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Exercise modulates microRNAs that affect the PTEN/mTOR pathway in rats after spinal cord injury

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Abstract

We investigated microRNAs (miRs) associated with PTEN/mTOR signaling after spinal cord injury (SCI) and after hind limb exercise (Ex), a therapy implicated in promoting spinal cord plasticity. After spinalization, rats received cycling Ex 5 days/week. The expression of miRs, their target genes and downstream effectors were probed in spinal cord tissue at 10 and 31 days post injury. Ex elevated expression of miR21 and decreased expression of miR 199a-3p correlating with significant change in the expression of their respective target genes: PTEN mRNA decreased and mTOR mRNA increased. Western blotting confirmed comparable changes in protein levels. An increase in phosphorylated-S6 (a downstream effector of mTOR) within intermediate grey neurons in Ex rats was blocked by Rapamycin treatment. It thus appears possible that activity-dependent plasticity in the injured spinal cord is modulated in part through miRs that regulate PTEN and mTOR signaling and may indicate an increase in the regenerative potential of neurons affected by a SCI.

The mammalian target of rapamycin (mTOR) is a key factor in an intracellular signaling pathway that regulates protein synthesis, cell growth and proliferation (Takei et al., 2001; Gingras et al., 2004; Hay and Sonenberg, 2004; Lenz and Avruch, 2005; Jaworski and Sheng, 2006). Studies focused on intrinsic changes within the damaged neuron and its axon have identified the mTOR signaling pathway as a critical regulator of process outgrowth, regeneration and synaptic plasticity in the damaged central nervous system (Park et al., 2010). Conditional deletion of PTEN, an upstream inhibitory mediator of mTOR, led to increased mTOR expression and robust axonal elongation and regeneration in the injured optic nerve (Park et al., 2008). Using similar genetic manipulation of PTEN after spinal cord injury elicited extensive regeneration of corticospinal tract axons through the lesion (Liu et al., 2010b). Exercise (Ex) is an effective, non-invasive therapy that maintains hindlimb muscle mass (Houle et al., 1999), stabilizes rhythmic firing patterns of lumbar motoneurons (Beaumont et al., 2004; Ollivier-Lanvin et al., 2010) and improves functional motor and sensory recovery after SCI (Hutchinson et al., 2004; Sandrow-Feinberg et al., 2009). Anatomical and biochemical plasticity in the spinal cord (Tillakaratne et al., 2000), increased levels of neurotrophic factors in muscle and spinal cord tissue (Gomez-Pinilla et

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al., 2002; Dupont-Versteegden et al., 2004; Hutchinson et al., 2004; Ying et al., 2005) and decreased inflammation in the spinal cord (Sandrow-Feinberg et al., 2009) all are positive features of this therapeutic intervention.

Micro RNAs (miRs) are a class of small, non-coding RNAs whose mature products are ~18-25 nucleotides long that control mRNA expression, protein production and cell function by silencing translation or by destabilization of target mRNAs (Filipowicz et al., 2008). Protein production is decreased and the ultimate consequences depend upon the function of the targeted mRNAs. SCI alters miR expression involved in many of the secondary injury responses including oxidative stress, inflammation and apoptosis (Liu et al., 2009; Liu et al., 2010a) and modulates the expression of their target genes. Recent data from our laboratory revealed that cycling Ex after SCI influences the expression of microRNAs (miRs) associated with apoptotic pathways (Liu et al., 2010a), eventually leading to decreased levels of caspases in the injured spinal cord. Because we found that part of the apoptosis pathway affected by Ex included PTEN we tested whether this effect of Ex on the PTEN/mTOR pathway could be a possible mechanism for activity dependent plasticity that is observed with training of spinalized animals. We characterized gene and protein expression of mTOR, its upstream modulators TGF α , AKT, and PTEN; as well as its downstream effectors eif-4E, 4E-BP1, S6K1 and S6 (Figure 1) in the lumbar spinal cord after complete transection. In knockdown experiments, we administered Rapamycin to spinalized rats to block the Ex-induced activity of mTOR and observed changes in gene and protein expression similar to levels seen with SCI alone. These findings indicate that cycling Ex represents an alternative to genetic modulation of components of the PTEN/mTOR pathway that also may provide a means for non-invasive potentiation of the regenerative effort of neurons affected by SCI.

METHODS

Adult, female Sprague-Dawley rats (225-250g) were divided into 6 groups (n=6 for each group, 36 total): uninjured control, transected for 10 days (Tx10d), transected for 10d with cycling exercise (Tx+Ex 10d), transected for 31d (Tx31d), and transected for 31d with cycling exercise (Tx+Ex 31d), transected for 10d with cycling exercise and systemic Rapamycin treatment (Tx+Ex+Rap). The animal use protocol was approved by Drexel University's Institutional Animal Care and Use Committee.

Spinal cord transection

Complete spinal cord transection was performed at thoracic (T) 10 as described previously (Liu et al., 2010a). Briefly, rats were anesthetized with isoflurane (2% in O₂). Laminectomy of the ninth thoracic vertebra exposed the dorsal surface of the T10 spinal cord. Meningeal membranes were opened and gentle aspiration created a 2 mm long complete transection lesion cavity. The dura was closed with 10-0 sutures and overlying muscles were closed in layers. After surgery, bladders were manually expressed 2-3 times daily until reflex voiding returned. Ampicillin (100 mg/kg, sc) was administered daily for 7 days to prevent infection; Buprenorphin (0.05 mg/kg, im) was given for 3 days as an analgesic and lactated Ringer's solution (5 ml daily, sc) was given for 3 days post injury to maintain animal hydration.

Cycling exercise

Details of this passive form of hindlimb exercise have been provided previously (Houle et al., 1999). Rats received 2 thirty minute sessions of passive cycling exercise (45 rpm) with a 10 minute rest between sessions, starting 5 days after SCI and continuing for 5 days per week until the end of the experiment. Full range of motion of the hindlimbs occurred through each cycle of the pedals. Rats in the Tx+Ex 10d and Tx+Ex+Rap group were

exercised for 5d, while rats in the Tx+Ex 31d group were exercised for a total of 20 days. Animals in the Tx+Ex+Rap group received every other day injections of Rapamycin (i.p., 6 mg/kg, LC Laboratories, Woburn, MA) beginning on the day of injury.

Tissue collection for RNA and Protein Analyses

All rats were given a lethal dose of Euthasol (390 mg/kg, i.p., J.A. Webster, Sterling, MA.), and L4-6 spinal cord segments were dissected, divided at the midline into two equal pieces, and frozen in liquid nitrogen. Rats in the Ex groups were sacrificed approximately 1 hour after their final training session.

Total RNA isolation and quantitative cDNA and miRs Q-PCR

Total RNA from one half of the L4-6 spinal cord was isolated with an RNeasy Mini kit (QIAGEN, Valencia, CA). cDNA synthesis was carried out with 500ng of total RNA using a RT First Strand Kit (SA Biosciences, Frederick, MD). Setup for the cDNA Q-PCR reaction was standard for all samples: all primers were ordered from Integrated DNA Technologies Inc. (San Diego, CA) and are listed in Table 1. Each 25 μ L reaction contained 12.5 μ L of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 1.5 μ L (4 μ M) each of forward and reverse primers, 1 μ L of cDNA, and 8.5 μ L of nuclease free water. The miRs Q-PCR system from Applied Biosystems (Foster City, CA) was used in a 2-step reaction: reverse transcription (RT) followed by PCR. The RT reactions used 30ng of total RNA, 3 μ L 5X miR RT looped-primers, 1.5 μ L 10X RT buffer, 1 μ L reverse transcriptase, and 0.188 μ L RNase inhibitor and water to a 15 μ L final volume, RT reactions were incubated for 30 min at 16°C and at 42°C. The miRs Q-PCR reaction contained the following: 10 μ L of TaqMan 2X universal PCR master mix, 1 μ L of 20X TaqMan miR primers, 1.33 μ L RT product and 7.67 μ L nuclease free water. Q-PCR primers and their sequence are listed in Table 2. All samples were run in duplicate with a MyiQ RealTime Detection System (Bio-Rad) and crossing thresholds were averaged for each rat. Data was analyzed by software supplied with the MyiQ system which uses a modified $2^{-\Delta\Delta CT}$ method described by Liu et al. (Liu et al., 2010a). All mRNA expression data was normalized to reference RNAs GAPDH and 18s, and all the miR expression data was normalized to reference miR U6 (Applied Biosystems). Experimental group expression was normalized to control group levels which were set at a relative expression of 1.

Western Blot Analysis

Protein was extracted from the other half of the L4-6 spinal cord using ice cold RIPA buffer (Roche) as described previously (Liu et al., 2010a). Samples were homogenized by sonication and centrifuged at 14,000 rpm at 4°C for 30 min. Supernatants were collected and stored at -80°C. Standard Laemmli buffer was added and samples were boiled for 5 min. Equal amounts of protein were resolved in gradient 7.5 % SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad) overnight. Membranes were blocked with 5% non-fat milk in Tris buffer saline, 0.1% Tween (TTBS) for 1 hour, and were incubated at 4°C overnight with one of the following primary antibodies: anti-AKT / PKB (0.5 μ g/ml), anti-mTOR (1:1,000), and anti-S6K1 (1:1,000; AKT / mTOR / S6K Pathway Explorer Antibody Mini Pack; Millipore), Anti Phospho-S6 Ribosomal Protein (Ser235/236) (1:1000; Cell Signaling Technology), Anti-phospho-PTEN (Ser380) (1:1000; Millipore). Goat anti-rabbit or mouse HRP-conjugated immunoglobulins (Covance, Berkeley, CA) were used as the secondary antibody (dilution 1:5,000) for 1 hour at room temperature. Membranes were washed and immunopositive bands were visualized using Western Lightning ECL (PerkinElmer) and Blue Basic Autorad film (ISC Bio Express). After stripping, membranes were re-probed with mouse monoclonal antibody β -actin (dilution 1:15,000, Sigma, St. Louis, MO) as an internal control for loading and transfer of

proteins. Optical densities of immunopositive bands were analyzed using GeneSnap and GeneTools (Syngene, Frederick, MD) and normalized to β -actin levels.

Immunohistochemistry and Quantification

At 10 dpi, rats were perfused with 0.9% saline followed by 4% paraformaldehyde; lumbar spinal cords were dissected, cryoprotected in 30% sucrose, and sectioned on a cryostat (25 μ m). To detect phosphorylated S6, sections were incubated overnight with a primary antibody against p-S6 (1:250; Cell Signaling Technology), followed by goat anti rabbit secondary antibody (1:500; Sigma, St. Louis, MO), and visualized using DAB (Sigma). Immunopositive non-motoneurons in the intermediate gray and dorsal horn were counted on five sections/rat distributed throughout the L4-6 spinal cord. The mean number of non-motoneurons per section was calculated for each rat. To quantify the degree of ps6+ immunostaining within lamina IX of the ventral horn, we quantified using techniques adapted from Popovich et al. (1997). Optical density thresholds were manually selected for positively labeled tissue (MetaMorph Premier Imaging System, Universal Imaging Corporation, Downingtown PA) and were quantified as the proportional area of positively stained tissue within a specific region. Proportional area measurements of ps6 labeling within lamina IX of the lumbar cord were taken within the focal plane of the image.

Statistical Analysis

Statistical analysis was performed with PASW Statistics v.18 (SPSS Inc.). ANOVA was used to determine whether or not a significant interaction was present. Positive results were followed up with Tukey's post-hoc test with an alpha of less than 0.05 considered significant.

RESULTS

Activity-dependent regulation of miRs and expression of their target genes

Change in the expression of a single miR can affect many target genes, and one mRNA target can be regulated by several miRs (Filipowicz et al., 2008). SCI affects the expression of miRs at and several segments below the lesion epicenter (Liu et al., 2009; Liu et al., 2010a). We examined the role of SCI and Ex on the expression of three miRs; miR21, miR216a and miR217, which target PTEN. Spinal cord injury alone did not change the expression of miR21, miR216a or miR217 or PTEN at short (10d) or long (31d) post injury periods (Figure 2A). Ex significantly increased expression of only miR21 at 10d with subsequent significant decrease in the expression of PTEN. The longer exercise regimen had no effect on the expression of miR21 or PTEN suggesting a critical window for the efficacy of Ex in this context. The other half of spinal cord samples that were probed for protein indicated a significant elevation in PTEN levels at 10d after SCI alone, but levels were reduced to normal with exercise (Figure 2B, C). These results indicate that change in expression of a single miR (miR21) is sufficient to modulate PTEN expression and that SCI alone or followed by Ex does not modulate all miRs to an equivalent extent.

We next asked whether SCI alone or with Ex would modulate the expression of miR199a-3p, a miR that directly targets mTOR mRNA. SCI significantly increased expression of miR199a-3p at 10d and 31d after injury and Ex for a short or long period inhibited this change in expression (Figure 3A). This activity-dependent decrease in miR199a-3p correlated with a significant increase in the expression of mTOR mRNA compared to SCI alone (Figure 3A). Patterns of gene expression with SCI and Ex were reflected in an increase in mTOR protein over control and SCI alone (Figure 3B, C).

Expression of upstream mediators of mTOR with SCI and/or Ex

To determine if SCI and Ex affects the PTEN/mTOR pathway through growth factor receptor activation we measured change in gene expression of several upstream mediators of mTOR; transforming growth factor (TGF)- α and the subsequent activation of phosphoinositide 3-kinase (PI3K) and the serine/threonine kinase AKT (also known as protein kinase B or PKB). Figure 4A shows that Ex compared to Control and SCI only groups resulted in a significant increase in the expression of TGF α mRNA, PI3K mRNA and AKT mRNA. Ex induced a 4-fold increase in AKT gene expression 10 days after injury in the lumbar cord, but had no effect at 31 days (Figure 4C). Further examination revealed that short or long term cycling Ex after SCI significantly increased protein levels of AKT within the lumbar cord compared to control or SCI alone (Figure 4D, E).

Expression of downstream effectors of mTOR with SCI and/or Ex

Assessment of Ex effects on cap-dependent translation of mRNA in the lumbar spinal cord was accomplished by measuring gene expression of eif-4F and its inhibitor 4E-BP1 in rats after SCI or SCI and Ex. Significant increase in eif-4E mRNA was found at 10d but not 31d after SCI. Ex decreased the expression of eif-4E mRNA after 10d but increased it at 31 days (Figure 5). There was no change in the expression of 4E-BP1 after SCI or Ex (Figure 5).

Quantitative PCR revealed that expression of s6K1 mRNA was significantly increased 10 days after SCI but returned to control level by 31 days. Ex further increased the expression of S6K1 at 10 and 31 days (Figure 6A). Expression of S6, the target of S6K1 was significantly increased with exercise (Figure 6A). To determine whether these changes in gene expression were translated to protein, we probed the lumbar spinal cord and Figures 6B and C show a representative Western blot and histogram demonstrating the significant increase in both S6K1 and phosphorylated S6 in SCI and both Tx+Ex groups compared to control and injury alone.

Rapamycin reduces pS6 expression in Ex animals

Because S6 phosphorylation increased in the lumbar spinal cord with Ex, we next examined whether this increase in activity was directly related to change in mTOR activity. The significant increase in mTOR mRNA and protein was blocked by systemic administration of Rapamycin during the Ex period (Figure 7A, B). Rapamycin treatment also blocked Ex-induced protein expression of downstream effectors of mTOR. Protein levels of S6K1 and pS6 were significantly increased with Ex and this was blocked by Rapamycin (Figure 7C, D).

Ex enhanced pS6 expression in spinal cord neurons

Control animals expressed constitutive levels of pS6 in motoneurons (MNs) of the lumbar enlargement as determined by immunostaining of the cell soma and in some of the primary dendrites (Figure 8A, E, I). Few immunopositive (pS6+) neurons were located outside of the MN pool. There was no obvious change in the distribution of pS6+ neurons after SCI (Figure 8 B, F, J) however, in Ex animals there was a 6 fold increase in the number of pS6+ neurons (Figure 8C, M) in the intermediate gray and dorsal horn and MNs exhibited more extensive processes immunostained for pS6 (Figure 8K) that corresponded to a significant increase in pS6 immunostaining within lamina IX of the ventral horn (Figure 8N). Treatment of Ex animals with Rapamycin prevented the activity dependent increase in pS6 in non-MNs (Figure 8 D, H, L, M) and MNs (Figure 8N).

DISCUSSION

This study provides evidence that cycling exercise of spinalized rats is a potent mediator of gene expression and cellular processing of the growth-associated PTEN/mTOR pathway. This pathway is a critical regulator of protein synthesis, axon regeneration and plasticity in the damaged CNS (Park et al., 2008; Abe et al., 2010; Liu et al., 2010b) and is affected through changes in the expression of endogenous miRs associated with different components of this pathway. At least 3 miRs are known to modulate the expression of PTEN and expression of one of these (miR-21) is increased after SCI and Ex; while a different one (miR-199a-3p) is decreased significantly by Ex after SCI. Activity dependent change in miR21 expression correlated with a significant decrease in PTEN mRNA and protein while the decrease in miR199a-3p correlated with a significant increase in the expression of mTOR mRNA and protein. Importantly, Ex affected the gene and protein expression of upstream mediators (TGF α , AKT, and PTEN) as well as downstream effectors (eif-4E, S6K1, S6) of mTOR. These molecular changes manifest as an increase in activated S6 protein in spinal cord interneurons and processes of motoneurons in the lumbar enlargement, all of which could be blocked by treating exercising animals with Rapamycin. With recent reports that activation of the mTOR pathway through conditional deletion of PTEN promotes robust axonal elongation and regeneration in the damaged CNS (Park et al., 2008; Liu et al., 2010b), it is extremely exciting to define an effective, non-invasive approach to modulate the downstream effectors in the mTOR pathway. That this is accomplished through regulation of miRs provides yet another layer of regulation by molecular gene pathways involved in spinal cord plasticity after injury. Although we do not have direct evidence, the results of this study suggest that Ex after spinal cord injury may provide a rehabilitative strategy to improve the regenerative capacity of damaged axons and help regulate spinal cord plasticity after injury.

It is well-established that Ex increases the expression of many neurotrophic and growth factors in the lumbar spinal cord after injury (Gomez-Pinilla et al., 2002; Hutchinson et al., 2004; Ying et al., 2005) leading to spinal cord plasticity (Mendell et al., 2001; Petruska et al., 2010) and the recovery of spinal reflexes (Cote et al., 2011). However, the intrinsic signaling mechanisms responsible for this activity dependent plasticity are not well understood. Since the mTOR pathway can be activated by factors that bind to receptor tyrosine kinases including TGF α (as shown here) and neurotrophins, it seemed likely that Ex-induced plasticity within the lumbar spinal cord may be driven intrinsically by the PTEN/mTOR pathway. Here we show that cycling Ex increases TGF α expression within the lumbar cord after short or longer periods of exercise that correlated with intrinsic activation of genes like PI3K, PTEN and AKT which are upstream mediators of mTOR. We have also shown in these experiments that non-invasive and non-pharmacologic cycling exercise is a potent mediator of the expression of some miRs associated with the PTEN/mTOR pathway (21, 199a-3p) but not others (216a, 217), resulting in an increase in gene and protein expression necessary for protein synthesis and cell growth. Since miRs control the translation of specific target genes in a dose dependent manner, miR21 and miR199a-3p identified here represent another layer of regulation of the mTOR pathway that can be driven to promote plasticity. While the genes which mediate the transcription of miR21 or miR199a-3p are currently unknown, we have identified exercise as a mediator of both miRs and the canonical mTOR pathway to promote protein synthesis and plasticity (Fig 2, 3). Whether exercise directly regulates miR expression or whether it indirectly modulates expression through other gene pathways and transcription factors should be examined further.

mTOR signaling can promote activity-dependent plasticity of lumbar spinal cord neurons via two major mechanisms. First, mTOR is a key regulator of cap-dependent translation of

mRNA, the most common mechanism for protein translation in cells (Gingras et al., 2004). mTOR accomplishes this by phosphorylating the eukaryotic initiation factor 4E binding protein 1 (4E-BP1), which releases eukaryotic initiation factor (eIF)-4E, thereby initiating translation. Importantly, eIF-4E expression is also a downstream product of the receptor tyrosine kinase activation; however rather than being regulated by mTOR directly, its translation is the result of the ERK MAP kinase pathway (Sonenberg and Gingras, 1998). Our results show a transient increase of eIF-4E within the lumbar cord at 10 dpi that returns to normal one month after SCI and no change in the expression of 4E-BP1. Thus, cap-dependent translation is modulated by over expression of eIF-4E rather than a reduction in its inactivator 4E-BP1, and this transient increase in expression above normal at 10d post Tx may be a result of ERK pathway activation (Sonenberg and Gingras, 1998; Gingras et al., 1999).

Second, mTOR facilitates ribosome biogenesis and translation elongation through its downstream effector p70 ribosomal protein S6 kinase 1 (S6K1) and its substrate S6 (Dufner and Thomas, 1999; Gingras et al., 2004). Phosphorylation of S6 by S6K1 mediates unwinding and initiates translation of 5 terminal oligopyrimidine tract (TOP) mRNAs. These TOP mRNAs encode essential ribosomal proteins and elongation factors necessary for ribosome biogenesis and translation. Cycling Ex induced a 2-fold increase in phosphorylated S6 protein in the lumbar cord that was associated with an increase in immunopositive p-S6 spinal cord interneurons and increased expression in motoneuron processes. Importantly, this labeling pattern identifies a population of neurons that may be an anatomical substrate responsible for generating the physiological plasticity (Barbeau and Rossignol, 1987; Wolpaw and Tennissen, 2001), biochemical changes (Tillakaratne et al., 2002) and subsequent improved in motor performance that is known to occur with training (Barbeau and Rossignol, 1987). Rapamycin treatment blocked the effect of Ex on the phosphorylation of S6 indicating the relationship of Ex to mTOR expression in spinal cord plasticity. Rapamycin treatment has been shown to partially inhibit the conditioning response observed in sensory neurons (Abe et al., 2010) and is a potent suppressor of translation (Choo et al., 2008).

That Ex induces new protein synthesis and plasticity within intact neurons of the injured spinal cord by activating the mTOR pathway raises an important question as to whether Ex can be effective at initiating a regenerative response of damaged CNS neurons via activation of the mTOR pathway. Importantly, we see a robust Ex-induced increase in the phosphorylation of s6 a downstream effector of mTOR by 10 dpi by both Western blot and immunohistochemistry (Fig 6, 7, 8). These data suggest that early Ex may be important in activating protein synthetic machinery in the damaged CNS that is significant for neuronal survival, not to mention neuronal plasticity or regeneration. Prolonged Ex shows a similar increase in s6 phosphorylation, however, the increase at this time point may not be a cumulative effect of weeks of Ex, but instead may represent the same transient increase in expression. That is, daily exercise may be necessary to keep the expression of the mTOR pathway (and possibly others as well) elevated. This is especially important as we strive to improve the regenerative capacity of damaged CNS neurons. Daily exercise may be critical promoter and provider of constant/consistent protein synthesis that may be necessary to potentiate long distance growth of axons and regeneration to targets. Indeed, Liu et al. (2010b) showed that conditional activation of the intrinsic neuronal mTOR pathway by knockdown of PTEN in corticospinal neurons is sufficient to drive remarkable axonal regeneration into and beyond a spinal cord lesion (~4 mm), and inhibition of mTOR with Rapamycin suppresses neuronal plasticity and growth (Tang et al., 2002). Additionally, mTOR is also present in axons and has been implicated in the control of local protein translation in axons and dendrites suggesting that mTOR activity in the axonal and dendritic

compartments may be critical for successful axonal regeneration and/or plasticity (Zheng et al., 2001; Klann and Dever, 2004; Piper and Holt, 2004; Verma et al., 2005).

We report that Ex also affects the expression of PTEN, PI3K and AKT that consequently may affect many other vital cellular processes, including cell proliferation, differentiation, motility and survival, in addition to protein synthesis and activity dependent plasticity as indicated in this manuscript. We have recently shown the involvement of PTEN regulation on programmed cell death and apoptosis (Liu et al., 2010a), and it is also important in cellular respiration and energy metabolism (Deas et al., 2009). Further studies are necessary to directly test for the influence of exercise on the regenerative effort of injured axons, either through modulation of miRs or through various other parameters. In this sense, it is of utmost importance to begin to define additional mechanisms by which rehabilitation therapies may provide beneficial effects to spinal cord injured patients.

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References

- Abe N, Borson SH, Gambello MJ, Wang F, Cavalli V. Mammalian target of rapamycin (mTOR) activation increases axonal growth capacity of injured peripheral nerves. *J Biol Chem.* 2010; 285:28034–28043. [PubMed: 20615870]
- Barbeau H, Rossignol S. Recovery of locomotion after chronic spinalization in the adult cat. *Brain Res.* 1987; 412:84–95. [PubMed: 3607464]
- Beaumont E, Houle JD, Peterson CA, Gardiner PF. Passive exercise and fetal spinal cord transplant both help to restore motoneuronal properties after spinal cord transection in rats. *Muscle Nerve.* 2004; 29:234–242. [PubMed: 14755488]
- Choo AY, Yoon SO, Kim SG, Roux PP, Blenis J. Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. *Proc Natl Acad Sci U S A.* 2008; 105:17414–17419. [PubMed: 18955708]
- Cote MP, Azzam GA, Lemay MA, Zhukareva V, Houle JD. Activity-dependent increase in neurotrophic factors is associated with an enhanced modulation of spinal reflexes after spinal cord injury. *J Neurotrauma.* 2011; 28:299–309. [PubMed: 21083432]
- Deas E, Plun-Favreau H, Wood NW. PINK1 function in health and disease. *EMBO Mol Med.* 2009; 1:152–165. [PubMed: 20049715]
- Dufner A, Thomas G. Ribosomal S6 kinase signaling and the control of translation. *Exp Cell Res.* 1999; 253:100–109. [PubMed: 10579915]
- Dupont-Versteegden EE, Houle JD, Dennis RA, Zhang J, Knox M, Wagoner G, Peterson CA. Exercise-induced gene expression in soleus muscle is dependent on time after spinal cord injury in rats. *Muscle Nerve.* 2004; 29:73–81. [PubMed: 14694501]
- Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet.* 2008; 9:102–114. [PubMed: 18197166]
- Gingras AC, Raught B, Sonenberg N. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu Rev Biochem.* 1999; 68:913–963. [PubMed: 10872469]
- Gingras AC, Raught B, Sonenberg N. mTOR signaling to translation. *Curr Top Microbiol Immunol.* 2004; 279:169–197. [PubMed: 14560958]
- Gomez-Pinilla F, Ying Z, Roy RR, Molteni R, Edgerton VR. Voluntary exercise induces a BDNF-mediated mechanism that promotes neuroplasticity. *J Neurophysiol.* 2002; 88:2187–2195. [PubMed: 12424260]

- Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev.* 2004; 18:1926–1945. [PubMed: 15314020]
- Houle JD, Morris K, Skinner RD, Garcia-Rill E, Peterson CA. Effects of fetal spinal cord tissue transplants and cycling exercise on the soleus muscle in spinalized rats. *Muscle Nerve.* 1999; 22:846–856. [PubMed: 10398201]
- Hutchinson KJ, Gomez-Pinilla F, Crowe MJ, Ying Z, Basso DM. Three exercise paradigms differentially improve sensory recovery after spinal cord contusion in rats. *Brain.* 2004; 127:1403–1414. [PubMed: 15069022]
- Jaworski J, Sheng M. The growing role of mTOR in neuronal development and plasticity. *Mol Neurobiol.* 2006; 34:205–219. [PubMed: 17308353]
- Klann E, Dever TE. Biochemical mechanisms for translational regulation in synaptic plasticity. *Nat Rev Neurosci.* 2004; 5:931–942. [PubMed: 15550948]
- Lenz G, Avruch J. Glutamatergic regulation of the p70S6 kinase in primary mouse neurons. *J Biol Chem.* 2005; 280:38121–38124. [PubMed: 16183639]
- Liu G, Keeler BE, Zhukareva V, Houle JD. Cycling exercise affects the expression of apoptosis-associated microRNAs after spinal cord injury in rats. *Exp Neurol.* 2010a
- Liu K, Lu Y, Lee JK, Samara R, Willenberg R, Sears-Kraxberger I, Tedeschi A, Park KK, Jin D, Cai B, Xu B, Connolly L, Steward O, Zheng B, He Z. PTEN deletion enhances the regenerative ability of adult corticospinal neurons. *Nat Neurosci.* 2010b; 13:1075–1081. [PubMed: 20694004]
- Liu NK, Wang XF, Lu QB, Xu XM. Altered microRNA expression following traumatic spinal cord injury. *Exp Neurol.* 2009; 219:424–429. [PubMed: 19576215]
- Mendell LM, Munson JB, Arvanian VL. Neurotrophins and synaptic plasticity in the mammalian spinal cord. *J Physiol.* 2001; 533:91–97. [PubMed: 11351017]
- Ollivier-Lanvin K, Keeler BE, Siegfried R, Houle JD, Lemay MA. Proprioceptive neuropathy affects normalization of the H-reflex by exercise after spinal cord injury. *Exp Neurol.* 2010; 221:198–205. [PubMed: 19913536]
- Park KK, Liu K, Hu Y, Kanter JL, He Z. PTEN/mTOR and axon regeneration. *Exp Neurol.* 2010; 223:45–50. [PubMed: 20079353]
- Park KK, Liu K, Hu Y, Smith PD, Wang C, Cai B, Xu B, Connolly L, Kramvis I, Sahin M, He Z. Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. *Science.* 2008; 322:963–966. [PubMed: 18988856]
- Petruska JC, Kitay B, Boyce VS, Kaspar BK, Pearse DD, Gage FH, Mendell LM. Intramuscular AAV delivery of NT-3 alters synaptic transmission to motoneurons in adult rats. *Eur J Neurosci.* 2010; 32:997–1005. [PubMed: 20849530]
- Piper M, Holt C. RNA translation in axons. *Annu Rev Cell Dev Biol.* 2004; 20:505–523. [PubMed: 15473850]
- Popovich PG, Wei P, Stokes BT. Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats. *J. Comp. Neurol.* 1997; 377:443–464. [PubMed: 8989657]
- Sandrow-Feinberg HR, Izzi J, Shumsky JS, Zhukareva V, Houle JD. Forced exercise as a rehabilitation strategy after unilateral cervical spinal cord contusion injury. *J Neurotrauma.* 2009; 26:721–731. [PubMed: 19489718]
- Sonenberg N, Gingras AC. The mRNA 5' cap-binding protein eIF4E and control of cell growth. *Curr Opin Cell Biol.* 1998; 10:268–275. [PubMed: 9561852]
- Takei N, Kawamura M, Hara K, Yonezawa K, Nawa H. Brain-derived neurotrophic factor enhances neuronal translation by activating multiple initiation processes: comparison with the effects of insulin. *J Biol Chem.* 2001; 276:42818–42825. [PubMed: 11551908]
- Tang SJ, Reis G, Kang H, Gingras AC, Sonenberg N, Schuman EM. A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus. *Proc Natl Acad Sci U S A.* 2002; 99:467–472. [PubMed: 11756682]
- Tillakaratne NJ, Mouria M, Ziv NB, Roy RR, Edgerton VR, Tobin AJ. Increased expression of glutamate decarboxylase (GAD(67)) in feline lumbar spinal cord after complete thoracic spinal cord transection. *J Neurosci Res.* 2000; 60:219–230. [PubMed: 10740227]

- Tillakaratne NJ, de Leon RD, Hoang TX, Roy RR, Edgerton VR, Tobin AJ. Use-dependent modulation of inhibitory capacity in the feline lumbar spinal cord. *J Neurosci.* 2002; 22:3130–3143. [PubMed: 11943816]
- Verma P, Chierzi S, Codd AM, Campbell DS, Meyer RL, Holt CE, Fawcett JW. Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration. *J Neurosci.* 2005; 25:331–342. [PubMed: 15647476]
- Wolpaw JR, Tennissen AM. Activity-dependent spinal cord plasticity in health and disease. *Annu Rev Neurosci.* 2001; 24:807–843. [PubMed: 11520919]
- Ying Z, Roy RR, Edgerton VR, Gomez-Pinilla F. Exercise restores levels of neurotrophins and synaptic plasticity following spinal cord injury. *Exp Neurol.* 2005; 193:411–419. [PubMed: 15869943]
- Zheng JQ, Kelly TK, Chang B, Ryazantsev S, Rajasekaran AK, Martin KC, Twiss JL. A functional role for intra-axonal protein synthesis during axonal regeneration from adult sensory neurons. *J Neurosci.* 2001; 21:9291–9303. [PubMed: 11717363]

HIGHLIGHTS

- > Exercise is an effective, non-invasive method to modulate spinal cord plasticity after injury.
- > We examine changes in the PTEN/mTOR pathway within the lumbar spinal cord after complete cord transection.
- > We show that exercise regulates small microRNAs that specifically target PTEN and mTOR.
- > These data suggest that exercise may help regulate plasticity after SCI.

Figure 1.
Schematic of the PTEN/mTOR signaling pathway.

Figure 2.

Cycling exercise regulates miRs and their target PTEN expression. **(A)** Relative gene expression of miR21, miR216a, miR217 and their target PTEN. Exercise significantly increased miR21 expression in the lumbar spinal cord but had no effect on miR216a or miR217 at 10 days post injury. The increase in miR21 was accompanied by a decrease in the expression of its target gene PTEN. At 31 days post transection, there was no change in the level of any miRs or PTEN relative to uninjured control ($n=6/\text{group}$; # $p<.05$; * $p<.01$; ** $p<.01$). **(B)** Representative Western blot of the L4-6 spinal cord for a control, Tx10d, Tx10d+Ex, Tx31d, Tx31d+Ex rat showing PTEN (top) and actin loading control (bottom). **(C)** Quantification of PTEN protein in the lumbar cord of exercised and non-exercised spinal cord injured rats relative to uninjured control at 10 and 31 days post injury determined by densitometric analyses illustrates that exercise reduced PTEN protein levels ($n=6/\text{group}$; mean $\pm$ sem; # $p<.05$).

Figure 3.

Cycling exercise facilitates mTOR expression by modulating miR199-3p. (A) Relative gene expression of miR199-3p and its target mTOR. Spinal cord transection causes at least a 2 fold increase in miR199a-3p expression at 10 or 31 days after injury. Exercise (Ex) significantly reduced the level of miR199a-3p, a natural inhibitor of mTOR translation, while increasing the level of the mTOR gene at both time points ($n=6/\text{group}$; $\#p<.05$; $\#\#p<.01$; $**p<.01$). (B) Representative Western blot of the L4-6 spinal cord for a control, Tx10d, Tx10d+Ex, Tx31d, Tx31d+Ex rat showing mTOR (top) and actin loading control (bottom). (C) Quantification of mTOR protein in the lumbar cord of exercised and non-exercised spinal cord injured rats relative to uninjured control determined by densitometric analyses shows that exercise significantly increased the level of mTOR in the lumbar spinal cord after short (10d) or longer (31d) bouts of exercise ($n=6/\text{group}$; mean $\pm$ sem; $\#p<.05$; $\#\#p<.01$).

Figure 4.

Effect of cycling exercise on the expression of upstream mediators of mTOR. Quantitative PCR revealed that cycling exercise after spinal cord transection significantly increased the expression of three upstream mediators of mTOR: TGfα (**A**), Phosphoinositide 3-kinase (PI3K; **B**) and AKT (**C**) in the lumbar spinal cord. Sustained exercise for longer times after injury did not affect the gene expression of AKT. (n=6/group; *p<.05; **p<.01). (**D**) Representative Western blot of the L4-6 spinal cord for a control, Tx10d, Tx10d+Ex, Tx31d, Tx31d+Ex rat showing AKT (top) and actin loading control (bottom). (**E**) Quantification of AKT protein in the lumbar cord of exercised and non-exercised spinal cord injured rats relative to uninjured control determined by densitometric analyses revealed that exercise significantly increased the level of AKT protein in the lumbar spinal cord after short (10d) or longer (31d) bouts of exercise (n=6/group; mean ± sem; *p<.05; **p<.01).

Figure 5.

Effect of cycling exercise on the gene expression of mTOR's downstream effectors eukaryotic initiation factor (eif)-4E and its initiator 4E-BP1. Spinal cord transection significantly increased the expression of eif-4E at 10 but not 31 days. Exercise for short (5d) periods reduced the expression of eif-4E to within control values. Sustained exercise for longer times after injury increased the gene expression of eif-4E. Interestingly, the expression of its initiator 4E-BP1 was not affected by either spinal cord transection or exercise at 10 and 31 days. (n=6/group; **p<.01).

Figure 6.

Cycling Exercise (Ex) increased expression of downstream effectors of mTOR, S6K1 and S6 is increased with exercise. **(A)** Relative gene expression of S6K1 and its substrate S6 in spinal cord injured rats with and without exercise training relative to uninjured control. Spinal cord transection does not affect the gene expression of S6K1 or S6. Exercise (Ex) after injury significantly increased the levels of both S6K1 and S6 genes at both time points ($n=6/\text{group}$; $\#p<.05$; $\#\#p<.01$; $*p<.05$; $**p<.01$). **(B)** Representative Western blot of the L4-6 spinal cord for a control, Tx10d, Tx10d+Ex, Tx31d, Tx31d+Ex rat showing S6K1 (top), phosphorylated (P)-S6 (middle) and actin loading control (bottom). **(C)** Quantification of S6K1 and P-S6 protein in the lumbar cord of exercised and non-exercised spinal cord injured rats relative to uninjured control determined by densitometric analyses shows that exercise significantly increased the level of both proteins in the lumbar spinal cord after short (10d) or longer (31d) bouts of exercise ($n=6/\text{group}$; mean $\pm$ sem; $\#p<.05$; $\#\#p<.01$; $**p<.01$).

Figure 7.

Inhibition of mTOR with Rapamycin decreases expression of downstream effectors of mTOR. (A) Rapamycin (Rap) treatment of spinal cord injured rats receiving exercise reduced the expression of miR199a-3p compared to transection alone and Tx10d+Ex groups. The expression of mTOR gene is increased with exercise. This effect of exercise is blocked by Rap ($n=5-6/\text{group}$; $\#p<.05$; $**p<.01$). (B) Representative Western blot of the L4-6 spinal cord for a control, Tx10d, Tx10d+Ex, Tx10+Ex+Rap rat showing mTOR (top) and actin loading control (bottom). (C) Densitometric analyses of mTOR protein in the lumbar cord revealed that Rap treatment was sufficient to block the exercise-induced increase in mTOR ($n=5-6/\text{group}$; mean $\pm$ sem; $\#p<.05$). (D) Quantitative analysis of gene expression of the downstream effector S6 revealed that rapamycin (Rap) treatment was sufficient to block the exercise-induced increase in S6 ($n=5-6/\text{group}$; $*p<.05$; $**p<.01$). (E) Representative Western blot of the L4-6 spinal cord for a control, Tx10d, Tx10d+Ex, Tx10d+Ex+Rap rat showing phosphorylated-S6 (top), and actin loading control (bottom). (F) Densitometric analysis of P-S6 protein revealed that inhibition of mTOR with Rap was sufficient to block the exercise-induced increase of S6 phosphorylation ($n=5-6/\text{group}$; mean $\pm$ sem; $*p<.05$; $**p<.01$).

Figure 8.

Exercise increases activation of S6 in spinal cord intermediate grey neurons. Representative low magnification images of the L4 spinal cord of uninjured control (A), spinal cord transection (Tx10d; B), a transected rat receiving cycling exercise (C), and a transected rat receiving exercise and the mTOR inhibitor Rapamycin (Tx10d+Ex+Rap, D). Uninjured controls express constitutive levels of phosphorylated (p)-S6 in motoneurons of the lumbar enlargement (I, arrow) and only a few pS6+ neurons were located outside the motoneuron pool (E, arrowhead). Spinal cord transection did not change the distribution of p-S6+ neurons (F, J) relative to control. With exercise, the motoneurons had many more processes positively labeled for P-S6 (K, L). Interneurons of the intermediate grey of rats receiving

exercise were P-S6 positive, and the number of non-motoneurons expressing activated S6 was significantly more than Control and Tx10d (**G, M**; * $p < .01$ vs. all other groups). Treatment of exercised rats with Rapamycin inhibited this increase in immunopositive interneurons (**H, M**) and had no effect on S6 activity within motoneurons (**L**).

Table 1

Q-PCR Primers Sequences

Genes / Nucleotide Ref #	5' Primer Sequence	start	3' Primer Sequence	start	Product Size
TGPa/NM_012671	gca agt tet gcc tgt tgc tc	1875	aaa ggg agc cag ggt taa ga	1986	112
PI3K/KM 022213	gct tgt tct gtg gtt gca ga	1174	tag tgg agc acc agc tcc tt	1295	121
PTEN/NM_031606	acc agg acc aga gga aac ct	840	ttt gtc agg gttg agc aca ag	965	126
AtoVPKB/NM_031575	ggg cct agg tgt tgt cat gt	1036	aag aga gtg ttc gggg gga at	1157	121
mTOR/NM_019906	agg aag gac gtt tgc tca ga	8249	tcc ctc act gaa cac agc ag	8351	102
S6K1/NM_001010962	aag agg gct tct cct tcc ag	1268	aac ccc tca aag gga gag aa	1370	102
S6/NM_017160	ctg ctg atg ctc ttg gtg aa	125	act ctg cca tgg gtc aaa ac	233	108
4E-BP1/NM_053857	cta gcc cta cca gcc atg ag	294	tgg cgg cta tgt tcc tta ac	417	123
elf-4E/NM_025829	ttc ccc tcc cca taa gat tc	990	tgg cgg cta tgt tcc tta ac	1116	126
ISSRNA/NM_001008373.1	aag tcc cct aac acc ctc gt	2009	agt ttg tgg aag agc gga ag	2124	116
GAPDH/NM_017008	cca tcc cag acc cca taa c	1194	gca gcc aac tta ttg atg g	1271	78

Table 2
Q-PCR iniKN'A Primers Sequences

Assay Name	Part Number	Target Sequence
hsa-miR-21	397	UAGCUUAUCAGACUGAUGUUGA
bsa-miR-216a	4427975	UAAUCUCAGCUGGCAACUGUGA
hsa-miR-217	1733	UACUGCAUCAGGAACUGACUGGAU
hsa-miR-199a-3p	2304	ACAGUAGUCUGCACAUUGGUUA
U6 snRNA	1973	GUGCUCGCUUCGGCAGCACAUUAUACUAiAAUUGGAACGAUACAGAGAAGAUUAG CAUGGCCCCUGCGCAAGGAUGACACGCAAAUUCGUGAAGCGUCCAUUUUUUA CUGCCCUCCAUGCCUGCCCCACAAACGCUCUGAUAAACAGUCUGUCCUGUCUCU CUCCUGCUGCUCCUAUGGAAGCGAAGUUUCCGCUCCUGCAGAAAGCAAAGUUA CGACUCAGAGACGGCUGAGGAUGACAUCAGCGAUGUGCAGGGAACCCAGCGCCU GGAGCUUCGGGAUGACGGGGCCUUCAGCACCCCCACGGGGGGUUCUGACACCCU GGUGGGCACCUCCUGGACACACCCCCGACCUCCGUGACAGGCACCUACAGAGGAG CAAGUGAGCUGGUGGGGCAGCGGGCAGCGGUCCUGGAGCAGGAAGCGGGCAGU GGGGGUGGCACCCGCCGCCUCCCGGGCAGCCCAAGGCAAGCACAGGCAACCGGGG CCGGGCCACGGCACCUGGGGGUGGAGCCGCUUGGUGCGGGCAUCX7CGAGCUAAUC UGGUGGG