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ORIGINAL ARTICLE

Modafinil normalized hyperreflexia after spinal transection in adult rats

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Study Design: Hyperreflexia occurs after spinal cord injury and can be assessed by measuring low frequency-dependent depression of the H-reflex in the anesthetized animal.
Objective: To determine the effects of Modafinil (MOD), given orally, following a complete SCI compared with animals receiving MBET and transected untreated animals and examine if changes exist in Connexin 36 (Cx-36) protein levels in the lumbar enlargement of animals for the groups described.
Setting: Center for Translational Neuroscience, Little Rock, AR, USA.
Methods: Adult female rats underwent complete transection (Tx) at T10 level. H-reflex testing was performed 30 days following Tx in one group, and after initiation of treatment with MOD in another group, and after MBET training in the third group. The Lumbar enlargement tissue was harvested and western blots were performed after immunoprecipitation techniques to compare Cx-36 protein levels.
Results: Statistically significant decreases in low frequency-dependent depression of the H-reflex were observed in animals that received MOD and those that were treated with MBET compared with the Tx, untreated group. Statistically significant changes in Cx-36 protein levels were not observed in animals treated with MOD compared with Tx, untreated animals.
Conclusion: Normalization of the loss of low frequency -dependent depression of the H-reflex was demonstrated in the group receiving MOD and the group receiving MBET compared with the Tx, untreated group. Further work is needed to examine if Cx-36 protein changes occur in specific subregions of the spinal cord.
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Introduction

The deficits resulting from spinal cord injury (SCI) include hyperreflexia and spasticity below the level of the lesion. The mechanisms of hyperreflexia and spasticity are unknown, although numerous theories have been postulated.[1] One of the mechanisms that has been proposed is that SCI leads to the loss of descending pathways that provide presynaptic inhibition to the motor system. However, Hasegawa and Ono[2] suggest that noradrengergic descending pathway tonically suppresses spinal presynaptic inhibition and may not contribute to hyperreflexia. The H-reflex or Hoffman reflex has been used to quantify hyperreflexia,[3] and several investigators have utilized frequency-dependent depression of the H-reflex to examine the changes in spinal cord circuitry after SCI.[4–6] We previously examined the use of motorized exercise bicycle training (MBET) in normalizing the loss of frequency-dependent depression of the H-reflex that occurs following the complete spinal cord transection (Tx) in the rat[5,7] as well as the effects of passive exercise in the acute as well as the chronic phase of injury.[8]

Recently, we found that Tx transiently decreased levels of the neuronal gap junction protein Connexin 36 (Cx-36).[9] Cx-36 levels decreased 30% 7 days after injury, and returned to control levels over the next 2–4 weeks. The onset of hyperreflexia was coincident with the recovery of Cx-36 to control levels. We hypothesized that a change in electrical coupling occurs after Tx that contributes to the hyperreflexive state, although the nature of this change is unknown.

The stimulant modafinil (MOD) is approved for the treatment of excessive sleepiness in narcolepsy, obstructive sleep apnea and shift work disorder. A recent landmark study found that the mechanism of action of MOD is to increase electrical coupling between cortical interneurons, thalamic

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reticular neurons and inferior olivary neurons.[10] The literature revealed limited studies of MOD as a treatment of hyperreflexia or spasticity induced from SCI. Mukai and Costa[11] reported positive effects from MOD on self esteem in two patients with SCI. Hurst *et al.*[12] described a retrospective study of the use of MOD in a population of children diagnosed with cerebral palsy. These authors reported that 76% of the patients studied ($n=30$) showed decreased spasticity after treatment with MOD, and showed decreased tone after physical examination. Hurst and Lajara-Nanson[13] conducted a pilot study to examine the benefit of MOD on spasticity and went on to hypothesize that MOD reduces spasticity of central origin. An additional study in 2006 by Hurst *et al.*[14] reported that 29/59 pediatric patients with spastic cerebral palsy that were treated by MOD showed improvements in gait during the treatment.

This study was undertaken to determine whether MOD, administered orally, would normalize the loss of frequency-dependent depression of the H-reflex that is observed in spinally Tx rats, and how this treatment compares to passive exercise of the hindlimbs that is initiated in the acute phase of exercise. We also wanted to examine the changes in Cx-36 protein in the lumbar tissue following Tx and after treatment. Preliminary results were presented in abstract form.[15]

Methods

The methods employed have been published previously.[5,7–9] All animal procedures were approved by the Institutional Animal Care and Use Committee of Univesity of Arkansas for Medical Sciences (UAMS).

Surgery

Adult female Sprague–Dawley rats ($n=48$, 200–300 g, Harlan) underwent a lower thoracic laminectomy under ketamine (60 mg kg^{-1}, i.m.) and xylazine (10 mg kg^{-1}, i.m.) anesthesia. A complete Tx of the spinal cord was made by aspiration and the Tx ends of the cord retracted, producing a 2–3-mm cavity. Surgery and postsurgical care were performed as described previously.[5] We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

One group of intact rats served as non-TXcontrols ($n=10$) and another group of Tx animals (Tx only 30 days, $n=16$) underwent no further treatment until H-reflex testing was carried out 30 days after complete Tx. The remaining rats were divided into two groups. One group ($n=16$) was treated with MOD beginning 7 days after Tx. The MOD was given orally at a dose of 4 mg kg^{-1}. The second group ($n=16$) began passive exercise training 7 days after Tx and exercised 5 days per week for 30 days. This group exercised for two 30-min exercise sessions per day with a 10-min rest period in between the two sessions. Specific methods for MBET have been described previously.[5,7]

Reflex testing

H-reflex testing was measured as described previously.[7,9] Recordings were made using amplifier (Grass P511) filter settings of 3 Hz to 3 KHz with the 60 Hz notch filter in use. Responses to the stimulus were digitized and averaged using a GW Instruments (Somerville, MA, USA) digitizer module and SuperScope software.

The reflex first was tested at 0.2 Hz to determine threshold and maximal response levels. After discarding the first five responses at each frequency to obtain an average of the stabilized reflex, averages of 10 responses were obtained and they were compiled following stimulation at 0.2, 1, 5 and 10 Hz. The change in the response at various frequencies was calculated as the percent of the response at 0.2 Hz to determine the depression of H-reflex as a function of stimulation frequency. Following the frequency series testing, the H-reflex amplitude was confirmed at 0.2 Hz for consistency. If the amplitude at recheck was less than 90% of the initial amplitude, the data were discarded.

At the end of the experiment, animals were euthanized with an overdose of barbiturate (Nembutal) and the Tx confirmed either visually or histologically following transcardial perfusion with paraformaldehyde (4%) and sucrose (20%).

Measurement and statistics

The amplitude of the H-wave was measured from peak to peak of the two components. For comparison of data between the different groups in each experiment, measures were tested using one factor, two factor or multifactor analysis of variance to conclude whether any of the factors had a significant effect on the magnitude of the variable and also whether the interaction of the factors significantly affected the variable. Differences were considered significant at values of $P<0.05$. If statistical significance was present, posthoc tests were used to compare the groups.

Cx-36 protein analysis

The methods employed for Cx-36 protein analysis have been published previously.[9,16] At the end of recording, cores (3–4 mm^3, 300–600 mg) from the lumbar enlargement were removed using a 3–4-mm dermal biopsy punch after performing laminectomies in anesthetized rats. Tissue was homogenized in 600 μl ice-cold radio immunoprecipitation assay buffer with HALT protease inhibitors (Pierce Thermofisher Scientific, Rockford, IL, USA) and centrifuged to remove debris. Tissue and lysate were kept on ice at all times. In all, 5 μg of anti-Cx-36 antibody (37–4600, Zymed, Invitrogen, Carlsbad, CA, USA) per sample was covalently coupled to a gel support (Seize Primary, Pierce). Then, 800 μg of protein from spinal cord lysates was mixed with antibody-coupled gel in a total of 1 ml radio immunoprecipitation assay (with protease inhibitors) and incubated 4 °C, overnight, with gentle end-over-end mixing. Immunoprecipitates were washed twice with radio immunoprecipitation assay buffer and resolved by SDS-polyacrylamide gel electrophoresis.[9] The amount of Cx-36 immunoprecipitated was determined by western blot as described previously.[9]

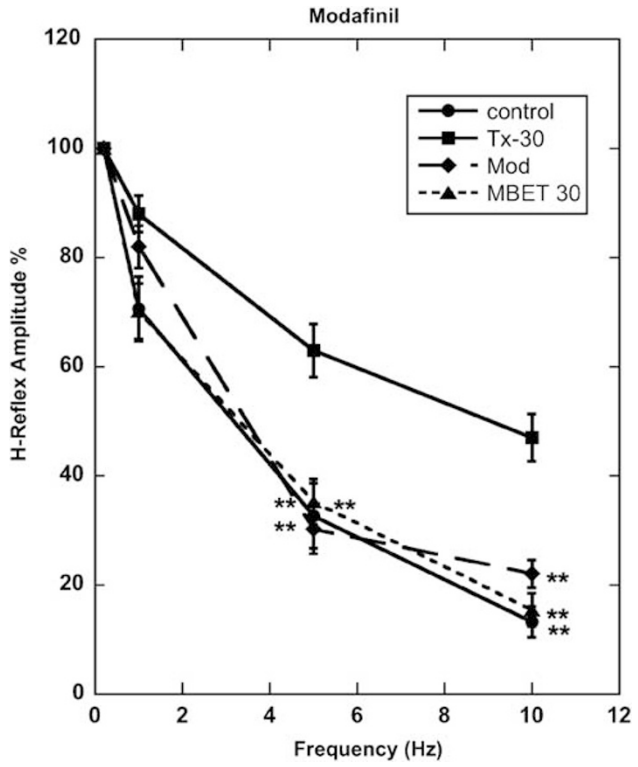

Figure 1 H-reflex amplitude at 0.2, 1, 5 and 10 Hz for intact animals (control, black circles), Tx only 30 days (Tx-30, square), MBET 30 days (Tx + Ex-30, triangle) and modafinil 30 days (MOD, diamond). Frequency-dependent depression of the H-reflex at 0.2 Hz was designated 100%, and statistical comparisons made against the Tx-30 group. At 5 and 10 Hz, the Tx-30 group differed from the motorized exercise bicycle training (MBET) group (triangle; $P<0.01$), the MOD group (diamond; $P<0.01$) and the control group (black circle; $P<0.01$).

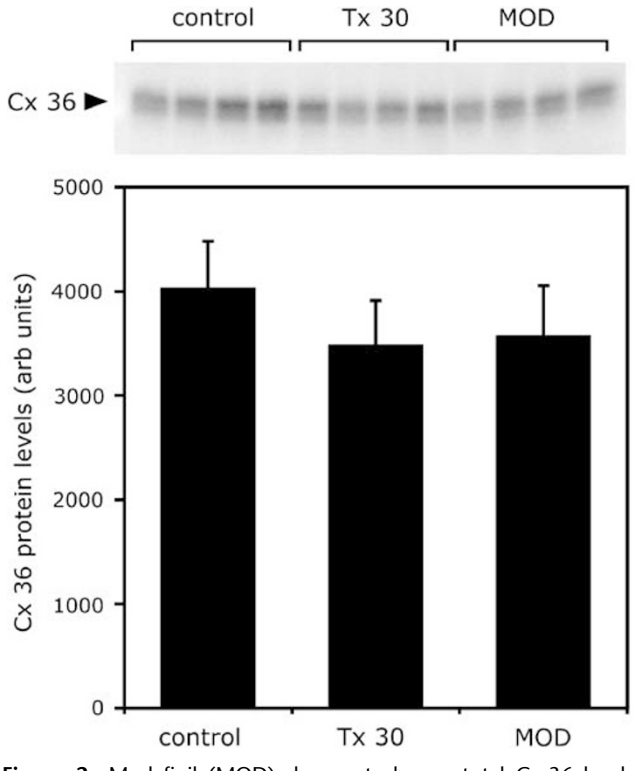

Figure 2 Modafinil (MOD) does not change total Cx-36 levels. Upper panel, western blot of immunoprecipitated Cx-36 from spinal cord. Lower panel, Quantification of Cx-36 from western blot. Bars show mean + s.d. Tissue was taken from control animals, animals 30 days after transection (Tx-30), and Tx animals after 30 days of MOD treatment that was initiated 10 days after injury.

Results

The habituation of the H-reflex was examined following stimulation at 0.2, 1, 5 and 10 Hz in the following groups of animals: Tx and untreated for 30 days (Tx only 30 days), Tx and exercised for 30 days (Tx + Ex 30 days Tx and treated with (MOD and intact control. The analysis of variance of these groups showed statistically significant differences across experimental groups for stimulation at 5 Hz (d.f. = 3, F = 7.662; $P<0.0002$), and 10 Hz (d.f. = 3, F = 18.647; $P<0.0001$). *Post hoc* comparisons between all groups were undertaken using the Newman–Keuls test and those against the Tx only 30 days (TX, untreated) group were considered the most relevant at 5 and 10 Hz.

Comparison against the Tx only 30 days group revealed significant differences to the intact control group at 5 ($P<0.01$) and 10 Hz ($P<0.01$), the passive exercise (MBET) group (Tx + Ex 30 days) at 5 ($P<0.01$) and 10 Hz ($P<0.01$) and the MOD group at 5 ($P<0.01$) and 10 Hz ($P<0.01$). The Control group revealed significant differences compared with the unexercised, untreated group (Tx only 30 days) at 5 Hz ($P<0.01$) and 10 Hz ($P<0.01$). Figure 1 is a graph of the habituation of the H-reflex following stimulation at 0.2, 1, 5 and 10 Hz for all groups described.

We hypothesized that the improvements in the H-reflex after MOD treatment were due to increased electrical coupling. To investigate this, we determined the amount of Cx-36 protein in the lumbar enlargement of the spinal cord. We measured total Cx-36 in control animals that had not undergone Tx or exercise, animals that had undergone Tx 30 days previously but remained untreated (Tx only 30 days) and animals that had undergone Tx and were then treated with MOD, starting 7 days after surgery. Earlier studies have shown that total Cx-36 protein transiently decreased 7 days after Tx, and then returned to control levels over the next few weeks.[9] The results of this study (Figure 2) showed that in animals that received MOD, total Cx-36 protein levels are not different to animals that did not receive MOD.

Discussion

The results of this study indicate that both passive exercise (MBET), if initiated in the acute phase of injury for 30 days, and treatment with MOD (without exercise), normalized the loss of frequency-dependent depression of the H-reflex that occurs after SCI.

Some methodological considerations and limitations of this study should be considered. It has been shown that MOD increases electrical coupling between GABA neurons.[10] Therefore, improvements in H-reflex attenuation at high

frequencies in animals that had undergone MOD treatment could be explained by increased coupling of spinal neurons. We found that there was no change in total Cx-36 protein in animals that had MOD treatment compared with the animals that had not, indicating that MOD did not change Cx-36 gene expression in whole spinal cord tissue. It is not known how MOD increases electrical coupling, but it has been suggested that MOD might induce translocation of Cx-36 from intracellular stores to the plasma membrane.[10] In this study, we used western blots to measure total tissue protein. As this technique is unable to detect translocation events, we cannot support or refute a translocation hypothesis.

In an earlier study, we showed that after Tx, Cx-36 protein transiently decreased by 7 days post-Tx, but returned to control levels within 30 days. The return of Cx-36 control levels paralleled the onset of hyperreflexia.[9] In another study, we showed that when Tx rats are passively exercised, hyperreflexia was normalized and Cx-36 levels were slightly higher than in unexercised rats, suggesting that exercise may change Cx-36 protein levels.[8] Hyperreflexia can also be normalized with MOD, as shown in this study, although there was no evidence for changes in protein levels. Therefore, although passive exercise and MOD could both target electrical coupling, they may do so in different ways.

The focus of this study was on measuring how the treatments tested influenced hyperreflexia as manifested in frequency-dependent depression of the H-reflex. This study did not address the symptom of spasticity that is observed in the rat following spinal cord Tx. Spasticity is classically referred to as resistance to passive limb movement in proportion to the velocity of movement.[17] This velocity-dependent resistance is thought to be due to increased stretch reflex responses in the lengthened muscle.[18] Additional studies are ongoing that include stretch reflex measures that would allow insights into the effects of passive exercise or MOD on spasticity compared with hyperreflexia in the Tx rat.

An additional consideration is that this study utilized MOD treatment to rats beginning 7 days following Tx (in the acute phase of injury). The effects of MOD on normalization of the H-reflex in the chronic phase of injury (after hyperreflexia has been established) are unknown. Further studies are needed to address the effects of MOD in the chronic phase of SCI and the long-term effects of normalization of the H-reflex in the acute model.

A final issue is the potential site of action of MOD at the level of the spinal cord. It is known that motoneurons are extensively coupled during development, but coupling decreases by 14 days postnatally in the rat.[19] There are a number of interneurons that were found to be electrically coupled in the ventromedial region of the spinal cord and appear related to locomotor control.[20] It is not known whether MOD directly affects electrical coupling in the adult Tx rat, but if it does, it may do so by influencing motoneurons, locomotion-related interneurons or perhaps even GABAergic spinal interneurons, as it does in the brain.[10] Although a number of new questions are raised by this study, our results do strongly suggest that MOD may represent a valuable therapeutic adjunct to the treatment of SCI, and supports earlier results in cerebral palsy, but suggest that some of the gains made may have been due to direct effects on spinal circuitry rather than cerebral in origin.

Another potential mechanism is the possibility that MOD influences monoamine regulation of persistent inward currents in motoneurons. Recent results suggest that motoneurons in the adult sacral cord of the rat reacquire the ability to generate PICs and plateau potentials within 1–2 months after spinal Tx.[21] MOD appears to modulate monoamine transporters and receptors.[22–24] Therefore, additional studies are needed to verify how exactly might MOD have a salutary effect on hyperreflexia, but this does not preclude clinical testing.

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