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Novel mechanism for hyper-reflexia and spasticity

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Abstract

We established that hyper-reflexia is delayed after spinal transection in the adult rat, and that passive exercise could normalize low frequency-dependent depression of the H-reflex. We were also able to show that such passive exercise will normalize hyper-reflexia in patients with spinal cord injury (SCI). Recent results demonstrate that spinal transection results in changes in the neuronal gap junction protein connexin 36 below the level of the lesion. Moreover, a drug known to increase electrical coupling was found to normalize hyper-reflexia in the absence of passive exercise, suggesting that changes in electrical coupling may be involved in hyper-reflexia. We also present results showing that a measure of spasticity, the stretch reflex, is rendered abnormal by transection and normalized by the same drug. These data suggest that electrical coupling may be dysregulated in SCI, leading to some of the symptoms observed. A novel therapy for hyper-reflexia and spasticity may require modulation of electrical coupling.

Keywords

electrical coupling; gap junctions; H-reflex; hyper-reflexia; spasticity; spinal cord injury; stretch reflex

Introduction

Electrical coupling in breathing, walking and chewing

Oscillatory activity mediated by electrical coupling via gap junctions has been demonstrated in a number of regions including the neocortex (Blatow *et al.*, 2003; Roopun *et al.*, 2006), hippocampus (Traub *et al.*, 2003), olfactory bulb (Friedman and Strowbridge, 2003), amygdala (Sinfield and Collins, 2006), inferior olive (Leznik and Llinas, 2005; Long *et al.*, 2002), and locus coeruleus (Christie *et al.*, 1989). In addition, electrical coupling is evident in respiratory brainstem regions (Bou-Flores and Berger, 2001; Rekling and Feldman, 1997) and spinal cord (Connors and Long, 2004; Kiehn and Tresch, 2002). Gap junction blockers have been reported to depress rhythmogenesis in the respiratory pre-Botzinger complex (Elsen *et al.*, 2008), the retrotrapezoid nucleus (Hewitt *et al.*, 2004), and the nucleus of the tractus solitarius (Parisian *et al.*, 2004). Although few studies on chewing and swallowing have been carried out using gap junction blockers, there is little doubt that gap junctions are present in the trigeminal nucleus (Honma *et al.*, 2004), and hypoglossal (Cifra *et al.*, 2009) neurons are electrically coupled. Jim Lund, in collaboration with Verdier and Kolta, was instrumental in determining that at least some electrical coupling is present in the trigeminal mesencephalic nucleus (Verdier *et al.*, 2004). As far as locomotion is concerned, electrical

coupling has been found to modulate swimming in tadpoles (Li *et al.*, 2009) and below we discuss evidence in mammals.

How do spinal cord gap junctions influence motor coordination? Electrical synapses may contribute to the generation and maintenance of synchronized neuronal bursting firing patterns (Kistler *et al.*, 2002). Others demonstrated that motor patterns in the neonatal rat spinal cord were observed during blockade of chemical synapses, probably through the synchronization of bursting through gap junctions (Tresch and Kiehn, 2000). They suggested, “Gap junction-mediated neuronal coordination contributes to the basic function and organization of spinal motor systems.” These authors also suggested the existence of numerous independent rhythms in distinct motor pools. However, the modulation of gap junction communication in the adult spinal system and the adult system after injury are poorly understood.

Hyper-reflexia and spasticity in spinal cord injury (SCI)

Spinal cord injury (SCI) results in numerous deficits of motor and sensory systems, including paralysis, anesthesia, hyper-reflexia, and spasticity below the level of the lesion. Many of these individuals will require lifetime care due to motor deficits suffered as a result of the injury. Hyper-reflexia and spasticity are evident in both humans and animals following SCI and can contribute to significant functional limitation experienced by patients following SCI. The mechanisms responsible for hyper-reflexia are not known, however, several observed physiological changes that have been postulated to contribute to hyper-reflexia include alpha motoneuron hyperexcitability (Magladery *et al.*, 1952; Landau and Clare, 1964; Milanov, 1994), changes in the intrinsic properties of alpha motoneurons (Bennett *et al.*, 2001; Eken *et al.*, 1989; Li and Bennett, 2003), reduced post-activation depression of transmission from Ia fibers (Hultborn, 2003; Nielsen *et al.*, 1995), synapse growth (Little *et al.*, 1999), alterations in morphology of alpha motoneurons (Kitzman, 2005), and decreased presynaptic inhibition of Ia terminals (Angel and Hofmann, 1963; Calancie *et al.*, 1993; Faist *et al.*, 1994; Nielsen *et al.*, 1995; Pierrot-Deseilligny, 1990; Schindler-Ivens and Shields, 2000). The time course of spinal changes after injury has been proposed to include an early postsynaptic mechanism, possibly involving an increase in excitability and/or receptor up-regulation, and a late change involving presynaptic mechanisms possibly involving synaptic growth in spared descending pathways and in reflex pathways (Kitzman, 2005).

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 responses in the lengthened muscle (Ju *et al.*, 2000; Kuhn, 1950; Powers and Rymer, 1988), although increased stiffness in limb compliance is also a factor (Dietz *et al.*, 1981; Sinkjaer *et al.*, 1993). A test of excitability in general, and of frequency-dependent depression can also be applied to the stretch reflex (Thompson *et al.*, 2001). Therefore, the use of both H-reflex and stretch reflex would yield assessments of hyper-reflexia and spasticity. However, the utility of the H-reflex test is limited by the invasive nature of the test (direct exposure of the tibial nerve to provide stimulation), typically a terminal procedure, and the inability to assess the velocity-dependent nature of spasticity. The development of: a) a preparation in which reflexes are measured longitudinally, and b) a mechanical stretching device, would overcome these limitations and enable studies of the emergence of hyper-reflexia and spasticity post SCI and effects of interventions. Such developments are discussed below.

The H-reflex

One measure used by numerous investigators to quantify hyper-reflexia is the electrical analogue of the classic tendon jerk reflex, the Hoffman or H-reflex (Angel and Hofmann, 1963; Faist et al., 1994; Little and Halar, 1985; Olsen and Diamantopoulos, 1967; Yablon and Stokic, 2004). The H-reflex is a compound electromyographic (EMG) response elicited by the synaptic activation of motoneurons by muscle afferents following stimulation of muscle nerves. Thompson et al. (Thompson et al., 1992) investigated four measures of H-reflex excitability in a contusion model of SCI in the rat. Results of their studies led these researchers to conclude that rate-sensitive depression of the H-reflex was of particular import in the assessment of hyper-reflexia following SCI. Other groups have reached similar conclusions regarding the importance of changes in H-reflex rate-sensitive depression as a measure of the effects of SCI (Chen et al., 2001). In spinally intact individuals, the H-reflex demonstrates depressed amplitude, due to marked frequency-dependent depression, once stimulus frequencies reach or exceed 1 Hz (Ishikawa et al., 1966; Kiser et al., 2005). However, frequency-dependent depression of the H-reflex is less evident in patients or animals with chronic SCI (Ishikawa et al., 1966; Calancie et al., 1993; Skinner et al., 1996; Kiser et al., 2005).

Schindler-Ivens and Shields (Schindler-Ivens and Shields, 2000) examined changes in the human H-reflex in a longitudinal study over 44 weeks and concluded that attenuation of frequency-dependent depression occurs gradually. They also reported gradual changes from high-rate sensitivity to low-rate sensitivity between 6–18 weeks in the human that correlated with the transition from flaccid to a spastic state (Schindler-Ivens and Shields, 2004). Thompson et al (Thompson et al., 1992) examined changes in reflex excitability in the rat contusion model and determined that there was no difference in control animals compared with animals 6 days post contusion regarding the threshold for reflex initiation.. They also concluded that animals that were 28 days post contusion were significantly different than control animals in rate dependent depression at 1–5 Hz. Animals continued to be significantly different than controls at 60 days post contusion.

The fact that hyper-reflexia does not set in immediately after transection, and that reflexes can be elicited with normal habituation at 6 days, suggests that a major component of hyper-reflexia involves reorganization and perhaps changes in structure, rather than the elimination of presynaptic effects by the lesion. That is, whatever changes take place in the spinal cord after transection require >1 week to become manifested.

The stretch reflex

The noninvasive measurement of EMG and torque response to a movement perturbation has been reported in the human (Schmit et al., 1999; Schmit et al., 2002). Thompson et al. (1996) have also developed a device to quantify the stretch reflex in rats and documented the velocity dependent response in normal rats (Thompson et al., 1996) and in rats with a contusion injury of the spinal cord (Bose et al., 2002). Furthermore, Thompson documented the time course of the response post injury. However, the stretch reflex response to a complete transection injury has not been reported, and evidence from human studies indicates that responses differ based on completeness of injury (Calancie et al., 2002; Nakazawa et al., 2006). From a methodological standpoint, single session quantification of the EMG response to imposed stretch is possible, but longitudinal comparison of EMG responses in rodents is problematic because of the inability to normalize the EMG signal. Validity of non-normalized comparisons can be compromised because measured differences could be due to altered electrode location or spacing rather than a true treatment effect (Soderberg and Knutson, 2000).

One paradigm that overcomes this limitation is the study of the windup of repeated stretches. In a windup protocol, the EMG response to the first stretch is used to normalize subsequent responses. Windup behavior is characterized by temporal facilitation that results in increased amplitude and duration of the reflex responses. This has been demonstrated for flexor reflexes (Hornby et al., 2003), and recently in stretch reflexes in humans post SCI (Hornby et al., 2006). It has been suggested that the mechanism for this prolonged reflex response is due to alterations in intrinsic motoneuron properties, namely persistent inward currents (PICs) (Bennett et al., 1998). In reduced preparations, PICs demonstrate the ability to amplify and prolong the response to brief inputs (Crone et al., 1988; Hounsgaard et al., 1988), and their re-emergence is linked to the onset of hyper-reflexia (Bennett et al., 1999, 2001). Therefore, we developed an actuator that is capable of producing controlled stretches using a) a windup protocol to provide another measure of hyper-reflexia, and b) a velocity protocol to simultaneously provide a measure of changes in spasticity due to interventions such as exercise and pharmacological agents. Coupled with the ability to carry out these measures longitudinally, the serious limitations in this type of research can be overcome, and we can now assess the effects of passive exercise and pharmacological interventions as abnormal symptoms develop and are reversed.

Passive exercise

Role

We developed a method of providing continuous passive alternating movement of the hind limbs, known as motorized bicycle exercise training (MBET), to rats that had undergone T10 spinal cord complete transection. We reported the ability of long-term passive exercise therapy to produce frequency-dependent depression of the H-reflex in adult rats with complete spinal cord transection. We find that a period of 3 months of MBET, performed in sessions of 1 hr/day, 5 days/week, was capable of producing frequency-dependent depression of the H-reflex equivalent to that of intact animals (Reese et al., 2006; Skinner et al., 1996). Most of the H-reflex work in rats has involved acute testing where the animals are anesthetized, undergo H-reflex testing, and sacrificed at the end of the experiment (Meinck, 1976; Skinner et al., 1996; Thompson et al., 1992). Recently, we developed a method of testing the H-reflex longitudinally in the same rats using percutaneous electrodes (Arfaj et al., 2007). This method allows us to perform the testing in unanesthetized animals, more closely mimicking the testing conditions employed with human subjects. In addition, the same animals can be tested on multiple occasions throughout the course of the experiment. Thus, use of the percutaneous electrode method of testing requires fewer animals and allows one to track changes in the same animal over time in response to the intervention being applied. Our studies employ groups of spinally transected animals that are followed over time to determine the time course of the decrease in hyper-reflexia (assessed using the H-reflex) and spasticity (assessed using the stretch reflex) seen with exercise, and if a shorter duration of exercise is capable of producing similar degrees of H-reflex frequency-dependent depression or stretch reflex habituation in adult rats following spinal cord transection.

Hyper-reflexia and spasticity are present following a multitude of upper motor neuron disorders. Pharmacological agents such as Baclofen, Diazepam, Tizanidine, Dantrolene, and others have been used in human subjects in an attempt to decrease spasticity following SCI. However, all of these drugs have some undesirable side effects on patients and some have no effects on hyper-reflexia (Kita and Goodkin, 2000). The effects of modafinil (MOD) on hyper-reflexia and spasticity have not been investigated extensively, despite the fact that we found it is effective in treating patients with neglect after stroke injury (Woods et al., 2006). Moreover, recent reports suggest MOD may be useful in the treatment of spasticity arising from cerebral palsy (Hurst and Cedrone, 2006; Hurst et al., 2006). Why would a stimulant

used as an antinarcotic agent have salutary effects on spasticity? The mechanism of action of MOD was unknown until recently, but was credited with increasing glutamate, acetylcholine, noradrenaline and serotonin release, and decreasing GABA release (Ballon and Feifel, 2006). However, MOD was recently found *to increase electrical coupling* between nerve cells in the inferior olivary nucleus, cortical interneurons, and thalamic reticular neurons (Urbano et al., 2007). Following pharmacological blockade of connexin permeability, MOD restored electrotonic coupling within 30 min. The effects of MOD were counteracted by the gap junction blocker mefloquine. These authors proposed that MOD may be acting in a wide variety of cerebral areas by increasing electrotonic coupling in such a way that the high input resistance typical of GABAergic neurons is reduced. This “shunting effect” of MOD may activate the entire thalamocortical system by slightly diminishing inhibitory networks and, at the same time, increasing synchronous activation of both interneurons and non-inhibitory neurons. We confirmed that MOD increased electrical coupling in cell groups in the reticular activating system, thus accounting for its stimulant effects on arousal (Garcia-Rill et al., 2007; Heister et al., 2007). Studies described below show that daily oral administration of MOD can also be used to prevent the loss of frequency-dependent depression of the H-reflex in acutely spinalized rats. These findings raise a number of intriguing questions regarding the mechanisms behind hyper-reflexia and spasticity.

Effects on the H-reflex

Using the complete transection model in the adult rat, we recently reported that rate-dependent depression in spinally-transected animals, like contusion model animals, become significantly different from non-transected controls when tested 30 days post transection (Reese et al., 2006). We also found that hyper-reflexia does not set in immediately post-transection and that it may be present as early as 14 days post transection (see Figure 1) (Yates et al., 2008a). Determination of the exact time course for the onset of hyper-reflexia provide us with the framework around which to a) examine the molecular and cellular mechanisms involved, and b) determine optimal parameters for interventions following SCI.

We measured group mean H-reflex amplitudes at 0.2, 1, 5, and 10 Hz for intact animals (Control, open circles, n=10), in rats 7 days (Tx+ 7D), 14 days (Tx + 14D), and 30 days post transection (Tx + 30D). Frequency-dependent depression of the H-reflex at 0.2 Hz was designated as 100%, and all statistical comparisons in this figure were made against the transection only group (Tx + 30D). At 10 Hz, the Tx + 30D group differed ($p<0.01$) from the Tx + 7D, and Control groups. These results suggest that the onset of hyper-reflexia may occur between 7 and 14 days, although the variance of means at 14 days (note error bar) was greater than at 7 days while reflexes were normal in all animals, and 30 days when they were increased in all animals. This suggests that 14 days is a transition period during which the reflexes in some animals were only marginally increased (Yates et al., 2008a).

Passive exercise for 30 days instituted 1 week after transection (Tx + Ex) was able to prevent the loss of low frequency-dependent depression of the H-reflex (Reese et al., 2006). Further studies (data not shown) demonstrated that passive exercise instituted after hyper-reflexia had set in required a longer duration of exercise therapy, and longer durations of exercise produced greater savings in the reduction of hyper-reflexia once therapy ceased (Yates et al., 2008b).

In addition, results from our lab indicate that oral administration of modafinil (MOD, 4mg/kg, p.o.) over a period of 30 days, was able to prevent the loss of frequency-dependent depression of the H-reflex when tested at 10 Hz (Tx + MOD). At 5 Hz and 10 Hz, the H-reflex habituation in the Tx + 30D group differed ($p<0.01$) from the Control group, the MBET 30 days (Tx+ Ex), and the MOD group (Tx+ MOD). These results for the first time

suggest that pharmacological intervention with an agent that increases electrical coupling may be useful in reducing hyper-reflexia. These data indicate that MOD was as effective as passive exercise in normalizing low frequency-dependent depression of the H-reflex. These results also suggest the possibility that at least some of the changes occurring between 1 and 2 weeks after transection involve changes in gap junction function (Yates et al., 2009).

Effects on the stretch reflex

Using changes in stretch reflex windup to track the effects of transection, we found that the stretch reflex became hyperactive much later than the H-reflex, with the windup of plantarflexion torque and gastrocnemius EMG emerging 7 weeks after transection (Figure 2). Plotting the responses from various stretch intervals revealed that windup peaked approximately one second from the first stretch with habituation occurring by 2 seconds. Figure 3 shows peak windup of the stretch reflex torque for three groups. Animals not receiving an intervention (Tx) demonstrated significant windup at 7 weeks ($p=0.01^*$). In contrast, neither MBET (Tx + Ex) nor MOD treated animals (Tx + MOD) developed windup, and normalized torque at 7 weeks was significantly lower than in the animals with transection only (Tx) ($p=0.01$, and $p=0.05$, respectively).

These data demonstrate that a) the stretch reflex habituation becomes abnormal much later after transection than the H-reflex, b) MBET nevertheless could normalize the stretch reflex, and c) MOD was also able to prevent the changes in the stretch reflex in the absence of MBET. These findings suggest that, although the mechanisms behind hyper-reflexia and spasticity undergo changes with different time courses, the stretch reflex is responsive to MBET and MOD in a manner similar to the H-reflex. Are passive exercise (MBET) and MOD addressing the same underlying mechanism? The answer is not known. We do not yet know if passive exercise has its salutary effects by modulating electrical coupling and Cx-36, along with other mechanisms. The fact that the onset of hyper-reflexia (H-reflex) and exaggerated stretch reflex are different suggests that they are mediated by different mechanisms. Other studies have shown differences in these two measures in regard to sensitivity to GABAergic presynaptic inhibition (Morita et al., 1998), temporal dispersion of the afferent volley (Enriquez-Denton et al., 2002), and post activation depression (Grey et al., 2008). However, both measures are normalized in response to passive exercise and to MOD, suggesting related mechanisms. Much work needs to be undertaken before we can answer these questions, but these need not await the application of these novel therapeutic avenues to the SCI patient. We were successful in demonstrating that passive exercise using MBET could normalize hyper-reflexia in an ASIA B C7 SCI patient (Kiser et al., 2005). We believe this technology may also be fruitful in restoring muscle mass after prolonged (>6 months) MBET. Moreover, we filed a patent for the use of MOD for the treatment of hyper-reflexia and spasticity, and hope to institute clinical trials in the near future.

Electrical coupling

Role

The role of electrotonic coupling in modulating motor behavior has not been extensively studied. Interestingly, if the spinal cord of the rat is transected before 15 days of age, the animal regains the ability to generate locomotor movements on a treadmill, but if the Tx is performed after 15 days of age, this function is lost (Stelzner et al., 1975). The period in which the spinal cord is optimal for plastic changes in the rat has been determined to be 18 days of age (Fawcett, 2006). What is occurring in development that could contribute to the changes in plasticity at the end of the critical period? Electrical coupling of neurons via gap junctions have been shown to exist in early postnatal development of spinal motoneurons (Fulton et al., 1980). Motoneuron electrotonic coupling has been shown to decrease with

postnatal age in the rat from postnatal day 0 through postnatal day 14 (Walton and Navarrete, 1991). Such coupling may allow stronger synchronized contractions in weak muscles during development, for example, when chicks must break the eggshell at hatching (Wenner and O'Donovan, 2001). Does a correlation exist between the end of the critical period of developmental plasticity in the rat spinal cord and the decreased gap junction coupling observed? The decrease in coupling has been attributed to the development of fine motor control by independent recruitment of motor units (Bradley, 1990). These authors suggested that coupling of motoneurons by gap junctions is function specific with the most prevalent coupling between motoneurons innervating the same muscle, reduced with synergistic muscles, and absent with antagonist muscles (Bradley, 1990). A recent important study showed that there are populations of locomotion-related interneurons in the ventromedial gray matter that are electrically coupled (Hinckley and Ziskind-Conhaim, 2006), suggesting that not only motoneuron pools may be coupled.

In general, electrical synapses allow the reciprocal flow of ionic currents and small molecules between neurons, often providing synchronization of subthreshold and spiking activity (Connors and Long, 2004). Connexins form clusters of channels that allow direct cell to cell communication. Electrical communication between neurons has been attributed to gap junctions made up of Connexin 36 (Cx-36). In mammals, Cx-36 is specifically expressed in neurons (Condorelli et al., 1998). Deans et al. (Deans et al., 2001) studied the knockout mouse of Cx-36 and determined that many physiologic properties of the knockout were similar to the wild-type with the exception that electrical coupling in the knockout mouse was rare and weak compared with the wild-type supporting the theory that gap junctions comprised of Cx-36 are responsible for electrical coupling. The best-studied electrical synapses are in the inferior olive, where the oscillatory properties of single neurons endow the system with important dynamics, but it is the gap junctions that are needed for synchronized neuronal ensemble activity (Leznik and Llinas, 2005).

What function functional significance can be accorded to electrical coupling if Cx-36 knockout (KO) mice breathe, chew and walk? Several studies showed that while macroscopic motor activity patterns appeared normal in the Cx-36 knockout mouse, detailed analysis of motor patterns showed a 10–20 msec degradation in coordination (Placantonakis et al., 2004), and a delay >20 msec in the optokinetic reflex (Kistler et al., 2002). These studies taken together suggest that gap junctions confer an advantage in timing, probably due to their ability to promote coherence in brain rhythms.

We analyzed the level of Cx-36 protein in cored samples from the lumbar enlargement of these animals (Yates et al., 2008a, 2008b, 2009). Figure 4 shows that Cx-36 protein levels decreased compared to levels in intact animals at 7 days after transection, but slowly returned to control levels over the next time points. Immunoprecipitation followed by western blot was used to quantify the amount of Cx-36 in spinal cord. A transient decrease below the level of the lesion in Cx-36 protein levels after transection was observed. Figure 4 shows the Cx-36 western blot following immunoprecipitation from spinal cord from control rats or 7, 14 or 30 days after transection (Tx + 7D, Tx + 14D, or Tx + 30D) and after passive exercise or MOD (Tx + Ex, Tx + MOD). This figure also shows quantification of the data shown in the upper panel. Note that Cx-36 protein level decreased significantly at 7 days ($p > 0.01$).

We showed that after transection, Cx-36 protein transiently decreased by 7 days, but returned to control levels within 30 days. The return of Cx-36 control levels paralleled the onset of hyper-reflexia (Yates *et al.*, 2008a). In another study, we showed that when spinal transected rats were passively exercised, hyper-reflexia was normalized and Cx-36 levels were slightly higher than in unexercised rats, suggesting that exercise may change Cx-36

protein levels (Yates et al., 2008b). Hyper-reflexia could also be normalized with MOD (Yates et al., 2009), although there was no evidence for changes in protein levels. Therefore, while passive exercise and MOD could both target electrical coupling, they may do so in different ways.

Another 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 (Walton and Navarrete, 1991). As mentioned above, 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 (Hinckley and Ziskind-Conhaim, 2006). It is not known if MOD directly affects electrical coupling in the transected adult rat, but if it does, it may do so by influencing motoneurons, locomotion-related interneurons, and/or perhaps even GABAergic spinal interneurons, as it does in the brain (Garcia-Rill et al., 2007; Heister et al., 2007; Urbano et al., 2007). We are exploring the possibility that overall levels of Cx-36 protein may not yield an accurate picture of changes after spinal transection and treatments like exercise and MOD, but regional measures within the spinal cord may reveal differential changes in different regions. While a number of new questions are raised by these studies, our results strongly suggest that MOD may represent a valuable therapeutic adjunct to the treatment of SCI, and supports previous results in cerebral palsy. However, some of the gains noted in other studies may have been due to direct effects on spinal circuitry rather than cerebral in origin (Hurst and Cedrone, 2006; Hurst et al., 2006).

Future directions

The data summarized suggest a number of experiments to confirm the role of gap junctions in chewing, breathing and walking. While the involvement of electrical coupling has been tested in these systems using gap junction blockers, each of which has side effects, the role of MOD has not been tested. Our hypothesis would predict that MOD will increase the amplitude of oscillations in all of these systems, an effect that should be blocked by carbenoxolone and mefloquine (we recommend the use of multiple gap junction blockers that have different side effects). We would also predict that changes in Cx-36 protein should be evident in preparations in which each of these functions, such as synchronization, is dysregulated. Interestingly, a number of very disparate pathological conditions could be amenable to MOD therapy.

A more general direction is emerging from these results suggesting that it is rhythmicity that is the common factor in such disparate functions as breathing, chewing, and walking. Years ago, we proposed that regions of the brainstem that could be electrically (Atsuta et al., 1988) and chemically stimulated (Garcia-Rill et al., 1985; Atsuta et al., 1991), were not so much induced as “recruited” to result in stepping (Garcia-Rill and Skinner, 1988; Garcia-Rill, 1991). That is, these regions were proposed to be rhythmogenic rather than function specific. By this we mean that these centers are not “locomotor” per se, but rather rhythmogenic regions that are electrically recruited, and it is the synchronization across rhythms that result in overt movement. The idea is that the oscillatory coupling between cell groups in these regions is responsible for generating various frequencies. The same can be said for regions involved in breathing and chewing, and the differences between these systems may arise because of the differing biomechanical requirements. This would help explain the considerable similarities in the structure of the premotor neurons as well as the pattern generators modulating these rhythmic functions.

Conclusions

The role of electrical coupling in rhythm generating systems is essential. The data presented suggest that electrical coupling may be dysregulated in SCI, leading to some of the symptoms observed. A novel therapy for hyper-reflexia and spasticity may require modulation of electrical coupling using an agent that increases electrical coupling.

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Abbreviations

Cx-36	connexin 36
EMG	electromyogram
GABA	gamma amino-butyric acid
MBET	motorized bicycle exercise trainer
MOD	modafinil
SCI	spinal cord injury
Tx	transection

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Figure 1. H-reflex

H-reflex amplitude at 0.2, 1, 5 and 10 Hz for intact animals (Control, open circles, n=10), in rats 7 days (Tx + 7D, open squares, n=7), 14 days (Tx + 14D, filled triangles, n=7), 30 days after transection (Tx + 30D, filled squares, n=16), in rats 30 days after MBET treatment (Tx + Ex, open triangles, n=16), and in rats 30 days after daily Modafinil (Tx + MOD, filled circle, n=16). Frequency-dependent depression of the H-reflex at 0.2 Hz was designated 100%, and statistical comparisons made against the transection only - 30 days group. Note that a) hyper-reflexia does not set in until 14 days after transection, b) both passive exercise and MOD independently prevent hyper-reflexia after transection.

Figure 2. Stretch reflex windup after spinal transection in rats (n=8)

A series of ten dorsiflexion stretches ($600^{\circ}/s$, 20°) were performed at different intervals (1 sec, 0.4 sec and 0.25 sec) and over-plotted to show the time course of the windup and habituation of the stretch reflex. Integrated EMG of the lateral gastrocnemius and peak plantarflexion torque responses to repeated stretch were normalized to the first stretch of the series and plotted for 7, 21, 35, and 49 days post transection. Although some variance was observed, a consistent pattern of windup was not present until 49 days.

Figure 3. Effects of exercise and Modafinil on windup of the stretch reflex

Group averages are plotted for peak windup of plantarflexion torque at 7, 21, 35, and 49 days post transection (Tx). Mean values ($\pm$ SD) were plotted as a percentage of the baseline (first) stretch for data from trials with 0.4 sec interval between stretches. Animals in the transection only (Tx, $n=8$) group demonstrated significant windup at 49 days ($p=0.01^*$). The exercise (Tx + Ex, $n=8$) group did not develop significant windup but were significantly different than the Tx group ($p=0.01$). Animals treated with Modafinil (Tx + MOD, $n=8$) also failed to develop windup of the stretch reflex by 49 days, and peak torque was significantly lower than the Tx group ($p=0.05$).

Figure 4. Cx 36 protein levels transiently decrease after SCI

Upper panel, immunoprecipitation followed by western blot of Cx 36 protein. Lower panel, quantification of the western blot (arbitrary units). Adult rats underwent spinal cord transection. After 7 (Tx + 7D), 14 (Tx + 14D), or 30 (Tx + 30D) days following transection, the entire lumbar region of the spinal cord was assayed for Cx 36 protein levels. 7 days following transection, one group of rats was treated with passive exercise for 30 days (Tx + Ex), while another group was given MOD for 30 days (Tx + MOD), and their Cx 36 protein levels assayed. There was a transient decrease in Cx 36 protein after 7 days, but it returned to normal levels over the course of a few weeks.