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Research article

Effect of voluntary wheel running on neuroactive steroid levels in murine experimental autoimmune encephalomyelitis

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ABSTRACT

Increasing evidence from both clinical and animal research has implicated changes in neuroactive steroids (rapid acting steroids that act as allosteric modulators at NMDA and/or GABA-A receptors) in multiple sclerosis. These changes have been linked to clinical differences in disease severity, prevention of disease development, as well as the disease state (relapsing vs progressive) in patients with multiple sclerosis. Previous research has also linked changes in neuroactive steroid levels to the beneficial effects of exercise in certain disorders such as traumatic brain injury and post-traumatic stress disorder. The present study therefore examined whether voluntary wheel running could modulate any of the reported changes in neuroactive steroids associated with the EAE model of multiple sclerosis. Female mice with EAE who ran were found to have significantly increased levels of brain pregnenolone compared to male EAE mice who ran. In contrast, male mice with EAE were found to have significantly higher levels of brain allopregnanolone compared to female mice with EAE regardless of exercise. Overall, these results indicate that exercise has moderate beneficial effects on brain neuroactive steroid levels in EAE. These changes may be related to other beneficial responses to exercise, such as improvements in disease severity, in EAE and/or multiple sclerosis.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease primarily associated with motor dysfunction. Its etiology and biological mechanisms are not yet fully understood. Neuroactive steroids (NASs) have recently been implicated in several aspects of MS symptomology and progression [1–5]. NASs are rapidly acting steroids that produce nongenomic effects by acting as allosteric modulators at neurotransmitter receptors, especially the NMDA subtype of glutamate receptors and/or gamma-aminobutyric acid-A (GABA-A) receptors [6,7]. NASs are synthesized from cholesterol in the nervous system. While some NASs such as progesterone can also be synthesised in other tissues (e.g. the ovaries), it is only when these steroids are synthesised in the brain, spinal cord or peripheral nerves that they are considered NASs [7]. Various NASs have been reported to have neuroprotective actions and may have effects on cell growth, differentiation and myelination [6]. Some NASs have also been reported to have anti-inflammatory actions and have been proposed to play a role in a number of neurologic, psychiatric and gastrointestinal disorders (see [6] for a review).

Clinical reports have found that there are significant differences in the levels of NASs in the cerebrospinal fluid (CSF) of healthy controls and those with relapsing remitting multiple sclerosis (RRMS) [3]. Both pregnenolone (PREG) and dehydroepiandrosterone (DHEA) are significantly elevated in the CSF of MS patients of both sexes [3]. Furthermore, it was found that these changes were regulated by disease state [3]. Disease state dependent changes in NAS levels have been seen in an animal model of MS, experimental autoimmune encephalomyelitis (EAE) [2,5]. It was found that in the acute stage of the EAE, spinal cord levels of progesterone (PROG) and isopregnanolone (3β,5α-tetrahydroprogesterone; ISO) are decreased, but at the chronic stage, there is no change in spinal cord levels of these NASs [2,5]. These studies suggest that different NASs might have different roles in disease

Abbreviations: ALLO, allopregnanolone (3α5α-tetrahydroprogestone); CNS, central nervous system; CSF, cerebrospinal fluid; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; EAE, experimental autoimmune encephalomyelitis; ISO, isopregnanolone (3β,5α-tetrahydroprogesterone); MS, multiple sclerosis; NAS, neuroactive steroid; PREG, pregnenolone; RRMS, relapsing remitting multiple sclerosis; THDOC, tetrahydrodeoxycorticosterone

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progression [2,3,5]. This is further highlighted by studies showing different changes in the levels of NASs, such as testosterone, between RRMS and progressive MS, with males showing decreased testosterone in progressive MS compared to RRMS [4].

Major sex dependent differences in the development, disease course, and symptomology of MS have been seen both clinically and in animal models, making this an important variable to consider in pre-clinical research [8–10]. Sex has also been found to be a significant factor underlying the changes in NAS levels associated with MS [2–5]. For example, in a study examining changes in NASs from the CSF of patients with MS, it was found that female patients had lower levels of ALLO compared to male patients, and that in general, there were more changes in NAS levels in females compared to males [3]. Additionally, PREG levels were found to be increased in the active disease state in males compared to stable MS, but these state-dependent differences in PREG levels were not seen in female patients [3]. Interestingly, a separate study examining CSF from male MS patients also found decreased levels of the testosterone metabolite, DHT, compared to non-diseased control subjects [1].

Sex dependent differences in NAS levels have also been reported in the EAE model. For example, it was found that female mice with EAE had larger increases in ISO and PROG in the spinal cord compared to males with EAE. On the other hand, males with EAE had increased levels of dihydrotestosterone (DHT) compared to females [5]. Furthermore, another study examining NAS levels in acute EAE found that NAS levels change not only in a sex dependent way, but also varied between plasma and different CNS regions (cerebrum, cerebellum, and spinal cord) [2].

We have become interested in exploring the role of NASs as potential mediators of the beneficial effects of exercise in MS. To our knowledge, no previous study has examined the role of NASs in relation to the benefits of exercise in MS, but changes in NAS levels in response to exercise in other diseases have been observed. For example, it has been shown that ALLO levels are increased after brief, but intense exercise. These changes in ALLO levels are associated with post-exercise pain tolerance in patients with post-traumatic stress disorder who also have chronic pain [11].

While there is only a small number of studies examining the role of exercise on NAS levels in general, the results to date suggest that exercise may be able to modulate NASs in a beneficial way. The present study therefore explored the relationship between an exercise therapy, voluntary wheel running, and NASs associated with EAE. Given the reported sex dependent differences in NAS levels in EAE, we also assessed NAS levels in both male and female mice with the disease.

2. Methods

2.1. EAE induction and assessment

All animal studies were conducted in compliance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Animal Care and Use Committee: Health Sciences for the University of Alberta. Male and female C57BL/6 J mice were obtained from Charles River Canada at age 6–8 weeks for all studies. Mice were housed in same-sex groups of 5 in standard shoebox cages with crinkle bedding with standard enrichment on a 12:12 light/dark cycle. Mice were given free access to standard chow food and water throughout all experiments. After a two week habituation period to the housing facility, mice were induced with EAE using myelin oligodendrocytes glycoprotein 35–55 (MOG_{35-55}). Mice were randomly assigned to Complete Freund's Adjuvant (CFA; control) or EAE (experimental) groups for all experiments EAE induction was done by giving a subcutaneous injection of 50 µg of MOG_{35-55}emulsified in CFA at a concentration of 1.5 mg/mL, followed by an intraperitoneal injection of 300 ng of pertussis toxin in 0.2 ml sterile phosphate buffer saline on the day of injection and again two days later. CFA control mice did not receive MOG injections, only injections of CFA and pertussis toxin. Mice were then kept in quarantine for 72 h after the induction of EAE as per university regulations. Weight and clinical score (graded on a four-point scale) was assessed daily. The four-point clinical scale is as follows: grade 0, normal mouse; grade 1, flaccid tail (disease onset); grade 2, mild hindlimb weakness with quick righting reflex; grade 3, severe hindlimb weakness with slow righting reflex; grade 4, hindlimb paralysis in one hindlimb or both. Mice who lost greater than 50% of their baseline body weight were euthanized to prevent unnecessary pain and discomfort.

2.2. Wheel running

For the hour of daily running, mice were removed from their home cage and placed in a similar, standard cage with only bedding and a running wheel (Living World®-Deluxe Exercise Wheel, 5″/12.5 cm #61701) for 30 consecutive days. Distance run was recorded using a Schwinn ® 20 Function bike computer (model 04SW654C6PK) fitted to the wheels. Running mice underwent 3 consecutive days of training prior to induction. Mice started daily running on day 4 post induction after a mandatory quarantine period. (EAE male n = 5, EAE female n = 5). Non-running EAE control mice spent an hour a day in a standard cage with only bedding and a fixed running wheel to control for any environmental enrichment (EAE male n = 5, CFA male n = 5, EAE female n = 5, CFA female = 5).

Gas Chromatography – Mass Spectrometry (GC–MS)

Mice were euthanized via a sodium pentobartibal (340 mg/ml) injection prior to dissection. After cardiac perfusion with 0.9% saline, whole brains were then dissected out fresh, flash frozen in liquid nitrogen, and stored at -80 ˚C until homogenization. Cerebrums were them separated from the cerebellum, brainstem, and olfactory bulbs and then prepared for gas chromatography–mass spectrometry (GC–MS) analysis to measure levels of NASs. Methods used were similar to those described previously by Ahboucha et al., 2008 [12] with minor modifications. Briefly, protein was first precipitated using methanol and then centrifugation. Supernatants were retained and NASs isolated using solid-phase extraction with Oasis HLB cartridges (Waters). Samples were then derivatized with heptafluorobutyrylimidazole (HFBI), and the resulting derivatives were analysed by gas chromatography combined with negative ion chemical ionization mass spectrometry using an Agilent 6890 gas chromatograph coupled to a 5973 N mass selective detector. Standard curves were prepared for each steroid and deuterated (d_4) pregnenolone was used an as internal standard for all samples.

2.3. Statistical analysis

Statistical analysis was carried out using two-tailed t tests or two-way repeated measures ANOVAs where appropriate with Tukey's multiple comparison *post hoc* tests. All values compared were a calculation of percent control of non-running CFA animals using the following formula: (experimental raw value/average of total raw CFA value) $\times 100$. Significance was set at $p < 0.05$. All statistical analysis was performed with GraphPad Prism 6.0 software.

3. Results

All endogenous NASs originate from cholesterol. Fig. 1 outlines the metabolic pathways involved in NAS synthesis and degradation. Those NASs that were detectable in the collected brains are outlined in red (Fig. 1). Isopregnanolone, however, was only detectable in the brains of male mice (see Fig. 2D). NAS levels were measured at a chronic timepoint (30 days post-induction), meaning that all mice were on average at a clinical score of at least 2 or higher.