ORIGINAL RESEARCH

# Activation of Neuroinflammation via mTOR Pathway is Disparately Regulated by Differential Target Multiplexed and Traditional Low-Rate Spinal Cord Stimulation in a Neuropathic Pain Model

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**Introduction:** Spinal cord stimulation (SCS) has been used for decades to treat neuropathic pain conditions with limited understanding of its mechanisms of action. The mTOR pathway is a well-known co-factor in chronic pain and has not been previously linked to SCS therapy. Proteomic and phosphorylation analyses allow capturing a broad view of tissue response to an injury model and subsequent therapies such as SCS. Here, we evaluated the effect of differential target multiplexed SCS programming (DTMP) and traditional low-rate spinal cord stimulation (LR-SCS) on the mTOR pathway using proteomic and phosphoproteomic analyses.

**Methods:** The spared nerve injury (SNI) model of neuropathic pain in animals was established followed by continuous treatment with either DTMP or LR-SCS for 48 hours. Control groups included sham-stimulated (No-SCS) and uninjured animals (No-SNI). Proteins were extracted from spinal cord tissue removed post-stimulation and subjected to liquid chromatography/tandem mass spectrometry to assess changes in protein expression and states of phosphorylation. Bioinformatics tools and literature were used to identify mTOR-related proteins in the various groups.

**Results:** Over 7000 proteins were identified and filtered to find 1451 and 705 proteins significantly affected by DTMP and LR-SCS ($p < 0.05$), respectively, relative to No-SCS. Literature and bioinformatic tools yielded 192 mTOR-related proteins that were cross-referenced to the list of DTMP and LR-SCS affected proteins. Of these proteins, 49 were found in the proteomic dataset. Eight of these proteins showed a significant response to the pain model, 25 were significantly modulated by DTMP, and 8 by LR-SCS. Phosphoproteomic analyses yielded 119 mTOR-related phosphoproteins affected by the injury model with a 66% reversal following DTMP versus a 58% reversal by LR-SCS.

**Conclusion:** Proteomic and phosphoproteomic analyses support the hypothesis that DTMP, and to a lesser extent LR-SCS, reverse injury induced changes of the mTOR pathway while treating neuropathic pain.

**Keywords:** proteomic analysis, phosphoproteomic analysis, nerve injury, central sensitization, inflammation

## Introduction

Chronic neuropathic pain creates major disruption in the lives of those suffering it and is detrimental to both the individual and society.[1] Those affected are unable to work productively, if at all, while incomes are used towards treatments. Chronic neuropathic pain is characterized, at the level of the spinal cord, by increased levels of inflammatory mediators and signaling molecules that produce distorted neuronal signaling.[2] Altered neuronal signals can result in persistent perception of pain, inducing a sensitized state in which non-painful stimuli are perceived as painful, and/or pain is perceived without stimuli. Inflammatory mediators are released by glial cells as well as neurons in the process of sensitization, and the resulting abnormal interactions between these two cell types promote and maintain a painful state.[3] While the activation of the NFkB signaling pathway promotes the release of pro-inflammatory mediators, there are other

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important pathways, such as the mammalian target of rapamycin (mTOR) pathway, that also contribute to neuroinflammation and regulation of pain.[4]

mTOR is a core protein that binds and interacts with a variety of other proteins to make two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Both complexes play a role in the regulation of numerous processes including inflammation and pain. Both complexes contain the inhibitory protein DEP Domain Containing mTOR Interacting Protein (DEPTOR) and the adaptor protein target of rapamycin complex subunit LST8. However, mTORC1 specifically contains the regulatory-associated protein of mTOR (RAPTOR), which regulates mTORC1 assembly and acts as scaffolding for substrates, such as the eukaryotic initiation factor-4E-binding protein 1 (4E-BP1). Similarly, mTORC2 involves other proteins such as the rapamycin-insensitive companion of mTOR (RICTOR), the protein observed with RICTOR (PROTOR), and the stress-activated protein kinase-interacting protein 1 (mSIN1). Much like RAPTOR, RICTOR is necessary for mTORC2 assembly with mSIN1, for mTORC2′s catalytic activity, and may play a similar role in recruiting substrates. It has been shown that rapamycin is an inhibitor of mTOR in mTORC1, but it does not affect mTORC2[5]. Regarding pain pathways, mTORC1 is more highly regulated than mTORC2 and has a more direct effect on inflammatory pathways, whereas mTORC2 serves as a feedback regulator of mTORC1. Addition of rapamycin to reduce mTORC1 activity and pain has been observed in a variety of injury models, further emphasizing the importance of mTORC1, and not mTORC2, in regulating pain.[6–8] Various upstream regulators of mTORC1, such as pyruvate dehydrogenase kinase isoform 1 (PDK1) and GTP-binding protein RHEB, can activate and promote a pain state whereas other proteins, such as tuberin (TSC2) and AMP-activated protein kinase (AMPK), can inhibit it. Pathways for mTORC1 regulation are found in both glial cells and neurons throughout the nervous system and can regulate cytokines, growth factors, and other signaling molecules that extend the effect of mTOR outside the originating cell. Studies using various neuropathic pain models have shown that mTOR and its complexes undergo increased presence and activity within both microglia and astroglia. For example, the neuroinflammatory agent lipopolysaccharide (LPS) was shown to activate microglia cells via mTOR pathway.[9] Signs of activated astrocytes expressing nestin, vimentin, glial fibrillary acidic protein (GFAP), and the epidermal growth factor (EGF) receptor were co-observed with increased mTOR activity in reactive astrocytes following induction of an injury model which became reduced upon rapamycin administration.[5]

Spinal cord stimulation (SCS) is an effective and safe treatment for chronic intractable neuropathic pain.[10–12] A recent SCS modality inspired by multiplexing electrical pulsed signals to target glial cells and neurons differentially (DTM SCS) provided ≥50% relief of chronic neuropathic back pain in 84% of patients over 12-months, an outcome superior to that provided by traditional SCS treatment.[13] Preclinical work has demonstrated that the mode of action of this differential target multiplexed programming (DTMP) approach may involve the modulation of both glial and neuronal activity more effectively than conventional SCS.[14] This work has confirmed, with proteomic and phosphoproteomic analyses, along with prior transcriptomics,[15–18] the ability of DTMP to reverse changes in gene and protein expression levels induced by a neuropathic pain model.

Considering the role of mTOR in neuroinflammation and neuropathic pain, and the hypothesized mechanism of action of DTMP, which is thought to involve the regulation of pathways that balance disrupted neuron-glial interactions, this works evaluated the ability of DTMP and LR-SCS to regulate several up- and down-stream proteins and phosphoproteins involved in the mTOR pathway in an animal model of neuropathic pain.

## Materials and Methods
### Animals, Surgical Manipulations and SCS

A detailed description of the experimental design is provided elsewhere.[14] The proteomic analysis presented in this work has been conducted on samples from animals subjected to behavioral testing reported in that work. This study furthers molecular biology-based analyses started with that study. Briefly, the study was approved by the Institutional Animal Care and Use Committee at Illinois Wesleyan University according to the USDA Animal Welfare Act and the NIH Public Health Service Policy on the Humane Care and Use of Animals. Male Sprague-Dawley rats (Envigo RMS, Indianapolis, US) weighing in the 275–315 g range were housed individually in a temperature and humidity-controlled room with a 12-hour light/dark cycle. Food and water were supplied *ad libitum*. After acclimation to the environment, animals were randomly assigned to either No-SCS (untreated, n = 10), DTMP (n = 10), LR-SCS (n=10), or No-SNI (uninjured, n = 10). Animals in the No-SCS, DTMP, and LR-SCS groups were implanted with a miniaturized cylindrical quadrupolar SCS lead (Pt/Ir, 1 mm long, 0.62 mm diameter,

Evergreen Medical, St. Paul, MN, USA) at the L1-L2 level and subjected to the spared nerve injury (SNI) model of neuropathic pain as described previously.[14] Five days after SNI surgery, animals in the DTMP and LR-SCS groups, which had developed mechanical hypersensitivity, were stimulated continuously for 48 hours. No-SCS animals were connected to the SCS device for the same duration but were not stimulated. The No-SNI group did not receive either the SNI model or SCS. All groups were behaviorally assessed in parallel before surgical intervention, as well as before starting and ending the stimulation period by a researcher blinded to the assignments. DTMP uses multiplexed charge-balanced pulsed signals with components at 50 Hz (150 μs pulse width, PW) and 1200 Hz (50 μs PW) that are distributed over the contacts of the lead. LR-SCS uses single pulses at 50Hz (150 μs PW). Signal intensities corresponded to about 70% of the motor threshold, assessed at the stimulation rate and PW, and were in the 0.03–0.10 mA range. Signals were not duty cycled and initial intensities were unchanged throughout stimulation.

## Protein Isolation and Quantification

After 48 hours of SCS, animals were assessed and euthanized. The ipsilateral dorsal quadrant of the spinal cord underneath the SCS lead was harvested, washed with cold saline, snap frozen and stored at −80°C until analyzed. Four representative samples (per group) from responders to treatment (paw withdrawal threshold increased to at least 30% of the pre-SNI measurement) were used in this study. Of the remaining samples, 4 were used for transcriptomics analyses reported elsewhere.[14] Tissues were homogenized and suspended in a urea (9M) buffer enriched with protease inhibitors and free of ionic detergents to separate the proteins out. After determination of total protein concentration, proteins were digested with trypsin followed by alkylation of cysteine residues under appropriate buffering using standard methods.[19] Tryptic peptides in a pool of biological specimens of each experimental group were isotopically labeled using a tandem mass tag (TMT) system for simultaneous identification, quantification, and comparison of a protein among the experimental groups.[20] Peptides were combined and loaded onto a capillary column (50 cm x 100 μm PicoFrit) packed with C18 reversed-phase resin and fractionated via reverse column liquid chromatography (LC). The column was developed with a linear gradient of acetonitrile in 0.125% formic acid delivered at 280 nL/min for 150 minutes. Fractions were combined non-sequentially to 12 fractions, and mass spectra obtained in a LC/TMS instrument via multi-notch MS3 methodology, with parameters optimized under protocols developed at Cell Signaling Technology (Danvers, MA) allowing the highest number of identifications possible and most accurate quantification. Mass spectra were evaluated using SEQUEST and the Core platform from Harvard University.[21] The Uniprot rat database (uniprot.org) was used to search for proteins. Search results were filtered with mass accuracy of ±5 ppm on precursor ions and further filtered to a 1% protein level false discovery rate (FDR). Fold changes were obtained from comparison of the normalized spectral intensities ($log_2$ scale) of the tagged peptides uniquely assigned to each protein. Two tailed *t*-tests for each protein identified and quantified were used to determine the significance of fold changes. Proteins significantly modulated ($p < 0.05$) between No-SNI and No-SCS were isolated, while the effect of either DTMP or LR-SCS treatment relative to No-SCS was followed. Protein-protein interaction networks for significantly modulated proteins were built using the StringDB v11.0 bioinformatics tool.[22] Significantly enriched biological processes modulated by the pain model and either DTMP or LR-SCS were obtained via Gene Ontology Enrichment Analyses (GOEA) within the online Panther database.[23] Also, the Kyoto Encyclopedia of Genes and Genome (KEGG)[24] was used for secondary confirmation of the target proteins.

For phosphoproteomics, proteins were digested with trypsin and peptides purified by reversed-phase solid-phase extraction, followed by phospho-enrichment using immobilized metal affinity chromatography (IMAC) with iron-based magnetic beads (PTMScan® Fe-IMAC, Cell Signaling Technology, Danvers MA).[25] Immobilized phosphopeptides were eluted with basic pH buffer after washing out unbound peptides. Phosphopeptides were purified using a reversed-phase column prior to LC/TMS analysis as described above following standard techniques developed at Cell Signaling Technology. Due to the limited amount of protein available for phosphoproteomics after the whole proteomic analysis, technical assays were run for each pool of biological samples only in duplicate. Therefore, phosphoproteomic results based on spectral intensities of phosphoproteins filtered as described above, although reliable, could not be assessed for statistical significance. Instead, the coefficient of variation (CV) was used to measure the relative variability of fold changes from the variability of protein expression levels for individual groups. The CV, defined as the ratio of the standard deviation to the mean for spectral counts for a given experimental group, was used to obtain the coefficient of

variation of the fold changes No-SCS/No-SNI and SCS/No-SCS. The CV of the ratio is given by the square root of the sum of the square of the individual CVs of the ratioed values.[18]

# Results

## Proteomic Analysis of mTOR Pathway-Related Proteins

The proteomic analysis yielded over 7000 total identified proteins. This dataset was filtered to obtain 1451 and 705 proteins significantly affected by DTMP or LR-SCS (p<0.05) respectively, relative to No-SCS animals. An initial list of 158 proteins associated with the mTOR pathway was generated using the KEGG and gene ontology enrichment analyses. An additional 34 proteins were identified from peer-reviewed literature searches, which ensured additional up-to-date coverage of known mTOR-related proteins. These 192 identified mTOR-related proteins were cross-referenced to the list of proteins significantly affected by DTMP and LR-SCS, yielding 49 proteins. Figure 1 shows a list of these proteins and differential expression levels. Out of these 49 proteins, expression level of 8 proteins showed a significant change in response to the pain model relative to uninjured animals, while expression levels in 25 and 8 of these proteins were significantly changed with DTMP and LR-SCS treatment, respectively, relative to the pain model.

Of the 8 shown to be significantly affected by the pain model, 7 and 5 had expression levels increased back toward levels found in uninjured animals following DTMP and LR-SCS treatment, respectively. Of the 25 mTOR-related proteins significantly affected by the DTMP, expression levels of 20 were increased and 5 were decreased (Figure 1). Figure 2 depicts a simplified cartoon of the pathways affected by some of these identified proteins involved in the mTOR-related signaling, and their effects on specific biological processes. For simplicity, only the effect of the SNI model and DTMP, not LR-SCS, on protein expression are shown.

PDK1, glycogen synthase kinase-3 alpha (GSK3A), TSC2, cAMP-dependent protein kinase catalytic subunit alpha (PKACA), protein kinase CAMP-dependent type I regulatory subunit beta (PKAR1B), protein kinase CAMP-dependent type II regulatory subunit beta (PKAR2B), and ribosomal protein S6 kinase alpha-2 (RSK3) are proteins upstream of mTORC1 that showed increased expression levels following DTMP relative to the No-SCS group (Figure 1), with only PKACA and PKAR2B affected by LR-SCS. Two upstream mTORC1 pathway proteins, p90 ribosomal S6 kinase (P90RSK) and ribosomal protein S6 kinase alpha-3 (RSK2), showed reduced expression levels following DTMP therapy; of which only RSK2 was affected by LR-SCS. Of the proteins downstream of mTORC1, levels of glycogen synthase kinase-3 homolog MSK1 were increased whereas expression levels of signal transducer and activator of transcription 3 (STAT3) and ribosomal protein S6 kinase C1 (RSKL1) were decreased following DTMP treatment with no significance of these 3 being observed in LR-SCS. Interestingly, only 3 of these mTORC1 modulators, PKACA, PKAR2B, and
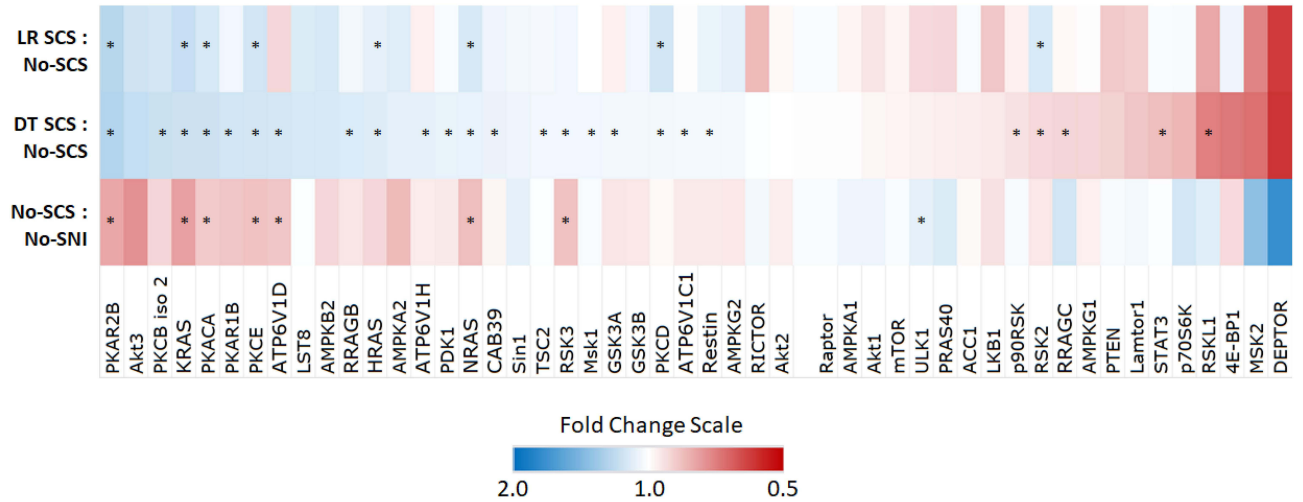


**Figure 1** Heatmap of proteins significantly affected by SNI, DTMP, and LR-SCS groups (n = 49). (*Denotes significant change in protein expression for No-SCS (SNI) animals relative to No-SNI and both DTMP and LR-SCS relative to SNI; p < 0.05).
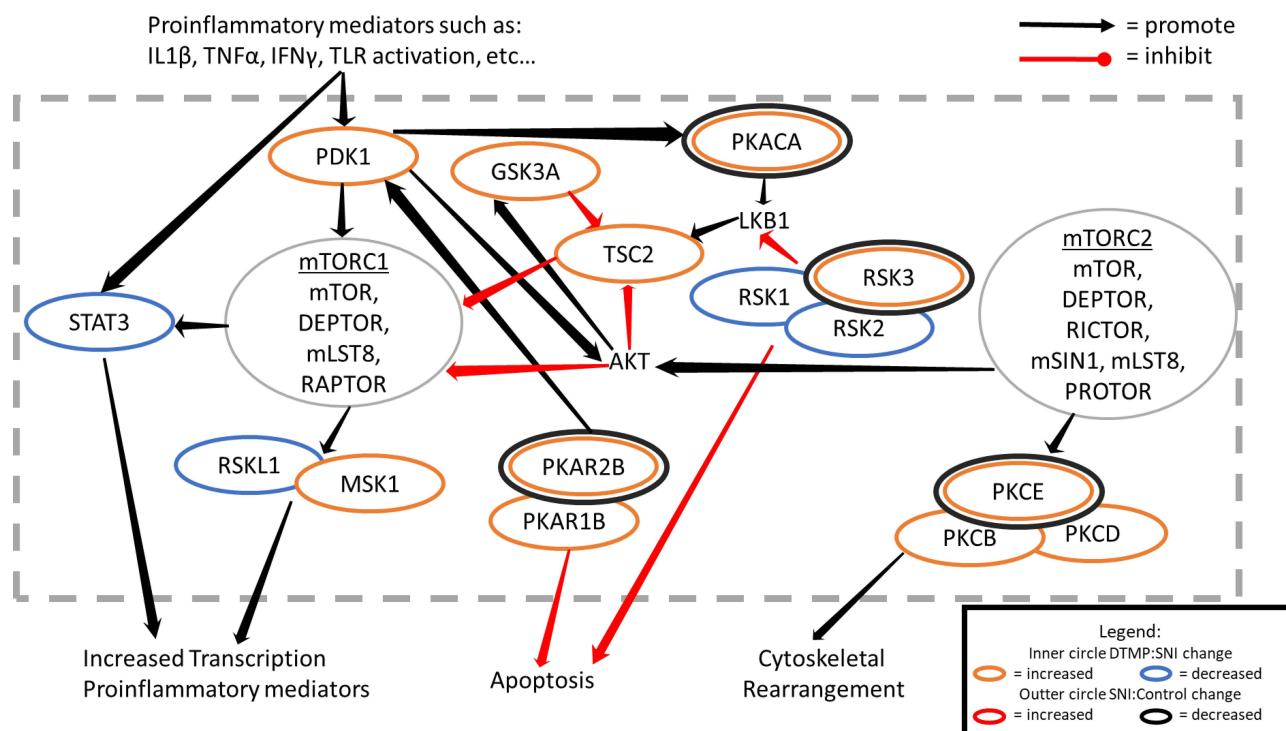
**Figure 2** Proteomic pathways of mTOR related proteins commonly associated with inflammatory mediators involved in the activation and maintenance of inflammatory state affected by DTMP.

RSK3, had expression levels altered by the pain model. These 3 proteins showed decreased expression levels following induction of the pain model. DTMP treatment back-regulated expression levels in the direction of those found in uninjured animals, whereas LR-SCS only back-regulated PKACA and PKAR2B.

The mTORC2 pathway has a smaller range of targets to regulate cellular activities thus it was unsurprising that there were fewer up- or down-stream proteins of mTORC2 identified in the proteomic data. In terms of the mTORC2, all three protein kinase C (PKC) isoforms, PKCB, PKCD, and PKCE, downstream of mTORC2 showed increased expression levels after DTMP treatment with only PKCD and PKCE being affected by LR-SCS. Expression levels of PKCE were the only ones significantly decreased by the pain model, though they were back regulated by DTMP. Expression levels of AKT, a primary target protein regulated by mTORC2, were not significantly altered by the pain model or treatment.

## Phosphoproteomic Analysis of mTOR Pathway-Related Proteins

Protein phosphorylation is the primary way by which regulatory changes are rapidly induced within the cell. This is more efficient than waiting on transcription and translation of desired proteins. As such, multiple phosphorylation sites often exist on a protein depending on the pathways and targeted cell structures to be regulated. The phosphoproteomics-based analysis identified 57 phosphorylated proteins out of the 192 identified to be mTOR-related. Figure 3 lists 141 different phosphorylated isoforms for these 57 proteins, with their corresponding differential changes in expression levels. The SNI pain model affected expression levels, by 10% or more, in 84.4% of these isoforms. Treatment with DTMP back-regulated (by 10% or more) expression levels of 65.5% of the isoforms affected by the pain model, while LR-SCS treatment back-regulated 58.0% of them.

Proteins that were phosphorylated the most consisted of kinases, such as PKC, protein kinase A (PKA), serine/threonine-protein kinase AKT, and AMPK, which are part of feedback loops and are heavily regulated as seen in Figure 4. In total, 16 p-PKC isoforms of p-PKCA, p-PKCB p-PKCD, p-PKCE, and p-PKCG were identified, with 12 of them affected by the pain model by at least 10% relative to expression level in No-SNI group. DTMP back-regulated 9 and LR-SCS 8 of these, relative to expression levels in the pain model towards levels found in uninjured animals. There
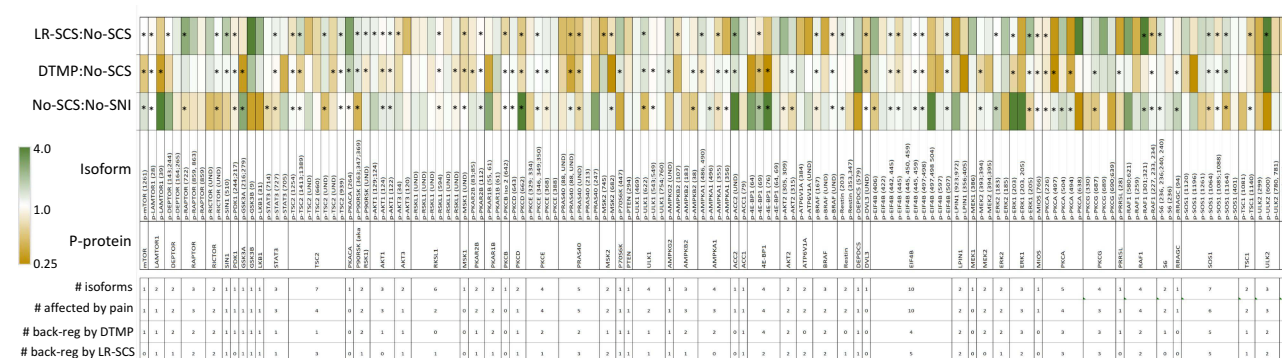
Figure 3 Phosphoproteins associated with the mTOR pathway. The heat map shows fold changes of 141 isoforms of the 57 phosphoproteins for the comparisons indicated to the left. *Denotes isoforms in which the fold change determination has a CV of 20% or less. The number of isoforms (# isoforms) for each phosphoprotein, the number of these affected by the pain model (# affected by pain) by at least 10%, and the number of these back-regulated by DTMP (# back-reg by DTMP) or LR-SCS (# back-reg by LR-SCS) by at least 10% in the direction of expression levels in uninjured animals are tabulated below the heat map.
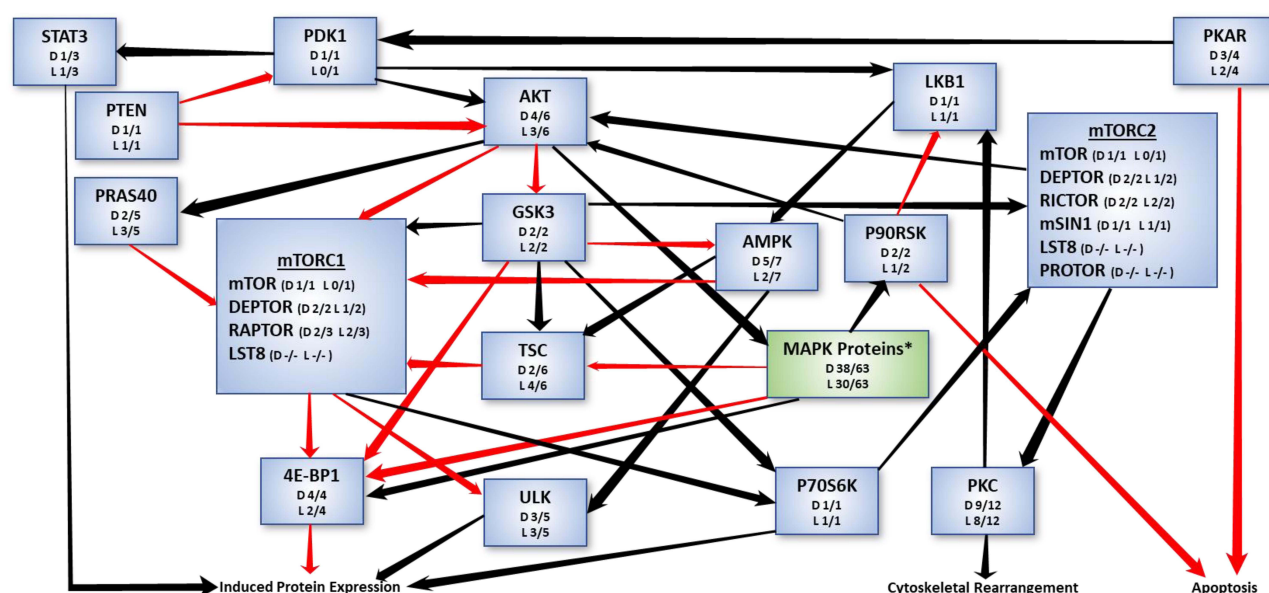


Figure 4 Phosphoproteomics of mTOR pathway-related proteins. The number of isoforms in each class of phosphoproteins back-regulated by DTMP (D) or LR-SCS (L) relative to the number of these affected by the pain model is included as a fraction. *Denotes a summary of p-MAPKs affected as previously published.[18] Black arrows indicate promotion, while red arrows indicate inhibition.

were 5 p-PKA isoforms with 4 being affected by SNI relative to the uninjured animals. Of the 4 SNI-affected p-PKA isoforms, 3 were reversed by DTMP, and 2 by LR-SCS, relative to the No-SCS group. Six out of 7 p-AKT isoforms identified were affected by the pain model, with expression levels of 4 of them being back-regulated by DTMP, and 3 by LR-SCS toward levels found in uninjured animals. There were 7 out of 8 p-AMPK isoforms affected by the pain model, with 5 of them back-regulated by DTMP and 2 of them back-regulated by LR-SCS. In addition to these more heavily regulated kinases, many of the mTORC proteins have undergone phosphorylation changes which can inhibit the mTORC as a whole or may prevent proteins from binding to proteins in the mTORC and thus inactivating this complex.

Of the non-specific mTORC proteins, DEPTOR and LST8, only 2 isoforms of p-DEPTOR were identified. Both isoforms had expression levels affected by the pain model and back-regulated by DTMP in the direction of expression levels found in uninjured animals, while LR-SCS back-regulated one of them. Among the mTORC1-specific proteins, DTMP and LR-SCS back-regulated 2 out of the 3 p-RAPTOR isoforms that had been affected by the pain model. DTMP back-regulated 2 and LR-SCS 3 out of 5 p-PRAS40 isoforms affected by the pain model. Expression levels of three

mTORC2-specific isoforms (2 p-RICTOR and 1 p-mSIN1) that had been down-regulated by the pain model relative to No-SNI, all 3 were up-regulated by both DTMP and LR-SCS.

The mTOR pathway can phosphorylate proteins that regulate protein synthesis at either the level of transcription, such as STAT3, or translation, such as 4E-BP1. We observed a p-STAT3 isoform affected by the pain model that had reversed expression in both DTMP and LR-SCS relative to SNI induced changes. There were 4 isoforms of p-4E-BP1 found, of which all 4 were affected by the SNI model. Of the p-4E-BP1 isoforms, all 4 were reversed by DTMP and only 2 were reversed by the LR-SCS treatments, relative to the SNI induced phosphorylation changes.

## Discussion

The role of mTOR and its related proteins spans a wide variety of functions. Dysregulation of mTOR signaling has been observed in various disease states such as diabetes, cancer, obesity, and neurodegeneration.[26] In the context of pain, the mTOR pathway is a major regulator of transcription and protein synthesis of inflammatory mediators, modulators of cell morphology (such as in glial cells), and metabolic processes. The mTOR pathway functions as a regulatory player able to activate or inhibit mitogen activated protein kinase (MAPK) pathways and nuclear factor kappa B (NFκB)-linked transcription of inflammatory mediators (see Figure 2). Studies have shown that targeting the mTOR pathway is a viable option to treat chronic pain.[27] While mTOR modulates activity levels of MAPK, it is relevant to recognize that mTORC1 activation can be induced by upstream activators, including the MAPK-pathway, primarily through phospho-regulation and can determine the downstream targets of mTORC1 facilitating a variety of cellular outcomes. Given the mTOR protein is a kinase itself, much of its mechanism of action is manifested in post-translational modifications, specifically phosphorylation, and thus much of the mTOR pathway modulation following both induction of the injury model and subsequent stimulation with either DTMP or LR-SCS was observed in the phosphoproteomic data.

The SNI pain model appeared to have a limited effect on directly modulating the protein expression of mTOR pathway-related proteins. Out of the 49 proteins found related to mTOR in the proteomic analysis, only 8 showed a significant change in expression level as a result of the pain model as implemented in our work. As shown in Figure 1, the pain model decreased expression levels of seven of these, with DTMP treatment back-regulating levels towards those found in uninjured animals, while LR-SCS back-regulated expression levels of 5 of them. In total, DTMP significantly changed expression levels of 25 proteins, while LR-SCS significantly changed 8. This suggests that DTMP may have a direct effect on regulating the mTOR pathway outside of strictly reversing injury-induced changes and to a greater degree than the traditional LR-SCS. The physiological relevance of this finding is currently under investigation.

The phosphoproteomic analysis of mTOR-related pathway proteins showed that out of 119 phosphorylated isoforms with expression levels affected by the injury model, DTMP back-regulated expression levels of 78 of these towards levels found in uninjured animals, while expression levels of 69 were back-regulated by LR-SCS. An overview of these results can be seen in Figure 4. The activation of mTOR signaling, and therefore the pro-inflammatory cascade, can occur with the ERK1/ERK2 MAP kinases and their downstream target p90RSK[28] leading to phosphorylation of TSC2 and inhibition of its activity.[29] TSC2, is a potent inhibitor of mTORC1 thus MAPK activity leads to downstream RHEB and mTORC1 activation. Thus, TSC2 modulation by DTMP has the potential to hamper the neuroinflammatory response associated with the pain model induced by peripheral nerve injury.

AKT is also an activator protein in inflammatory-related pain and can activate mTORC1 by phosphorylating TSC2 leading to its inactivation.[29] However, AKT can also directly and indirectly inhibit mTORC1. Six out of 7 p-AKT isoforms identified were affected by the pain model, with expression levels of 4 of them being back-regulated by DTMP, and 3 by LR-SCS toward levels found in uninjured animals. Out of the p-mTORC1-related isoforms found in our phosphoproteomic analysis, we observed reverse-regulation of expression levels in 5 of the 6 by DTMP compared to 3 of 6 by LR-SCS. We also observed back-regulation of expression levels in 6 of the 6 p-mTORC2-related isoforms by DTMP and 4 of 6 by LR-SCS. Taken together, these changes may result in a significant steering toward an anti-inflammatory phenotype of the molecular machinery set in motion by the pain model.

While mTOR is a ubiquitous protein, its expression and activity are regulated based on environmental conditions. Furthermore, specific methods of activation appear to generate different outcomes for where and how mTOR pathway proteins will be activated in the central nervous system. Literature indicates that mTORC proteins in the spinal cord are

found in neurons and microglia following an acute peripheral injury,[30] in only neurons in a peripheral chronic pain model,[31] in neurons and astrocytes in a chemically induced injury model,[32] and only in astrocytes in a spinal cord injury model.[5] Regarding the current study using a neuropathic pain model associated with a peripheral injury, literature indicates neurons in the spinal cord dorsal horn are likely the source of mTOR activation.[31] Other studies have shown that either enhanced[33] or inhibited[34] neuronal mTORC1 activity induces astrocyte activation.

Although the exact cell type responsible for mTOR downstream effects is unclear, it appears that the mTOR pathway modulates the neuro-glial environment in neuropathic pain. Many studies have studied and identified mTOR as a possible target of pain therapy.[27] However, due to the crucial regulatory nature of mTOR beyond the boundaries of pain and hypersensitization, it would not be advisable to systemically inhibit mTOR activity. A better therapeutic approach could be to functionally guide mTOR activity towards non-pain related pathways at a relevant functional location as opposed to systemically. Here we show that DTMP, more so than LR-SCS, is capable of modulating protein expression and phosphorylation of mTOR-related proteins that are responsive to the injury model as well as proteins that were unaffected by the pain model at the spinal cord level. For example, it has been proposed that the activation of mTOR pathways contribute to the initiation, establishment, and maintenance of hypersensitivity in an animal model of bone cancer-induced pain.[35] In this case, the phosphorylation of mTOR occurs upon activation of the N-methyl-D-aspartate (NMDA) receptor via glutamate. Phosphorylation of mTOR, which is preceded by the phosphorylation of AKT, induces the phosphorylation of 4EBP1. Our phosphoproteomic analysis found elevated levels of 3 p-AKT1 and 1 p-AKT3 isoforms, 1 p-mTOR isoform, and 4 p-4EBP1 isoforms in the spinal cord tissue of untreated SNI animals. Treatment with DTMP reduced expression levels of 7 of these isoforms (1 p-AKT1, 1 p-AKT3, 1 p-mTOR, and 4 p-4EBP1), while LR SCS reduced levels of 3 of them (1 p-AKT3 and 2 p-4EBP1). These results support previous literature demonstrating the relevance of the mTOR signaling pathway in the SNI model of neuropathic pain. Geranton et al reported that intrathecal injection of rapamycin (2.3 μg) into the L4/L5 spinal cord of rats subjected to SNI (developed for 6 days) significantly reduced mechanical hypersensitivity.[36] They also found that mTOR and p-mTOR were expressed in subsets of primary afferent sensory fibers as well as in non-neuronal cells of the surrounding tissue. Furthermore, these authors also found that p-mTOR and p-4EBP1 were increased in the dorsal horns particularly within lamina I/III neurons, in which projection neurons have been shown to play a key role in the establishment and maintenance of chronic pain. They also found that intrathecal injection of rapamycin reduced expression levels of p-4EBP1.

While many proteins of the mTOR pathway were identified, some classical injury related proteins (such as growth factors, cytokines, etc.) known to promote mTORC1 activation were not identified in the analysis. This is a limitation of the experimental design for protein quantification, which may miss transient proteins or small peptides. Large proteins or those embedded in a membrane or attached to the cytoskeleton were most likely to be identified and quantified.

## Conclusion

The ability of electrical signals to modulate both protein expression and phosphorylation of the mTOR pathway by reversing injury-induced changes provided further evidence that SCS mechanism of action is based on affecting molecular pathways and not just electrical disruption of pain signals. Furthermore, DTMP, compared to conventional LR-SCS, appears to have a stronger effect on the mTOR pathway and the ability to reverse both proteomic and phosphorylation changes observed following onset of a nerve injury.

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## Disclosure

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