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The effects of passive exercise therapy initiated prior to or after the development of hyperreflexia following spinal transection

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Abstract

Hyperreflexia develops after spinal cord injury (SCI) in the human and in the spinal cord transected animal, and can be measured by the loss of low frequency-dependent depression of the H-reflex. Previous studies demonstrated normalization of low frequency-dependent depression of the H-reflex using passive exercise when initiated prior to the development of hyperreflexia. We examined the effects of passive exercise prior to compared to after the development of hyperreflexia in the transected rat. Adult female rats underwent complete transection (Tx) at T10. Frequency-dependence of the H-reflex was tested following passive exercise for 30 days, initiated prior to hyperreflexia in one group compared to initiation after hyperreflexia became established, and compared to intact and untreated Tx groups. An additional Tx group completed 60 days of exercise initiated after hyperreflexia was established. Lumbar enlargement tissue was harvested for western blot to compare Connexin-36 protein levels in control vs Tx animals vs Tx animals that were passively exercised. No differences in whole tissue were evident, although regional differences may still be present in Connexin-36 levels. Statistically significant decreases in low frequency-dependent depression of the H-reflex were observed following 30 days of exercise initiated prior to the onset of hyperreflexia, and also after 60 days of exercise when initiated after hyperreflexia had been established, compared with Tx only animals. We concluded that modulation of spinal circuitry by passive exercise took place when initiated before and after the onset of hyperreflexia, but different durations of exercise were required.

Keywords

H-reflex; hyperreflexia; passive exercise

Introduction

One of the numerous deficits resulting from SCI is hyperreflexia and spasticity below the level of the lesion. The H-reflex or Hoffman reflex has been used to quantify hyperreflexia (Angel

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and Hoffmann 1963, Olsen and Diamantopoulos 1967, Little and Halar 1985, Milanov 1994, Faist et al. 1994), and several investigators have utilized frequency-dependent depression of the H-reflex to examine changes in spinal cord circuitry after SCI (Thompson et al. 1992, Ishikawa et al. 1966, Chen et al. 2001, Skinner et al. 1996)

Previously, we reported the time course that the animal with a complete transection (Tx) transitions to a state of hyperreflexia (Yates et al. 2008). We also reported the ability of long-term passive exercise therapy to restore frequency-dependent depression of the H-reflex in adult rats with complete spinal cord Tx that began exercise prior to the onset of hyperreflexia (Skinner et al. 1996, Reese et al. 2006). A major area of interest is a comparison of the modulation of spinal cord circuitry using passive exercise prior to the onset of hyperreflexia (acute phase of injury) compared with initiation of exercise after the onset of hyperreflexia (chronic phase of injury). The literature is limited when examining exercise paradigms that compare exercise during the acute vs chronic phases.

The first objective of this study was to determine if passive exercise can be used to modulate the H-reflex in Tx animals that begin exercise after hyperreflexia has been established (after 30 days post Tx), therefore demonstrating a form of **rescue** from hyperreflexia. However, we hypothesized that the amount of exercise required to **prevent** hyperreflexia, when initiated prior to its onset, would differ compared to the amount of exercise required to **rescue** from hyperreflexia. This hypothesis was tested by comparing H-reflex frequency-dependent depression in a group of Tx animals in which exercise was initiated prior to the onset of hyperreflexia (acute phase) compared with a group of animals that were exercised starting after hyperreflexia was established (chronic phase). The third objective was to determine if spinal cord circuitry would retain its modulation when exercise was stopped. Therefore, the duration of **savings** on modulation of frequency-dependent depression of the H-reflex was examined. The mechanisms underlying the development of hyperreflexia and spasticity after SCI are currently unknown. In addition, the mechanisms influenced by passive exercise to induce reflex changes are also unknown. Two potential mechanisms that have been studied related to exercise training include changes in insulin-like growth factor-1 (IGF-1) and brain derived neurotrophic factor (BDNF). Recent attention has focused on the role of insulin-like growth factor-1 (IGF-1) in the recovery of locomotion after incomplete injury and the effects of exercise in complete injuries. (Bauman et al. 2006; Koopmans et al. 2006). An additional mechanism has focused on changes to BDNF following exercise. After several days of voluntary wheel running, normal rats were tested and found to have increased levels of BDNF mRNA in the hippocampus, cerebellum and cortex (Neeper et al. 1995). Other authors have investigated mRNA changes in BDNF after exercise in the lumbar spinal cord following SCI (Gomez-Pinilla et al. 2001). Treadmill training facilitated normalization of the peripheral BDNF levels and normalized sensory changes observed in incomplete spinal cord injuries in the rat (Hutchinson et al. 2004).

Recently we found that Tx transiently decreased levels of the neuronal gap junction protein Connexin 36 (Cx-36) (Yates et al. 2008). 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 co-incident with recovery of Cx-36 back to control levels. We hypothesized that a change in electrical coupling occurs after transection that contributes to the hyperreflexive state, although the nature of this change is unknown. In this study, animals were passively exercised and the levels of Cx-36 protein determined. We hypothesize that changes in gap junctions could contribute to both the onset of hyperreflexia and be influenced by passive exercise after injury.

Methods

Surgery

Adult female Sprague-Dawley rats (Harlan, 200–250 g, $n = 55$) underwent a lower thoracic laminectomy under ketamine (60 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.) anesthesia. A complete transection (Tx) of the spinal cord was made by aspiration and the transected ends of the cord retracted, producing a 2–3 mm cavity. Surgery and postsurgical care was performed as previously described in Reese et al. 2006 and Yates et al. 2008. All procedures were approved by the Institutional Animal Care and Use Committee at UAMS. 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-transected (controls, $n=10$) and another group of transected animals (Tx 30D, $n=16$) underwent no further treatment until H-reflex testing was carried out 30 days after complete Tx. The remaining rats ($n=39$) were divided into four exercise groups that received MBET daily. Exercise was initiated prior to the onset of hyperreflexia (started less than 7 days following surgery) in one group and exercised for 30 days (Tx+Ex 30 D, $n=16$). Two groups began exercise after hyperreflexia had been established, which was previously demonstrated to occur at >14 days (Yates et al. 2008). Lack of habituation in low frequency-dependent depression continued to occur until 30 days after injury, therefore exercise was not initiated until the full 30 days post surgery to ensure that the transition had occurred. One group exercised for a 30 day duration after the establishment of hyperreflexia (Tx+ Delay 30+ Ex 30 D, $n=7$), and the other group exercised for a 60 day duration after the establishment of hyperreflexia (Tx+ Delay 30+Ex 60 D, $n=9$). The final group examined changes in H-reflex after 30 days without exercise when exercise was stopped after modulation of the system had occurred (Tx+ Ex 30+Delay 30, $n=7$).

Reflex Testing

H-reflex testing was measured as previously described in Reese et al. 2006 and Yates et al. 2008. 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) digitizer module and SuperScope^R software.

The reflex first was tested at 0.2 Hz to determine threshold and maximal response levels. After discarding the first 5 responses at each frequency in order to obtain an average of the stabilized reflex, as previously demonstrated by Meinck (1976), averages of 10 responses were obtained. Averages 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 in order to determine depression of the 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 was 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 transcatheter 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 (ANOVA) 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 between groups.

Cx-36 protein analysis

At the end of recording, cores (3–4 cu mm, 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 μ l ice cold RIPA buffer (Heister et al. 2007; Garcia-Rill et al. 2007) with HALT protease inhibitors (Pierce) and centrifuged to remove debris. Tissue and lysate was kept on ice at all times. 5 μ g of anti-Cx-36 antibody (37–4600, Zymed, Invitrogen) per sample was covalently coupled to a gel support (Seize Primary, Pierce). 800 μ g of protein from spinal cord lysates was mixed with antibody-coupled gel in a total of 1 ml RIPA (with protease inhibitors) and incubated 4°C, overnight, with gentle end-over-end mixing. Immunoprecipitates were washed twice with RIPA buffer and resolved by SDS-PAGE (Yates et al. 2008). The amount of Cx-36 immunoprecipitated was determined by western blot as previously described. (Heister et al. 2007; Garcia-Rill et al. 2007).

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 30D), Tx and exercised for 30 days (Tx+Ex 30 D), Tx and exercised for 30 days then untreated for 30 days (Tx+Ex 30 D+ delay 30 D), Tx and untreated for 30 days, then exercised for 30 days (Tx + delay+ Ex 30 D), Tx and untreated for 30 days, then exercised for 60 days (Tx+ delay+ Ex 60 D), and intact Control. ANOVA of these groups showed statistically significant differences across experimental groups for stimulation at 5 Hz ($df=5$, $F= 7.4384$, $p<0.0001$), and 10 Hz ($df=5$, $F= 8.9337$, $p<0.001$). Post hoc comparisons between all groups were undertaken using the Newman-Keuls test and those against the Tx only 30 D group were considered the most relevant at 5 Hz and 10 Hz.

Comparison against the Tx only 30 D group revealed significant differences to the intact control group at 5 Hz ($p<0.01$) and 10 Hz ($p< 0.01$), the exercise group that initiated exercise prior to the onset of hyperreflexia (Tx+Ex 30 D) at 5 Hz ($p<0.01$) and 10 Hz ($p< 0.01$), the exercise group that initiated exercise prior to the onset of hyperreflexia and then had no treatment for 30 days (Tx+Ex 30 D+ delay 30 D) at 5 Hz ($p<0.05$) and 10 Hz ($p< 0.01$), and the exercise group that initiated exercise after the onset of hyperreflexia for 60 days (Tx+ delay+ Ex 60 D) at 5 Hz ($p<0.01$) and 10 Hz ($p< 0.01$). The Control group revealed significant differences compared to the unexercised Tx only group (Tx only 30 D) at 5 Hz ($p< 0.01$) and 10 Hz ($p< 0.01$), and the chronic exercise group that exercised for 30 days (Tx + delay+ Ex 30 D) at 5 Hz ($p<0.01$) and 10 Hz ($p<0.05$). There were no significant differences between the Tx only 30 D group and the exercise group that initiated exercise after the onset of hyperreflexia and exercised for 30 D. 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 previously described.

We measured total Cx-36 protein levels in the lumbar enlargement of intact control animals that had not undergone Tx or exercise, animals that had undergone Tx 30 days previously (Tx only 30), and animals that had undergone Tx and were then exercised for 30 days, starting 7 days after surgery (Tx + Ex 30D). Previous studies have shown that total Cx-36 protein decreased 7 days after Tx, and then returned to control levels over the next few weeks (Yates et al. 2008). The results of this study (Figure 2) showed that in animals that received passive exercise, total Cx-36 protein levels were not statistically different from animals that did not receive passive exercise, however, levels in Tx only animals were numerically lower than in intact and exercised groups.

Discussion

It has previously been demonstrated that if passive exercise is initiated prior to the onset of hyperreflexia (7 days post Tx) and the animal continues to exercise for 30 days, the animal demonstrates normalized reflexes compared to unexercised Tx animals (Reese et al. 2006). That is, passive exercise can be used to **prevent** hyperreflexia.

The results of the present study suggest that if passive exercise is initiated after the onset of hyperreflexia (30 days post Tx), and the animal exercised for 30 days, which was the minimum amount of exercise required to demonstrate changes in spinal cord circuitry when initiated in the acute phase of injury, significant changes were not observed. That is, the animal with a chronic SCI (hyperreflexia had been established) did not show beneficial reflex changes after only 30 days of exercise. These results are consistent with the work of Norrie et al. (2005) who reported that animals with a contusion SCI that received a three week training session after a delay, were less responsive to rehabilitation training than those immediately trained after injury. Therefore, a delay in training can impact potential functional recovery and reflex modulation.

Compared with the animals that exercised for 30 days after the onset of hyperreflexia, the animals that exercised for 60 days after the onset of hyperreflexia, did demonstrate significant normalization of reflex habituation at 5 and 10 Hz. Therefore, it required twice the duration of exercise to show modulation of the spinal circuitry sufficient to recover habituation of the H-reflex. Therefore, this experiment suggests that passive exercise can be used to **rescue** the spinal cord circuitry once hyperreflexia has been established, but requires a longer duration of therapy.

The results of the current experiment suggest that there is a difference in spinal plasticity in animals that are acutely injured compared to animals that are in the chronic phase of injury. Future studies need to investigate H-reflex changes after initiation of passive exercise at different time points in the chronic phase of injury. The results of this type of study would be important to determine if there are differences in spinal plasticity in a patient who is 1 year post SCI compared with a patient who is 5–20 years post SCI. All animals in this experiment that began their training after hyperreflexia had been established, started training 30 days post-transection which would be considered in the initial phase of chronic injury. Future work is needed to investigate the impact of longer delays after injury before passive exercise is instituted, to determine the ability of passive exercise to modulate spinal circuitry.

This experiment did examine the impact of cessation of passive exercise after initiation of exercise prior to the onset of hyperreflexia on normalization of the H-reflex. Results indicate that when exercise is initiated prior to the onset of hyperreflexia, the animal maintained the gains achieved when measured after not exercising for 30 days. That is, considerable **savings** were observed when hyperreflexia was prevented and therapy was discontinued. Further studies are needed to determine the duration of savings that can be gained if exercise is initiated after the onset of hyperreflexia, but continued for 60 days to achieve rescue from hyperreflexia, and then stopped to determine savings from such rescue. The H-reflex gains observed in a chronic patient (>1 year after injury) with a SCI who regained normalization of the H-reflex after a thirteen week training program only showed savings over a two week period after cessation of passive exercise (Kiser et al. 2005). That is, there was minimal savings after rescue in a human chronic patient with a SCI.

The current experiment suggests that it required shorter exercise duration to prevent hyperreflexia than was required to rescue from hyperreflexia once it had been established. Future studies warrant examination of the effects of different delay durations on the benefits of passive exercise.

Finally, this experiment highlights the difference that delaying exercise after injury induces on the reflex changes observed. When comparing the group in which exercise was delayed after injury and then exercised for 30 days with the group that exercised prior to the onset of hyperreflexia and then ceased for thirty days, they were significantly different. This implies that exercise provided greater benefit to the plasticity of spinal cord circuitry if it was initiated prior to the onset of hyperreflexia. However, longer durations of passive exercise were still effective in ultimately normalizing reflexes if exercise was initiated after the onset of hyperreflexia.

An additional potential mechanism involved in hyper-reflexia and/or spasticity may include electrical coupling of spinal neurons. Electrical coupling through Cx-36 gap junctions synchronizes coupled neurons (Heister et al. 2007; Garcia-Rill et al. 2007). Studies have also shown that following midthoracic spinal cord transection in the cat, electromyographic (EMG) recordings following stimulation to spinal cord segments revealed decreased synchronization of muscle activity with reduced burst duration in transected animals compared with control animals (Iwahara et al. 1991). This suggests that post-transection EMGs suffer from desynchronization. Improvements in H-reflex habituation in animals that had undergone passive exercise could be explained by changes in coupling of spinal neurons. Although the levels of Cx-36 protein in the transected spinal cord of animals that had undergone passive exercise were slightly higher than animals that were not passively exercised, this increase was not statistically significant. However, at this time we do not know if this increase is physiologically significant. The lumbar region of the spinal cord contains both motoneurons and interneurons and a recent landmark study described the presence of electrical coupling in locomotor-related interneurons in the spinal cord (Hinckley et al. 2006), suggesting that this mechanism is not only present in motoneurons but also interneurons. Perhaps the changes in gap junction protein Cx-36 after SCI reflect changes observed in synchronization of firing of motoneurons and/or interneurons and lead to the hyperreflexia observed. The subset of neurons that are involved in the hyperreflexive state could have significantly increased levels of Cx-36 after passive exercise, but these levels may be masked by insignificant changes in Cx-36 in other types of neurons that are not involved. Interestingly, there is less variability in Cx-36 protein levels between animals after passive exercise suggesting a link between Cx-36 and exercise, and importantly, we show that after passive exercise, Cx-36 is present to mediate electrical coupling. We have preliminary evidence suggesting that daily treatment of transected rats using modafinil, an agent whose mechanism of action appears to be to increase electrical coupling (Urbano et al. 2007), in the absence of passive exercise, normalizes hyperreflexia (Yates et al. 2007). Further work is needed to investigate the role of electrical coupling after SCI, for example, the differential Cx-36 expression in different types of spinal cord neurons after transection. While this mechanism may be in part responsible for hyperreflexia, it should be noted that other components of the more general symptom of spasticity have yet to be determined.

Finally, these experiments investigated the ability of passive exercise to normalize H-reflex changes that occur after spinal cord transection in order to address the issue of hyperreflexia, one component of spasticity. Additional studies need to address the effects of passive exercise on spasticity. Spasticity is classically referred to as resistance to passive limb movement in proportion to the velocity of movement (Lance 1980). This velocity-dependent resistance is thought to be due to increased stretch reflex (SR) responses in the lengthened muscle (Powers and Rymer 1988, Thilmann et al. 1991, Ju et al. 2000, and Kamper and Rymer 2000) although increased stiffness in limb compliance is also a factor (Dietz et al. 1981 and Sinkjaer 1983). Bose et al. (2002) described one technique of quantifying the ankle stretch reflex using EMG and torque responses to an imposed stretch following mid-thoracic contusion injury in the rat. These authors revealed the time course of the onset of spasticity and reported a significant level of spasticity in the ankle extensors that was velocity-dependent beginning week 4 post-injury

and continuing through the three month testing period. Additional studies that include stretch reflex measures would allow insight into the effects of passive exercise on spasticity vs hyperreflexia in the transected rat.

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Figure 1. Amplitude and habituation of the H-reflex in different groups

H-reflex amplitude at 0.2, 1, 5 and 10 Hz for intact animals (Control, black circles), MBET 30 days (Tx+ Ex 30D, red triangle), acute exercise for 30 days and then delay 30 days (Tx+ Ex 30D+ delay 30D purple square), Chronic animals that exercised for 30 days (Tx + delay 30D + MBET 30D, green triangle), and chronic exercise for 60 days (Tx + delay 30 D + Ex 60D, light blue diamond). Frequency-dependent depression of the H-reflex at 0.2 Hz was designated 100%, and statistical comparisons made against the Tx ONLY 30 D group (++). At 5 Hz, the Tx ONLY 30 D group (++) differed from the Tx+Ex 30 D + delay 30 D ($p<0.05$), and acute exercise group (Tx+Ex 30 D), the chronic exercise group for 60 days (Tx+ delay+ Ex 60 D), the acute exercise group (Tx+Ex 30 D), and the control group ($p<0.01$). At 10 Hz, the Tx ONLY 30 D group (++) differed from the Tx+Ex 30 D + delay 30 D ($p<0.05$), and acute exercise group (Tx+Ex 30 D), the chronic exercise group for 60 days (Tx+ delay+ Ex 60 D), and the control group ($p<0.01$).

Figure 2. Cx-36 levels in different groups

Upper panel, western blot of immunoprecipitated Cx 36 from spinal cord. Lower panel, quantification of Cx 36 from western blot. Bars show mean + SD. Tissue was taken from control animals, animals 30 days after transection (Tx 30), and transected animals after 30 days of motorized bicycle exercise training (MBET) that was initiated 7 days after injury.