine was administered periodically at the injection site for the first 24 h. We used a female cohort because we and others have used female mice as our research standard, for example with Theiler's virus challenges of C57Bl/6 mice (Moore et al., 2013). Mice were housed (12 h light/dark cycle) two to three animals per standard mouse cage. LabDiet 5010 (PMI Nutrition International, Richmond, Indiana, USA) and water were provided ad libitum. Behavioral testing began at age 14 weeks. During the five days of the Sucrose Preference test, mice were housed individually. Mean weights of mice in each injection condition were equivalent ($M_{PBS} = 21.82$ g, $SE = 0.33$, $M_{ATCV\text{-}1} = 21.19$ g, $SE = 0.30$), $F(1, 27) = 1.98$, $p = 0.17$, $\eta^2_p = 0.07$). All experimental procedures were approved by the Nebraska Wesleyan University Institutional Animal Care and Use Committee (Protocol #01-000015F) and the University of Nebraska Medical Center (Protocol #13-067-09-FC) Institutional Animal Care and Use Committee. After testing for behavioral and cognitive effects, mice were euthanized, whole brains were extracted, and dissociated in lysis buffer for RNA isolation and RNA subjected to RT-qPCR analysis. A separate cohort of mice (n = 5) was also i.c. injected with PBS or ATCV-1 and three days later, brains from individual mice were extracted, inverted, and a region cut from the midbrain to the basal forebrain was placed into lysis buffer for RNA extraction preceding RT-qPCR.

2.2. RNA preparation and qRT-PCR

RNA was extracted from brain tissue with the PureLink total RNA kit from Ambion/Invitrogen (Carlsbad, CA) according to the manufacturer's specifications. One hundred fifty nanograms of RNA was reverse transcribed as we have done before (Petro et al., 2015). The cDNA was diluted 1:2, and 1 μl was incubated with a 0.4 μM concentration of the following primer pairs: IL-6 sense (5′ ATGAAGTTCCTCTC TGCAAGAGACT 3′) and antisense (5′ CACTAGGTTTGCCGAGTAGATCTC 3′), iNOS sense (5′ CCCTTCCGAAGTTTCTGGCAGCAGC 3′) and antisense (5′ GGCTGTCAGAGCCTCGTGGCTTTGG 3′), CD11b sense (CAGATCAACAATGTGACCGTATGGG-3′) and antisense (5′ CATCATGTCCTTG TACTGCCGCTTG 3′), BDNF sense (5′ AGGCAACTTGGCCTACCCAGGTGTG 3′) and antisense (5′ TACTGTCACACACGCTCAGCTCCCC 3′), IFN-γ sense (5′ TGCATCTTGGCTTTGCAGCTC TTCCTCATGGC 3′) and antisense (5′ TGGACCTGTGGGTTGTGACCTCAAACTTGGC 3′), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense (5′ TTGTCAGCAATGCATCCTGCAC 3′) and antisense (5′ ACAGCTTTCCAGAGGGGCCATC 3′). ACTV-1 major capsid protein mRNA (gene z280l) levels were evaluated by qRT-PCR with sense primers (5′ ATGGCCGGAGGACTTTCACAGC 3′ and antisense (5′ AACGGAACCGTTGATGGTCTGC 3′ and ATCV-1 PCNA mRNA (gene z530l) was evaluated with sense primers (5′ ATGGACACGAGCTGTGCGATAA 3′) and antisense (5′ GCTCTTGATGACGGAACCCTGA 3′). Quantitative PCRs were run on an ABI Prism 7000 thermal cycler at 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 60 °C for 30 s. Cycle threshold (CT) values of samples were normalized to the CT of GAPDH and then normalized to the average CT of the control samples, after which data were expressed as relative mRNA levels by the $2^{-\Delta\Delta}$CT method.

2.3. Materials and procedures for behavioral assessment

Tests of anxiety, social processes and cognitive function were conducted using standard protocols in the following order: Open Field, Novel Object Recognition, Novel Object Location, Light-Dark Exploration Test, Non-Spatial Barnes Maze, Spatial Barnes Maze (with Reversal and Probe Trials), Sociability Test, and Sucrose Preference Test. Descriptions of the materials and procedures are organized by the behavioral domains the tests were intended to measure. Mice were tested individually. The apparatuses were cleaned with a 70% ethanol solution between trials.

2.3.1. Anxiety-related behaviors and locomotor activity

2.3.1.1. Open Field test. Mice were individually exposed to the open field apparatus (Med Associates ENV 510; Med Associates Inc., St. Albans, New York, USA). The open field (43 cm × 43 cm × 30 cm) was lit overhead and activity was measured using three 16 infrared beam arrays. The activity monitor provides data on locomotor activity by measuring the time and distance traveled (cm), average velocity (cm/s), time and counts of standing on hind legs. Anxiety-related measures obtained in the open field included time spent performing stereotypic behaviors (i.e. grooming), time spent with no movement (i.e. freezing behavior), thigmotaxic behavior (activity occurring within 10 cm of the walls (Bailey and Crawley, 2009), and number of boli excreted.

The subject was placed in a randomly designated corner of the field to begin the exposure period. Two exposures periods occurred. The first was for 30 min. However, due to equipment malfunction, data from half the mice were lost. Mice were given a second exposure two days later. The data from this 15 m exposure were used in the analyses.

2.3.1.2. Light-dark chamber. The light-dark chamber provided additional measures of anxiety-related behaviors. Rodents normally prefer to spend time in a dark protected area of a novel environment (Bailey and Crawley, 2009). The chamber was a square (40 cm × 40 cm × 40 cm), clear plexiglass box with a dark, covered insert (20 cm × 20 cm × 40 cm) of black plexiglass. The dark insert had an opening in the center (5 × 12.5 cm) to allow transitions from the light (780 lx) to the dark side (25 lx).

Each mouse was placed in the center of the light side, facing away from the opening to the dark side, to begin the 10 m trial. Shorter latencies to enter the dark side, spending a lower percentage of total time on the light side, and fewer number of transitions from the dark side (front half of body through opening) indicate heightened anxiety.

2.3.2. Social affiliation and anhedonia

2.3.2.1. Sociability test. To measure the social process of affiliation, a standard polypropylene rat cage (42 cm × 24 cm × 20 cm) was divided into three equal sized areas. Cylindrical wire cages (12 cm d × 15 cm h) were placed in each area. The stranger mouse (same age female C57Bl/6 mice with no prior contact with the subject mice) was placed under one of the end cages (randomly determined). The stranger mouse had been previously habituated to the wire cage. The subject mouse was placed under the center cage for a 5 m habituation period. The center cage was removed allowing the mouse to freely explore the entire apparatus for a 10 m trial. Total time the mouse's nose was within 1 cm of the wire cages were noted. To measure sociability, mean exploration ratio (time exploring stranger-mouse-containing cage/total time exploring both cages) was calculated (Crawley, 2004).

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