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Administration of Phosphodiesterase Inhibitors and an Adenosine A1 Receptor Antagonist Induces Phrenic Nerve Recovery in High Cervical Spinal Cord Injured Rats

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Abstract

High cervical spinal cord hemisection interrupts the descending respiratory drive from the medulla to the ipsilateral phrenic motoneurons, consequently leading to the paralysis of the ipsilateral hemidiaphragm. Previous studies have shown that chronic oral administration of theophylline, a phosphodiesterase inhibitor and an adenosine receptor antagonist, can restore function to the quiescent phrenic nerve and hemidiaphragm ipsilateral to hemisection. Both of these actions of theophylline result in an increase in 3'-5'-cyclic adenosine monophosphate (cAMP). Furthermore, the *chronic* theophylline-mediated respiratory recovery persists long after the animals have been weaned from the drug. To date, the precise cellular mechanisms underlying the recovery induced by theophylline are still not known. Since theophylline has two modes of action, in the present study we tested whether *chronic* administration of pentoxifylline, a non-selective phosphodiesterase inhibitor, rolipram, a phosphodiesterase-4 specific inhibitor, and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), an adenosine A1 receptor antagonist, would induce recovery similar to that induced by theophylline in male Sprague Dawley rats following a left C2 spinal cord lesion. Recovery of left phrenic nerve activity was assessed at 5 or 10 days after the last drug administrations to assess the persistent nature of the recovery. Pentoxifylline, rolipram and DPCPX, all capable of modulating 3', 5'-cyclic monophosphate (cAMP) levels, brought about long-term respiratory recovery in the phrenic nerve ipsilateral to the left C2 lesion at 5 and 10 days after the last drug administration. Therefore, these results suggest that compounds capable of regulating cAMP levels may be therapeutically useful in promoting functional recovery following spinal cord injury.

Keywords

Cervical spinal cord injury; Rolipram; Pentoxifylline; DPCPX; Adenosine receptors; Phosphodiesterase inhibitors; cAMP; Plasticity; Motor recovery

Introduction

Respiratory insufficiency is the leading cause of morbidity and mortality in human spinal cord injury. Phrenic motoneurons (C3–C6 in rats) which innervate the diaphragm, receive descending respiratory drive from the rostral ventral respiratory group (rVRG) in the medulla.

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Spinal cord hemisection at the C2 level interrupts these descending respiratory pathways to the ipsilateral phrenic motoneurons, subsequently leading to the paralysis of the ipsilateral hemidiaphragm (Aserinsky 1961; Guth 1976). However, respiratory function can be restored to the previously paralyzed hemidiaphragm by increasing respiratory drive (Lewis and Brookhart 1951) or by allowing a sufficient time after hemisection for plasticity in the respiratory pathways to occur spontaneously (Nantwi et al. 1999). The restoration of hemidiaphragmatic function is thought to be mediated via the activation of a latent respiratory motor pathway which arises bilaterally from the rVRG and crosses the spinal midline below C2 (Fig. 1). The latent pathway is known as the “crossed phrenic pathway” (Moreno et al. 1992). The exact mechanisms involved in the induction or the maintenance of activity in the phrenic nerve ipsilateral to the hemisection have not been clearly demonstrated (Vinit et al. 2007; Fuller et al. 2003; 2005).

The crossed phrenic pathway can be activated either by increasing respiratory drive or via the administration of a variety of chemical compounds such as serotonin receptor agonists, NMDA receptor antagonists and theophylline (Alilain and Goshgarian, 2007; Ling et al. 1994; Zhou et al. 2001; Basura et al. 2002; Fuller et al. 2005; Nantwi et al. 1996; Nantwi and Goshgarian 1998). The various methods mentioned above activate the latent respiratory pathway transiently; however, only the chronic administration of theophylline induces plasticity in the respiratory pathways which results in recovery that persists days after the animal has been weaned from the drug (Nantwi et al. 2003). Nantwi et al. (2003) administered theophylline (3 times per day) orally for as little as three consecutive days to left C2 hemisectioned rats and assessed ipsilateral phrenic nerve recovery at 3, 7, 12 and 30 days after the last theophylline administration. In the above study, 3 days of theophylline administration was sufficient to bring about and maintain respiratory recovery that persisted for 3, 7, 12 and 30 days after the last administration. No other drug has been shown to activate the latent crossed phrenic pathway to induce a long lasting recovery of the phrenic nerve activity days after the animal has been weaned from the drug.

Theophylline is a non-selective phosphodiesterase inhibitor and an adenosine receptor antagonist that blocks both the A1 and A2 adenosine receptors. Theophylline was shown to mediate its *acute* effects on the activation of the crossed phrenic pathway primarily via the antagonism of adenosine A1 receptors (Nantwi and Goshgarian 2002). However, the *chronic* theophylline-induced mechanisms leading to the persistent activation of the crossed phrenic pathway have still not been delineated. As such, understanding the precise molecular basis of the theophylline-induced recovery of respiratory function following spinal cord injury in our model could provide important clues for the development of treatments that strengthen spared axonal connections permanently and facilitate functional recovery after spinal cord injury.

Theophylline can non-selectively block phosphodiesterase enzymes which are responsible for the cellular breakdown of 3'-5'-cyclic adenosine monophosphate (cAMP) and 3'-5'-cyclic guanosine monophosphate (cGMP) (Horn and McAfee, 1977). Furthermore, theophylline can also modulate cAMP levels by inhibiting the adenosine A1 receptors which normally prevent the synthesis of cAMP (Marks et al., 2005; Hogan et al., 1998). Both of the above actions of theophylline could lead to an increase in cAMP. Elevated levels of cAMP have been shown to be a powerful mediator of synaptic plasticity and memory in the central nervous system (Alberini, 1995; Lee et al. 2000; Nguyen and Woo 2003; Inan et al. 2006; Tronson et al. 2006; Schacher et al. 1988; Park et al. 2005). Following spinal cord injury, administration of cAMP analogs as well as pharmacological agents capable of elevating cAMP levels in neurons have been shown to promote regeneration and functional recovery (Pearse et al. 2004; Nikulina et al. 2004; Qiu et al. 2002a). Furthermore, increased cAMP levels have been associated with improvements in many neural functions including respiration (Ruangkittisakul and Ballanyi, 2006; Ballanyi et al., 1997). Therefore, it is reasonable to hypothesize that theophylline-

induced respiratory plasticity in C2 hemisectioned rats leading to the persistent recovery of the ipsilateral hemidiaphragm may be mediated by the drug's non-selective inhibition of phosphodiesterase enzymes and/or by the blockade of adenosine A1 receptors, both of which can modulate cAMP levels (Beavo et al. 1994; Dryden et al. 1988).

In the present study, we evaluated whether the chronic administration of pentoxifylline, a non-selective phosphodiesterase inhibitor, rolipram, a phosphodiesterase-4 specific blocker, and DPCPX, an adenosine A1 receptor antagonist, would restore left phrenic nerve activity after a left C2 spinal cord lesion and whether this activity would persist at 5 and 10 days after the last drug administration. Results from this study indicate that all of the above drugs can bring forth respiratory recovery in the ipsilateral phrenic nerve that is maintained for several days after the last drug administration.

Materials and methods

Animal surgery protocol

Male Sprague-Dawley rats (age:3–6 months,295–480g) were deeply anesthetized with a mixture of ketamine (70mg/kg, i.p) and xylazine (20mg/kg, i.p) and the surgical site (dorsal aspect of the neck) was shaved and prepared for aseptic surgery. A midline incision was made through the skin and paravertebral muscles above the first four cervical vertebrae to expose the second vertebra. A C2 laminectomy and durotomy was performed to expose the spinal cord. A left spinal cord lesion below the C2 dorsal roots was made using microscissors. Care was taken to extend the lesion from the midline through the most lateral extent of the spinal cord. The paravertebral muscles were sutured with absorbable suture and the skin was closed with wound clips. Immediately after the spinal cord lesion, the functional effectiveness of the lesion in eliminating respiratory-related activity ipsilaterally was verified by electromyography of the diaphragm.

The abdominal surface of the diaphragm was exposed by making a horizontal incision caudal to the rib cage. Bipolar recording electrodes were inserted into the crural regions of left and right hemidiaphragms to assess respiratory-related diaphragmatic activity. Following the diaphragmatic assessment the muscles were sutured with absorbable suture and skin closed with wound clips. The wounds were cleaned and the animals were injected with buprenorphine (0.01 mg/kg s.c.) in 10mls of saline for post-surgical pain control. After a short period of recovery on a heating blanket the animals were returned to clean cages with food and water provided ad libitum. The Wayne State University Animal Investigation Committee approved all experimental procedures and animal care.

Experimental protocol

Left C2 lesioned rats were divided into 8 groups. Rats in group 1 (N=10) and 2 (N=10) were administered 20 mg/kg of pentoxifylline orally (3 times/day) for 3 days starting the day after the lesion. Electrophysiological assessments of the phrenic nerves were performed at 5 and 10 days following the last drug administration respectively. Rats in group 3 (N=10) and 4 (N=10) were injected with 2 mg/kg of rolipram (2 times/day i.p.) for 3 days and phrenic nerve assessments were again performed at 5 and 10 days following the last rolipram administration respectively. Rats in groups 5 (N=10) and 6 (N=10) were administered 0.1 mg/kg of DPCPX (2 times/day i.p.) for 3 days and assessed for respiratory recovery at 5 and 10 days after the last DPCPX administration respectively.

Finally, the last two groups of rats (Group 7 and 8, N=10/group) were subjected to a left C2 lesion and diaphragmatic assessments and were treated with vehicle control solutions. The rats in group 7 received oral administration of saline (3times/day) for 3 days and served as vehicle

control for pentoxifylline treated rats, while the rats in group 8 served as vehicle controls for rolipram and DPCPX administered rats and received 10% DMSO dissolved in saline injected intraperitoneally (2 times/day) for three days. These vehicle treated control rats were also assessed for respiratory recovery via phrenic nerve recordings at 5 and 10 days after the last vehicle administration.

Electrophysiology

Before electrophysiological assessments, animals were anesthetized with urethane (1.6 mg/kg, i.p.). Following a tracheotomy an endotracheal tube (PE 160) was inserted into the trachea and the animals were placed on a rodent mechanical ventilator (Harvard Apparatus, model 683). The rats were ventilated with a mixture of oxygen and air that yielded an FIO₂ value of approximately 50%. The femoral vein and femoral artery were cannulated (PE 50) to administer drugs and monitor blood pressure, respectively. The animals were vagotomized bilaterally to eliminate entrainment with the ventilator and paralyzed using pancuronium bromide (0.5 mg/kg, i.v.). Body temperature was maintained at 37°C with a rectal probe and a thermostatic pad (Harvard). Finally, the phrenic nerves were exposed in the neck via a ventral approach and placed on bipolar platinum electrodes for recording. The phrenic nerve signals were filtered (0.1–3 kHz) and amplified (10 K) using a Tektronix 502 AM amplifier.

The end-tidal CO₂ was monitored using a CO₂ monitor (Capnograd, Novamatrix, Model 1265, Wallingford, CT). The ventilator pump rate and volume were gradually increased until phrenic inspiratory bursting failed to appear and at that point the ventilator rate and volume were decreased until the inspiratory bursting reappeared. The rats were ventilated at 3–5 mmHg above the end-tidal CO₂ value at which phrenic inspiratory bursting returned by adjusting the ventilator for the entire experimental protocol. Bilateral phrenic nerve activity was recorded for approximately 60–90 minutes after obtaining the apneic threshold.

Data analysis

Right and left phrenic nerve recordings were rectified and integrated (time constant = 100ms) using Spike2 data analysis software (v 4.13, CED Cambridge, UK). Integrated phrenic nerve signals were analyzed for respiratory frequency, peak inspiratory burst amplitude, duration and area for a period of 30 seconds after waiting a minimum of 30 minutes. The 30 second window for analysis was selected between 30 and 90 minutes after the establishment of the baseline phrenic nerve activity. Peak burst amplitude, duration and area of the recovered left phrenic nerve respiratory activity was expressed as a percentage of right phrenic nerve activity in the same animal. In addition, raw values obtained for peak burst amplitude, duration and area of the inspiratory bursts on the right phrenic nerve of drug treated rats were compared to vehicle treated rats. Finally, peak burst amplitude and area of inspiratory bursts of the contralateral and ipsilateral phrenic nerve activity of the injured rats treated with drugs were expressed as a percentage of contralateral phrenic nerve activity of injured rats that were treated with vehicle solutions.

Statistical analysis was performed using commercially available Systat software (Systat 10.2, Richmond, CA). Statistical comparison of the effect of drug and weaning period on the expression of left phrenic activity was determined using a one-way multiple analysis of variance. Studentized residual (raw residuals divided by the standard error of estimate) was used to determine outliers and statistical comparisons were performed on results both with and without the outliers. Group differences were considered statistically significant when $p < 0.05$. All data is expressed as means $\pm$ SE.

Results

Functional effectiveness of the high cervical spinal cord injury was assessed using diaphragmatic EMG recordings immediately after the lesion. There was a total absence of respiratory related activity on the left hemidiaphragm in contrast to the right hemidiaphragm indicating the disruption of the major respiratory pathways from the rVRG to the ipsilateral phrenic nucleus (Fig 2). EMG recordings were used to assess the extent of the lesion rather than phrenic nerve recordings because a considerable amount of scar tissue would have invaded the exposed left phrenic nerve recording site and this may have affected the subsequent assessment of functional recovery after drug treatment.

All rats treated with pentoxifylline, rolipram and DPCPX demonstrated robust recovery of respiratory related motor activity in the left phrenic nerve, while the twenty rats in the control groups that were treated with either saline or 10% DMSO in saline failed to exhibit any respiratory related activity in the left phrenic nerve at both 5 and 10 days after the last vehicle administration. The latter result from the control animals suggests that our C2 lesion interrupted all of the descending bulbospinal input from the rVRG to the ipsilateral phrenic motoneurons. Representative tracings of right and left phrenic neurograms illustrating 10 seconds of recovered activity in an injured rat treated with pentoxifylline, rolipram and DPCPX and weaned for 5 and 10 days are presented in Figure 3. The left phrenic nerve of rats that were treated with saline or 10% DMSO plus saline did not display any inspiratory-related activity. Figure 3 illustrates representative tracings of integrated right and left phrenic neurograms taken 5 and 10 days after the last administration of saline. In addition, to demonstrate the stability of our preparation, we have also included an example of a condensed phrenic neurogram showing a 30 minute period of stable bursting activity recorded 5 days after saline (Fig. 4A) and rolipram treatment (Fig. 4B).

The respiratory frequency (mean $\pm$ SE) of pentoxifylline treated rats weaned for 5 and 10 days were 53 ± 6 and 54 ± 7 breaths/min respectively (Fig. 5). The respiratory frequency of injured rats administered saline (pentoxifylline control) and weaned for 5 and 10 days were 55 ± 3 and 59 ± 6 breaths/min respectively (Fig. 5). Thus, pentoxifylline treatment or the weaning period did not produce a significant difference in breathing frequency compared to vehicle treated control rats.

The respiratory frequency of injured rats administered with 10% DMSO dissolved in saline (rolipram and DPCPX control) and weaned for 5 and 10 days were 52 ± 5 and 54 ± 4 breaths/min respectively (Fig. 5) which was not significantly different from those administered saline alone at both time points. The breathing frequency of rolipram treated rats at 5 and 10 days were 61 ± 5 and 54 ± 6 breaths/min respectively. Thus, at 5 days, rolipram treated rats had a significantly higher respiratory rate compared to control as well as pentoxifylline treated rats at the same time point. However, 10 days after rolipram treatment, the frequency was not significantly different from control or pentoxifylline treated rats (Fig. 5).

Respiratory frequency of rats treated with DPCPX at 5 and 10 days after the drug treatment was 57 ± 5 and 55 ± 3 breaths/min respectively (Fig. 5). Breathing frequency was not significantly different as a result of DPCPX treatment or the weaning period. Also, DPCPX treated rats did not have a significantly different respiratory rate compared to those rats that received the other drug treatments.

In order to determine if one drug was more effective in inducing recovery than the others, the extent of left phrenic nerve recovery induced by the three drugs was quantified as a percentage of right phrenic nerve activity at 5 and 10 days after the last drug administration (Fig 6). Inspiratory burst amplitude, duration and area were not significantly different at either 5 or 10 days after the last pentoxifylline administration. Similar to the pentoxifylline treated rats, spinal

cord injured rats that were injected with rolipram also exhibited robust recovery in the left phrenic nerve. Quantitative analysis of rolipram induced phrenic nerve recovery failed to show any significant differences in peak burst amplitude, burst duration or burst area of integrated respiratory bursts compared to pentoxifylline treated rats at the two time points.

The adenosine A1 receptor antagonist, DPCPX, was also capable of restoring motor recovery in the left phrenic nerve. The peak burst amplitude, burst duration and burst area of DPCPX-induced left phrenic nerve activity expressed as a percentage of right phrenic nerve also failed to show significant differences when compared to the other two drugs at the two time points.

Rats that were administered pentoxifylline, rolipram, and DPCPX had 1, 2 and 2 outliers respectively. Statistical analysis was performed with the outliers and after the removal of the outliers. Group significances were not altered as a result of removing the outliers. These outliers were removed from the graphical representation of the results presented in the paper.

Lastly, inspiratory burst activity of the ipsilateral and contralateral phrenic nerve of rats treated with pentoxifylline, rolipram and DPCPX was compared to those that were treated with control solutions. There was no difference in right phrenic nerve output between the two vehicle treated control groups at both 5 and 10 days after treatment. At 5 and 10 days after pentoxifylline and DPCPX treatments, the contralateral phrenic nerve output was not significantly altered compared to vehicle treated controls. However, after 5 days of weaning, peak inspiratory burst amplitude and burst area of rats treated with rolipram were significantly higher than control ($p=0.006$). This difference in peak height and burst area of rolipram treated rats compared to vehicle treated rats persisted even at 10 days after the last drug administration. The effect of drug treatment on the contralateral and ipsilateral phrenic nerve activity is expressed as a percentage of the contralateral phrenic nerve activity of vehicle treated control rats (Fig 7).

In summary, rats treated with pentoxifylline, rolipram and DPCPX all brought about phrenic nerve recovery in left C2 spinal cord injured rats. Quantitative analysis of drug induced recovery in the ipsilateral phrenic nerve at 5 and 10 days of weaning failed to show any significant differences in peak burst amplitude, burst duration or burst area of integrated respiratory bursts with the various drug treatments (Fig. 6). However, rolipram treatment did result in increasing the phrenic nerve output of the contralateral phrenic nerve compared to those treated with vehicle controls at 5 and 10 days after the last drug administration. In contrast to the rolipram treated rats, the contralateral phrenic nerve output of those treated with pentoxifylline and DPCPX were not significantly different from the contralateral phrenic nerve activity of the vehicle treated controls. Furthermore, after 5 days of weaning, rolipram treated rats exhibited a significantly higher breathing frequency compared to the vehicle treated controls as well those rats that received pentoxifylline. This difference in respiratory frequency, however, did not persist 10 days after the last drug administration.

Discussion

The results from this study suggest that following a left C2 spinal cord lesion, ipsilateral phrenic nerve activity can be restored either by the antagonism of adenosine A1 receptors or by preventing the breakdown of cAMP with phosphodiesterase inhibitors. Specifically, three day administration of phosphodiesterase blockers, pentoxifylline (non-selective phosphodiesterase inhibitor) and rolipram (phosphodiesterase 4 specific inhibitor) as well as the adenosine A1 receptor antagonist, DPCPX in spinal cord injured rats all brought about equivalent respiratory recovery in the phrenic nerve ipsilateral to the lesion. While pentoxifylline and DPCPX were able to promote respiratory recovery in the ipsilateral (left) phrenic nerve, they failed to produce any significant effects on contralateral phrenic nerve output. Rolipram, however, enhanced the

right phrenic nerve output compared to vehicle treated controls, in addition to inducing recovery in the left phrenic nerve.

In this study, it was of interest to find that the phosphodiesterase-4 specific inhibitor, rolipram was the only drug that increased respiratory frequency and increased motor output in the contralateral phrenic nerve in addition to inducing ipsilateral phrenic nerve recovery. In support of our findings, using an *in vitro* brain stem-spinal cord preparation, Ruangkittisakul and Ballanyi (2006) demonstrated that rolipram application significantly reincreased respiratory frequency after nociception-induced respiratory depression. In our model of spinal cord injury the increase in frequency suggests that rolipram may be promoting plasticity concomitantly within the spinal cord and the brainstem to promote phrenic nerve recovery. This dual site of action may be related to the increase in right phrenic nerve activity which was seen only with rolipram and not the other drugs, but the precise mechanism for this effect is still not known.

As mentioned in the introduction, it has been well established that theophylline, a non-selective phosphodiesterase inhibitor and an adenosine receptor antagonist, is capable of activating a latent respiratory motor pathway to promote respiratory recovery following left C2 spinal cord hemisection (Nantwi, El-Bohy et al. 1996). It has been shown that a single administration of theophylline 24 hours after a left C2 hemisection will activate the latent respiratory motor pathways and restore phrenic nerve function, but this single drug dose lasts for only up to 3 hours. The predominant mechanism involved in this theophylline-mediated short-term phrenic motor recovery is thought to be via the antagonism of adenosine A1 receptors (Nantwi and Goshgarian 1998b; Nantwi and Goshgarian 2002). Later, it was also demonstrated that theophylline administered chronically for as little as 3 days (3 times per day) in C2 hemisectioned rats would restore activity in the ipsilateral phrenic nerve that persists for as long as 30 days after the last drug administration. Although extensive work by Nantwi and colleagues have implicated a key role for adenosine A1 receptor antagonism in the short-term recovery mediated by theophylline, the theophylline-induced long-term respiratory recovery has not been heretofore elucidated (Nantwi and Goshgarian 2002).

Although theophylline has been in use clinically for many years for the treatment of a number of pulmonary diseases, its mode of action is not very apparent (Milgrom and Bender 1993). Firstly, theophylline is a weak and non-specific inhibitor of phosphodiesterases, which can modulate intracellular levels of cAMP and cGMP by preventing the degradation of cyclic nucleotides (Horn and McAfee, 1977). Secondly, theophylline also non-selectively antagonizes the adenosine receptors with higher affinity for A₁ and A₂ receptors than A₃ receptors. Adenosine, by way of its G-protein coupled receptors, can either inhibit (A₁ and A₃) or stimulate (A_{2a} and A_{2b}) the synthesis of cAMP (Dunwiddie and Masino 2001). Adenosine acts at both pre- and post-synaptic neurons to modulate synaptic communications (Proctor and Dunwiddie 1987). Adenosine is also known for its tonic inhibitory effect on the excitability of neurons in the nervous system (Gundlfinger et al. 2007). Inhibition of adenosine A1 receptors can lead to enhanced synaptic excitability via increase in intracellular calcium concentration in the presynaptic cell (Schwartz et al., 2003). Therefore, it is plausible that theophylline induces respiratory plasticity in our model of spinal cord injury via adenosine receptor antagonism and/or by the non-specific blockade of phosphodiesterases. Both approaches could potentially lead to the strengthening of existing but inefficient synaptic connections by increasing cAMP levels in neurons (Dunwiddie and Masino, 2001; Beaumont and Zucker, 2000; Dunwiddie and Fredholm, 1989; Schacher et al., 1988).

Despite the fact that theophylline has been in use clinically for decades and is able to produce robust respiratory recovery in our animal model of spinal cord injury, it does not appear well-suited to restore respiratory muscle strength after spinal cord injury in humans. When theophylline was tested for its efficacy in improving respiratory function in human spinal cord

injured patients in a placebo-controlled, double blind crossover study, significant differences between placebo control and theophylline treated patients were not seen (Bascom et al. 2005). Failure to detect significant improvements may have been a result of the variability in the type and extend of spinal cord injury as well as the small number of patients that completed both phases of the study (Bascom et al. 2005). Therefore, elucidating the molecular mechanisms of the theophylline-induced recovery in our rat model of spinal cord injury could lead to the discovery of drugs that are better suited for the treatment of respiratory insufficiency that follows human spinal cord injury. In this study, we evaluated whether theophylline-induced phrenic recovery in our model could be reproduced by the following three approaches: 1) inhibiting adenosine A1 receptors 2) non-selectively blocking phosphodiesterases and 3) selectively blocking the cAMP-specific phosphodiesterase-4 isoenzyme. Indeed, all three of the above methods re-established motor function in the phrenic nerve ipsilateral to the injury. Although, it is possible that the theophylline mediated activation of the crossed phrenic pathway via adenosine A1 receptor antagonism may be cAMP-dependent, it should be pointed out that the results obtained in this study with DPCPX could be due to the ability of adenosine receptor antagonism to release intracellular calcium (Schwartz et al., 2003; Gundlfinger et al., 2007). Nonetheless, it is still reasonable to hypothesize that any compound capable of modulating cAMP levels in the central nervous system may be potentially targeted for promoting plasticity in our model.

The above hypothesis is supported by the findings of two subsequent studies in our lab currently being prepared for publication. In the first study, an acute single administration of rolipram after a left C2 spinal cord lesion was able to significantly increase cAMP levels in the cervical spinal cord as determined by ELISA analysis and induce ipsilateral phrenic nerve recovery. In the second study, direct intrathecal application of the cAMP analog, db-cAMP at the level of the phrenic nucleus in left C2 spinal cord injured rats resulted in restoring function to the phrenic nerve ipsilateral to the injury. Thus, manipulations which result in increasing either endogenous or exogenous cAMP will initiate recovery in our spinal cord injury model.

In fact, there is ample evidence in the literature that suggests that pharmacological compounds that increase cAMP will induce plasticity and recovery in other spinal cord injury models as well. Increasing cAMP levels by a variety of means has been shown to promote axonal regeneration and overcome the barriers to axonal growth in the injured axons of the central nervous system (Qiu et al. 2002a; Qiu et al. 2002b; Pearse, Pereira et al. 2004). Moreover, direct application of cAMP analogs have been shown to promote central sensory neuron regeneration (Neumann, Bradke et al. 2002). In particular, administration of rolipram after spinal cord injury has been shown to promote axonal regeneration and functional recovery while attenuating glial scar formation (Nikulina, Tidwell et al. 2004). It needs to be stressed here that in addition to promoting regeneration of injured axons, direct and indirect elevation of cAMP levels can increase the efficacy of existing synaptic connections. For instance, in the hippocampus, cAMP has been shown to play a pivotal role in memory formation, which requires the strengthening of existing synaptic connections (Barad et al. 1998; Navakkode et al. 2004). Several groups have shown that rolipram improves cognitive function (Zhang et al. 2000; Gong et al. 2004) and reverses memory deficits caused by scopolamine (Zhang and O'Donnell 2000; Rutten et al. 2006). Furthermore, respiratory depression in general can be reversed by the administration of cAMP elevating agents (Arata et al. 1993). Stimulation of the cAMP pathway reversed ventilatory depression in human studies as well as in vitro and in vivo animal studies (Lipworth, 2005; Ruangkittisakul and Ballanyi 2006; Sturton, 2002). In an *in vitro* brainstem spinal cord preparation, respiratory depression caused by opioids was reversed by applying the phosphodiesterase-4 specific inhibitor, rolipram (Ruangkittisakul and Ballanyi 2006). However, no one has previously examined the effectiveness of a specific PDE4 inhibitor for its ability to induce phrenic nerve recovery following spinal cord injury. Moreover,

modulating cAMP levels via the specific inhibition of PDE4 enzymes may be a promising strategy for promoting respiratory recovery after spinal cord injury in man.

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Figure 1.
Schematic illustrating the crossed phrenic pathways and the left C₂ spinal cord lesion.

Figure 2. A representative electromyographic (EMG) recording taken following a left C2 spinal lesion. A total absence of EMG activity in the left hemidiaphragm (LHD) compared to the presence of respiratory activity in the right hemidiaphragm (RHD) is indicative of a functionally complete lesion.

Figure 3. Representative contralateral (right, R) and ipsilateral (left, L) integrated phrenic neurograms from left C2 lesioned rats treated with pentoxifylline, rolipram, DPCPX and vehicle control at 5 and 10 days after weaning.

Figure 4. Examples of ipsilateral (Left) and contralateral (Right) phrenic nerve activity (30 minutes) from left C2 spinal cord lesioned rats treated with saline (A) and rolipram (B) recorded 5 days after the last drug administration.

Figure 5.

Graphs demonstrating the respiratory burst frequency of left C2 lesioned rats treated with vehicle controls, pentoxifylline, rolipram and DPCPX after 5 and 10 days of weaning. Respiratory frequency was not significantly different between the two control groups or those treated with pentoxifylline and DPCPX at both time points. 5 days after weaning rolipram treated rats exhibited a significantly higher respiratory frequency compared to control (saline + DMSO) (*) and pentoxifylline treated rats (#).

Figure 6.

Quantitative summary of drug-induced respiratory recovery in the left phrenic nerve expressed as a percentage of right phrenic nerve activity. Left C2 lesioned rats treated with pentoxifylline, rolipram, and DPCPX all exhibited equivalent respiratory recovery in the left phrenic nerve at 5 and 10 days after the drug treatments. Peak inspiratory burst amplitude (A), duration (B) and area (C) of the left phrenic neurograms were expressed as a percentage of the right phrenic neurograms of the same rat. Vehicle treated control rats failed to show any left phrenic nerve recovery at 5 and 10 days after the treatments.

Figure 7.

Inspiratory phrenic nerve burst amplitude (A and B) and burst area (C and D) of spinal cord injured rats treated with vehicle control (con), pentoxifylline (Ptx), rolipram, and DPCPX at 5 and 10 days after weaning. The peak amplitude and area of ipsilateral and contralateral integrated phrenic nerve activity of injured rats treated with pentoxifylline, rolipram and DPCPX are normalized to the peak amplitude and area of contralateral phrenic nerve activity of rats treated with vehicle control. A: Integrated peak amplitude after 5 days of weaning. B: Integrated peak amplitude after 10 days of weaning. C: Integrated burst area after 5 days of weaning. D: Integrated burst area after 10 days of weaning. * Different from contralateral phrenic nerve activity of vehicle treated control. $P < 0.05$.