Genome-wide translational profiling in a mouse model of neuropathic pain

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Abstract

Acute pain is a protective mechanism guiding the organism away from a potentially harmful stimulus. Contrarily, the pathophysiology of chronic pain manifests in ways which are not advantageous for survival and are rather debilitating. The development and maintenance of chronic pain rely on new gene expression which is in turn responsible for the morphological and biochemical alterations in different components of the pain pathway. Translational control of gene expression has emerged as a key mechanism in regulating different forms of long-lasting neuronal hypersensitivity. Recent studies showed that signaling pathways upstream of mRNA translation, such as mTORC1 and ERK, are upregulated in chronic pain conditions, and their inhibition effectively alleviates pain in several animal models. Translation is primarily regulated at the initiation stage via the coordinated activity of translation initiation factors. The mRNA cap-binding protein, eIF4E integrates inputs from the mTOR and ERK signaling pathways. However, mRNAs whose translation is altered in chronic pain conditions remain largely unknown. Here, we performed genome-wide translational profiling of dorsal root ganglion (DRG) and spinal cord dorsal horn tissues in a mouse model of neuropathic pain, spared nerve injury (SNI), using the ribosome profiling technique. We identified distinct subsets of mRNAs that are differentially translated in response to nerve injury in both tissues. We also discovered key converging upstream regulators and pathways linked to mRNA translational control and neuropathic pain. Our data are crucial for the understanding of mechanisms by which mRNA translation promotes persistent hypersensitivity after nerve injury.

Résumé

La douleur aiguë est un mécanisme protecteur guidant l'organisme hors de stimulus potentiellement dangereux. En revanche, la physiopathologie de la douleur chronique n'offre pas d'avantages pour la survie et reste plutôt débilitant. Le développement et le maintien de la douleur chronique reposent sur une nouvelle expression génétique à son tour responsable des changements morphologiques et biochimiques de différentes composantes de la voie de la douleur. Le contrôle translationnel de l'expression génétique s'est révélé être un mécanisme essentiel dans la régulation des différentes formes d'hypersensibilité neuronale de longue durée. Des études récentes ont montré que des voies de signalisation en amont de la traduction de l'ARNm, telles que mTORC1 et ERK, sont régulées positivement dans les douleurs chroniques et que dans plusieurs modèles animal leur inhibition diminue la douleur. La traduction est principalement régulée au stade d'initiation grâce à l'activité coordonnée de facteurs d'initiation de la traduction. La protéine de liaison à l'ARNm, eIF4E, intègre les entrées des voies de signalisation mTOR et ERK. Cependant, les ARNm dont la traduction est altérée dans les conditions de douleur chronique restent en grande partie inconnus. Dans le cadre de cette présente recherche nous avons effectué un profilage d'expression du génome entier des ganglions dorsaux (DRG) et de la corne dorsale de la moelle épinière dans un modèle de douleur neuropathique chez la souris impliquant la lésion nerveuse de type SNI au moyen de la technique de profilage de ribosomes. Nous avons identifié des sous-ensembles distincts d'ARNm traduits différentiellement en réponse à une lésion nerveuse dans les deux tissus. Nous avons également découvert des régulateurs clés convergents en amont ainsi que des voies liées au contrôle translationnel de l'ARNm et à la douleur neuropathique. Nos données sont essentielles pour comprendre les mécanismes par lesquels la traduction de l'ARNm favorise l'hypersensibilité persistante suivant une lésion nerveuse.

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Preface

This manuscript-based thesis includes the contents of a review paper and a research article. Information about the manuscript status, authors and their contribution are given below. The complete manuscripts from journal website are attached in Appendix B.

In chapter 1, section 1.2.3. (including its sub-sections) uses pertinent sections of the manuscript titled **"eIF4E-dependent Translational Control: A Central Mechanism for Regulation of Pain Plasticity"**

Authors: Sonali Uttam, Calvin Wong, Theodore J Price and Arkady Khoutorsky
Manuscript status: Under review in Frontiers in Genetics

Contribution of authors: S.U. and C.W. drafted the manuscript; T.J.P. and A.K. critically revised the manuscript.

Chapter 2 comprises of the manuscript titled **"Translational profiling of dorsal root ganglia and spinal cord in a mouse model of neuropathic pain"**

Authors: Sonali Uttam, Calvin Wong, Inês S. Amorim, Seyed Mehdi Jafarnejad, Shannon N. Tansley, Jieyi Yang, Masha Prager-Khoutorsky, Jeffrey S. Mogil, Christos G. Gkogkas and Arkady Khoutorsky
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Contribution of Authors: S.U. drafted the manuscript and carried out the majority of the experiments (SNI surgery, harvesting animal tissues and ribosome profiling), interpreted and analysed the data obtained from bioinformatic analysis and framed response to reviewer's comments; C.W. performed the von Frey assay on mice, helped in preparation and formatting of figures and proof-read the manuscript; I.S.A. performed the bioinformatic analysis on ribosome profiling data; S.M.J. helped in preparation of libraries, submission of samples for sequencing, proofread the manuscript and framed response to reviewer's comments; S.N.T., J.Y., and M.P.K. proofread the manuscript; J.S.M. provided training to S.U. for SNI surgeries in his lab and proofread the manuscript; C.G.G. supervised the bioinformatic analysis of ribosome profiling data, proofread the manuscript and framed response to reviewer's comments; A.K. contributed to the design and implementation of the study, analysis of results and critically revised the manuscript.

Chapter 1. Introduction and Review of Literature

1.1. Pain - Background

Any individual capable of feeling 'pain' commonly describes it in a dispassionate manner as physical suffering and discomfort. Scientifically, pain is known to be a crucial protective mechanism to detect damaging stimuli and thus, prevent further injury. Genetic disorders causing an inability to sense pain seen in individuals with congenital insensitivity or indifference to pain (CIP) lead to early death as these individuals fail to protect themselves from injurious activities (Nagasako *et al.*, 2003). However, as the understanding and classification of pain have evolved, it has been known that pain can manifest in ways which are no longer protective and can become chronic and debilitating. The first effort to describe the sensation of pain from a neurobiological perspective, dates back to more than 60 years ago (Livingston, 1953). The definition of pain has evolved multiple times since then as pain research has progressed. The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey & Bogduk, 1994).

Pain is classified into three general varieties, of which the first two (nociceptive pain and inflammatory pain) have an overall protective effect, while the third type, which results from either damage to the nervous system (neuropathic pain) or its abnormal functioning (dysfunctional pain), is not protective and becomes maladaptive (Woolf, 2010). This third kind of pain, also known as pathological pain is the most concerning, whereas the first, nociceptive pain, is crucial for survival by enabling us to avoid harmful stimuli. The following subsections explain neuropathic pain in greater details, with emphasis on its epidemiology, the process of transition from its acute to chronic phase, related animal models, and the importance of specific nervous tissues which play an important role in pain processing.

1.1.1. Neuropathic pain

Conventionally, neuropathic pain was defined in general terms as "pain initiated or caused by a primary lesion or dysfunction in the nervous system." This definition, although useful in distinguishing neuropathic pain from nociceptive pain, lacked defined boundaries. Hence in 2011, the IASP updated the definition of neuropathic pain as "pain caused by a lesion or a disease of the somatosensory system" (Jensen *et al.*, 2011). A clear and distinct definition of neuropathic pain

would foremost lead to the development of specific and precise diagnostic tests for neuropathic pain. Hopefully, this will accelerate research in elucidating relevant pain generating pathways and will further provide more effective treatment strategies. Although being distinct and specific to the somatosensory system, neuropathic pain encompasses a multitude of diseases and lesions affecting both the peripheral nerves and central neurons. Lesions to the peripheral nervous system caused by mechanical trauma, metabolic disorders, infection, and exposure to neurotoxic chemicals, along with diseases of the central nervous system like multiple sclerosis or stroke, bring about a range of pathophysiological changes that lead to and sustain neuropathic pain. The maladaptive plasticity attributable to these lesions and diseases can result in altered and dysfunctional sensory signal transmission to spinal cord and brain, which could lead to enhanced responses to innocuous or noxious stimuli (Costigan *et al.*, 2009).

1.1.2. Transition from acute to chronic pain: mechanism and timeline

Pain research in the past decade has come a long way in answering an important question – how and when does acute pain become chronic pain? Acute pain implies a rapid onset pain condition which lasts for a relatively short time span, while chronic pain is characterized as a pain that persists beyond the normal time of tissue healing. The progression of acute pain into more persistent pain involves three inter-related processes - peripheral sensitization, central sensitization, and descending modulation (McGreevy *et al.*, 2011). Understanding the processes that trigger the transition of acute to chronic pain is essential for proper prevention and management of chronic pain.

An acute injury associated with trauma or surgery can trigger pain by facilitating neuroplastic changes in the peripheral and central nervous system and is termed peripheral sensitization. The activation of peripheral nociceptors leads to changes in conduction, transduction, and neurochemical activity in the afferent fibres. Simultaneously, nerve or tissue injury also triggers the neurogenic inflammatory pathways releasing a host of inflammatory mediators (histamine, serotonins, etc.) and pro-inflammatory cytokines (interleukin-6, nerve growth factor, TNF-α) locally or by activated mast cells or neutrophils. This triggers a cascade of events that can promote enhanced ion-channel permeability and altered gene expression, eventually leading to peripheral nociceptor hyperexcitability, or in other words ‘peripheral sensitization’ (McGreevy *et al.*, 2011).

Following peripheral sensitization, the peripheral nerves carry the signals to the spinal cord which acts as a junction, where neurons integrate, amplify, and modulate the signal, which is eventually conveyed to the higher brain centers to be perceived as pain. Continuous activation of the central synapses leads to central sensitization, whereby, even weak noxious (pain evoking) stimuli can activate second and third order neurons. The complex inhibitory, excitatory, and modulatory mechanisms converge in the spinal dorsal horn. Interneurons, glial cells, and descending pathways play a crucial role in pain modulation in the dorsal horn, contributing to central sensitization and eventually causing allodynia (pain in response to an innocuous stimulus) and hyperalgesia (heightened pain response to a noxious stimulus).

As the damaged tissue recovers and the peripheral nociception terminates, normal homeostasis is restored, thereby ending the pain process. In some cases, continuous nociception may bring about pathological changes at all levels from the periphery to the brain resulting in chronic pain. Within the peripheral system itself, the changes may include upregulation of voltage gated sodium channels, phosphorylation of protein kinases, and activation of TRPV1 receptors (Feizerfan & Sheh, 2014). Continuous nociceptive stimulation results in altered gene and protein expression in the DRG and dorsal horn neurons of the spinal cord. The most prominent changes are an increase in mRNAs encoding for sodium channels and TRPV1 receptors leading to hyper-excitability state(Macintyre *et al.*, 2010).

1.1.3. Epidemiology of chronic pain

The lack of simple diagnostic screening tools has complicated the epidemiological surveys conducted for the estimation of incidence and prevalence of neuropathic pain. Thus, estimation of the incidence and prevalence of neuropathic pain is mainly based on focused studies involving a specific condition such as trigeminal neuralgia, postherpetic neuralgia (complications of shingles-caused by chicken pox virus), polyneuropathy (general degeneration of peripheral nerves), post-stroke pain, multiple sclerosis, diabetic neuropathy, cancer, etc. (Colloca *et al.*, 2017). The epidemiological significance of Chronic Post Surgery Pain (CPSP) is also enormous. For instance, it is estimated that for about 300 million surgeries performed worldwide every year, 11% will suffer from CPSP (Lavand'homme, 2017). Furthermore, the incidence and prevalence of chronic pain are also estimated to increase over time due to increased cancer survival rates following chemotherapy, increased incidence of diabetes mellitus and overall ageing global population.

The recent developments in simple questionnaire-based screening tools have enabled several countries to conduct epidemiological surveys providing valuable new information on the incidence and prevalence of chronic pain in the general population. Screening tools like the Leeds Assessment of Neuropathic Symptoms and Signs (LANSS) and Douleur Neuropathic 4 questions (DN4) have estimated the prevalence of chronic pain to be around 7-10% of the general population worldwide (Van Hecke *et al.*, 2014) and have found that associated risk factors include age, gender, and anatomical site of the injury. Chronic neuropathic pain is found to be more frequent in women (8.9% vs 5.6%) and in patients above 50 years of age, with lower back, lower limbs and upper limbs being the most commonly affected regions (Bouhassira *et al.*, 2008). Moreover, the most frequent cause of chronic neuropathic pain was found to be Lumbar and Cervical radiculopathies (Freynhagen *et al.*, 2006). Even though there have been recent advances in diagnosing chronic pain, a more comprehensive understanding of pathophysiology and molecular basis of chronic pain would refine the research efforts and provide better prevention and treatment strategies.

1.1.4. Animal models of pain and related assays

The use of animal models to understand the pathophysiology of pain is crucial to drive the pain research forward. Use of animal models have obvious advantages like ease of standardizing genetic and environmental backgrounds, safety, and cost (Mogil *et al.*, 2010). In the last 30 years, a number of animal models have been developed to study a variety of pain states ranging from acute to chronic, neuropathic, inflammatory, cancer pain, arthritic pain, muscle pain, postoperative pain, and visceral pain (Gregory *et al.*, 2013).

Like in humans, the outcome of pain is assessed by behavioural responses in animal models as well. An extensive battery of assays is developed to measure the outcome of pain in animal models. Pain assays can be broadly classified as reflexive and non-reflexive. Reflexive pain assays rely on a functionally intact motor system and measure responses to mechanical or thermal stimulus. Using these assays, one can measure hyperalgesia (enhanced response to noxious stimulus) or allodynia (response to innocuous stimulus). Assays for thermal testing include tail flick test (D'Amour & Smith, 1941), hot-plate test (WOOLFE & MacDonald, 1944), and Hargreaves test (Hargreaves *et al.*, 1988). The most widely used assay to measure mechanical allodynia or

hyperalgesia is measuring the paw withdrawal threshold of the paw using von Frey filaments (Chaplan *et al.*, 1994).

Recently, it has been acknowledged that measurement of evoked pain response alone does not completely reflect the features of pathological pain observed in clinal settings in human patients. Clinically, pain is reported as a complex mix of evoked pain and spontaneous pain (ongoing pain in absence of a stimulus) (Mogil, 2009). Thus, various non-reflexive pain assays are being widely incorporated in preclinical studies. Spontaneous pain behaviour can be assayed by formalin test (Dubuisson & Dennis, 1977), writhing test (Blumberg *et al.*, 1965), guarding of paw (Sluka & Westlund, 1993), or limb (Gabriel *et al.*, 2007). Behaviours depicting preference for analgesics or avoidance of painful stimuli can be measured by conditional place preference (CPP) (Shippenberg *et al.*, 1988) assay for preference for analgesics. On the other hand, thermal escape test (Shippenberg *et al.*, 1988), conditioned place avoidance (CPA) (Johansen *et al.*, 2001), and Place Escape Avoidance Paradigm (PEAP) (LaBuda & Fuchs, 2000; Moqrich *et al.*, 2005) are used for measuring the avoidance for pain-evoking stimulus. Further, it is known that chronic pain adversely affects the quality of life in patients. Hence, in animal models spontaneous pain is also assayed by monitoring their home-cage activity for parameters such as food and water intake, grooming and posture, gait, as well as social interaction with other animals (Cortright *et al.*, 2008).

There are numerous animal models developed to study neuropathic pain. They can be classified into 'peripheral nerve injury models', 'central pain models', 'drug-induced neuropathy models', and 'disease induced neuropathy models'; together, there are 27 identified models (Jaggi *et al.*, 2011). The peripheral nerve injury models are developed by a variety of surgical injuries preformed on the sciatic nerve, including complete (complete sciatic nerve transaction) or partial injury (chronic constriction injury [CCI], partial spinal nerve ligation, spinal nerve ligation [SNL], spared nerve injury [SNI], tibial or sural nerve transection [SNT], and ligation of common peroneal nerve (Jaggi *et al.*, 2011)) of the peripheral nerves.

The sciatic nerve is comprised of afferent nerve fibres whose cell bodies lie in the lumbar DRG (L3-5 in C57BL-6 mice) (Rigaud *et al.*, 2008). The peripheral nerves from these three DRG merge to form the sciatic nerve which runs along the hind limbs in rodents. In the thigh region, it divides into three branches – namely tibial, common peroneal and sural branches. These three branches further extend to innervate the hind paw. The spared nerve injury procedure involves a tight

ligation followed by axotomy (removal of a 2-3 mm segment of nerves, distal to the ligature) of the tibial and common peroneal branches (Decosterd & Woolf, 2000). This surgical procedure requires that the sural branch is completely spared of any injury due to stretching of the nerve. Following SNI, the animal develops long lasting (at least up to six months in mice) cold allodynia, mechanical allodynia and hyperalgesia starting from 2-3 days post SNI procedure, which is reflected by a significant drop in the paw withdrawal threshold (Bourquin *et al.*, 2006). The lateral region of the paw, innervated by the uninjured neurons of the sural branch, become hypersensitive. Thus, the SNI model of neuropathic pain is a persistent and robust model which can be easily replicated.

1.1.5. Pain processing

Pain processing is a result of integrated sub-processes, namely nociception and perception and encompasses a multitude of cognitive, psychological, and behavioural aspects (Garland, 2012). Under normal conditions, the sensation of pain arises on encounter with an unpleasant and potentially harmful stimulus, termed as noxious stimulus. Such stimuli can exist in the external environment or originate from within the body. Noxious stimuli, which can either be physical, thermal or chemical in nature, is detected by nociceptors (Sherrington, 1952), a type of sensory receptors, widely distributed in the skin. The pathophysiological process by which the nociceptors first sense the encounter with noxious stimuli prior to communicating this information to the brain via the spinal cord is called nociception (Melzack & Wall, 1965; Brooks & Tracey, 2005). The conception of this phenomenon began in 1964 as evident from the lectures of Descartes R. on the History of Physiology dating back to between the 16th to 18th centuries (Melzack & Wall, 1965). Nociceptors are capable of translating the encounter with noxious stimuli into electrical signals (action potentials) (Purves *et al.*, 2001) that can be relayed to the designated pain processing centers in the brain, where pain is perceived and the process is referred to pain perception (Dubin & Patapoutian, 2010) (Figure 1.1).

The idea of the involvement of nociceptors in sensing noxious stimulus was established more than a century ago by Sir C. S. Sherrington, in his book "The Integrative action of the nervous system". (Sherrington, 1907; Julius & Basbaum, 2001). Nociceptors are peripheral nerve endings, finely tuned to respond to only potentially damaging stimuli (for example high temperatures, or a skin cut). This characteristic threshold of nociceptors makes them distinct from other afferent nerve

fibres which are also activated by a stimulus of similar nature, but a harmless one (for example slightly warm temperature or light touch) (Burgess & Perl, 1967).

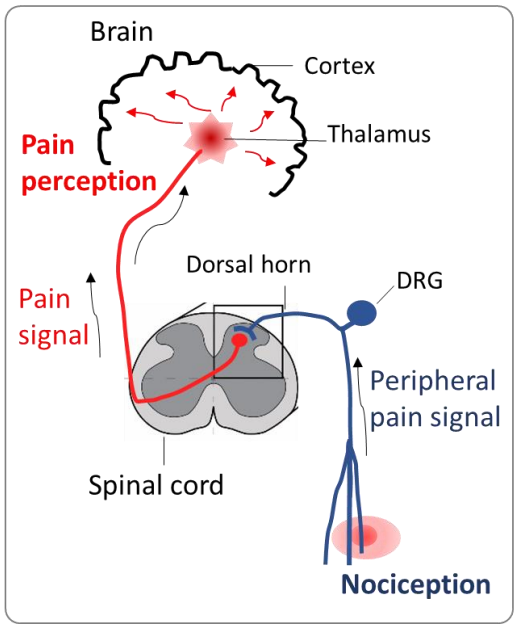

Figure 1.1. Schematic representation of pain perception
Pain receptors activated by tissue damage generate a signal (Nociception) which travels up to the spinal cord through the peripheral nerves and is further relayed to the brain. The processing of the pain signal in the brain results in the subjective experience of pain called pain perception.

The largest diameter, myelinated, and fastest conducting nerve fibres are known as Aβ sensory fibres; and a majority, but not all, respond to innocuous stimuli (e.g. touch or light rub), and thus do not participate in pain transmission (Djouhri & Lawson, 2004). Nociceptors are primarily comprised of slow conducting, thinly myelinated Aδ and unmyelinated C fibres. Aδ fibres are relatively fast conducting and respond to intense mechanical and/or thermal stimulus. They can be further classified as type I and type II based on adapting speed and threshold level. On the other hand, C fibres are relatively slow conducting but respond to a greater variety of noxious stimulus – mechanical, thermal or chemical (e.g. capsaicin) and are thus known as polymodal (Van Hees & Gybels, 1981). Aδ fibres result in the experience of sharp pain, while C fibres, which are recruited later as the stimulus persists or intensifies, result in a delayed pain, that is more intense and diffused.

Activation of nociceptors at the periphery causes the pain signal to travel along the peripheral axon toward the spinal cord via the dorsal root ganglion (DRG) (Figure 1.1.). Nociceptors, like other

somatosensory neurons are pseudo-unipolar neurons, whose cell bodies are clustered in dorsal root ganglion (nociceptors innervating the body) and trigeminal ganglion (for those innervating the head). A single process extending from the cell body bifurcates into two branches, forming a T-junction - the longer exonal branch extends to the peripheral organs, while the shorter central branch terminates at the dorsal horn of the spinal cord or to the trigeminal subnucleus caudalis, projecting to second order neurons (Basbaum *et al.*, 2009) (Figure 1.2).

1.1.6. DRG and spinal cord in pain processing

Housing the cell bodies of somatosensory neurons and nociceptors, dorsal root ganglion serves as a remarkably structure, with some of its peculiarities unexplained. DRG lacks blood brain barrier, and some of the cell somata in DRG are electrically excitable (Devor, 1999). Classically, it was referred to as a 'metabolic depot' providing support to the T-junction formed by the conducting axon and the peripheral and central branches. The formation of this T-junction was initially seen as a hurdle to sensory signalling; however, this morphology was later appreciated to help in preventing failure in signal propagation (Amir & Devor, 2003). Previous studies have established that the DRG is deeply involved in the peripheral processes that lead to the development of neuropathic pain. It has been demonstrated that changes in the DRG post-injury to the innervating neurons involve the release of cytokines, growth factors, and interleukins, as well as changes in influx of ions and gene expression changes which altogether establish hypersensitivity of the DRG neurons (Krames, 2014).

The afferent branch of the axon of DRG neurons extends to the dorsal horn of the spinal cord, wherein different afferent nerve endings project to different layers or laminae of the dorsal horn (Baron *et al.*, 2010) (Figure 1.2.). The Nociceptive C-fibres terminate in the superficial laminae, while the A-fibres project to deeper laminar levels (D'mello & Dickenson, 2008). The dorsal horn of the spinal cord acts as a gateway where this maladaptive plasticity from the peripheral nociceptive circuits is relayed to the spinothalamic tract which results in stimulation of various brain areas. The relay of plasticity from peripheral to central circuits is mediated by a repertoire of signaling molecules, which are not very well characterized. These signaling molecules are expected to be a result of differential gene expression taking place in peripheral neurons on induction of chronic pain. Knowledge about the comprehensive landscape of the DRG and dorsal

horn of the spinal cord can help us understand how hypersensitivity is maintained and relayed to the brain in chronic pain conditions.

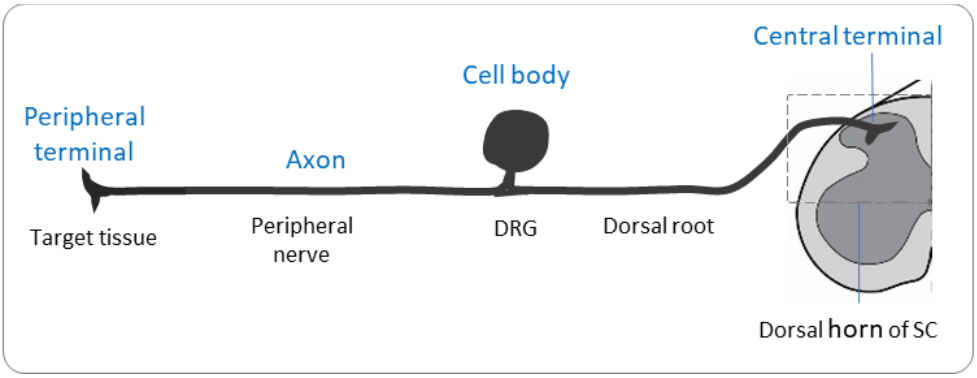

Figure 1.2. Schematic view of a nociceptor (modified from (Woolf & Ma, 2007))
A single process extending from the cell body bifurcates into two branches, forming a T- junction - the longer exonal branch extends to the peripheral organs, while the shorter central branch terminates at the dorsal horn of the spinal cord (SC).

1.2. Gene expression in the chronic pain state

Gene expression is a complex mechanism with multiple levels of regulation. Beginning from the last decade, it has been established that new gene expression underlies the phenotypic changes in nociceptors and establishment of abnormal plasticity, leading to hypersensitivity. Regulation of gene expression takes place at various levels starting from the organization of the genome, e.g. histone modifications regulate the packing of DNA into chromosomes and the opening of the chromatin to give access to various transcription factors. Another early level of regulation is exerted during DNA methylation. The assembly of DNA into the chromatin structure is largely influenced by the methylation pattern. It has been shown that nerve injury induces aberrant methylation patterns causing dysregulated expression of the affected genes (Garriga *et al.*, 2018). These early levels of regulation primarily affect transcription and are reflected by the level of mRNA transcripts corresponding to these genes.

Transcription is also regulated by various signalling pathways, through a group of transcription factors. In the soma of DRG neurons, posttranslational modifications of proteins, which are in turn able to regulate gene expression, have been detected and it is demonstrated that these changes are responsible for inducing changes in neuronal phenotypes contributing to the establishment of hypersensitivity (Woolf & Costigan, 1999). Phosphorylation and activation of signalling

molecules belonging to the mitogen activated protein kinase (MAPK) pathway, which itself regulates gene expression at the level of both transcription and translation, has been shown (Cheng & Ji, 2008). The mammalian target of rapamycin (mTOR) and extracellular-signal-regulated kinase (ERK) are known to be the master regulators of translation (Roux & Topisirovic, 2012). Activation of signalling molecules both upstream and downstream of ERK or mTOR pathways have also been found to be upregulated in response to noxious stimuli, and their inhibition leads to a rescue of hypersensitivity (Khoutorsky & Price, 2017). Together these findings highlight the role of gene expression regulation at both transcriptional and translational levels in the pathophysiology of chronic pain. The following sections describe the studies which provide evidence for the role of transcription and translation in the development of chronic pain. Pain related studies which have employed genome-wide gene expression profiling techniques to study the pain transcriptome and/or translatome are also discussed.

1.2.1. Evidence for involvement MAPK/ERK in establishment of chronic pain

The MAPK family responds to a variety of extracellular stimuli and transduce them into posttranslational (e.g. phosphorylation) and transcriptional responses. The prime members of the MAPK family include – ERK, p38 MAPK, and c-Jun N-terminal kinase (JNK); they have been demonstrated to play a role in neuronal plasticity (Ji *et al.*, 2009). These MAPKs are themselves activated by a series of subsequent phosphorylation events starting from MAPK kinase kinases (MEKKs) and followed by MAPK kinases (MKKs or MEKs) (Figure 1.3.).

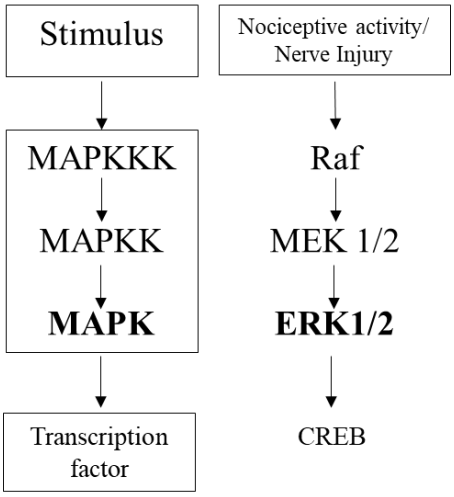

Figure 1.3. Overview of MAPK pathway and upstream activators of ERK1/2 (modified from (Ji et al., 2009))

Originally, regulation mediated by ERK was associated with cell growth and differentiation (Sun *et al.*, 2015). Eventually, accumulating evidence has highlighted the role of ERK in neuronal plasticity. The role of the ERK/MAPK cascade has been demonstrated in long-term facilitation (LTF) by regulating transcription factors ATF4 and C/EBP, long-term potentiation (LTP) and memory formation (Impey *et al.*, 1999). CREB, which is not a direct target of MAPK, was also found to be crucial for LTP formation in the brain (Impey *et al.*, 1998). Expression of CREB is up-regulated up to 300 days post SNI in rats (Herdegen *et al.*, 1992).

With the development of specific MAPK inhibitors, future research could resolve the role of specific MAPK pathways. Recently, a study (Qu *et al.*, 2016) assessed the effect of intrathecal inhibition of p38, JNK or ERK using their respective inhibitors, on mechanical allodynia in a neuropathic pain model in rats. It was found that mechanical allodynia developed after chronic constriction of DRG (CCD), but subsided following the administration of p38, JNK or ERK inhibitors. The group also showed an increase in the mRNA levels, and both the dephosphorylated and phosphorylated protein levels of p38, JNK, and ERK in the DRG of rats subjected to CCD, as assessed by real time quantitative PCR and western blot analysis, respectively. Administration of inhibitors (SB203580, SP600125 and U0126 respectively) significantly diminished the CCD-induced increase in both mRNA and protein levels of the target MAPKs.

Several other studies have shown the role of ERK in the development of hyperalgesia and allodynia in both inflammatory and neuropathic pain models. Wang *et al*., observed increased phosphorylated ERK [p-ERK]) in response to increased expression of a glycolytic enzyme, Pyruvate kinase isozymes M2 (PKM2), after nerve injury (Chronic constriction injury) in rat lumbar spinal cord (Wang *et al.*, 2018). It has also been suggested that the activation of ERK within the ipsilateral DRG and dorsal horn of the spinal cord is sequential (Zhuang *et al.*, 2005). More precisely, it was shown that there was an immediate, but very transient (from 10 min post injury, up to 6 h) activation of ERK (estimated by levels of p-ERK) in the spinal cord dorsal horn and DRG neurons. This was followed by activation of ERK in the microglia several days after injury, and finally in astrocytes after a few weeks.

Together these studies corroborated the therapeutic potential of MAPK and MEK inhibitors in alleviating chronic pain (Ma & Quirion, 2005). However, these studies did not provide insights on

which subset of genes were differentially expressed as a result of regulation by the various transcriptional and translational regulators.

1.2.2. Transcriptomic analysis in chronic pain

Transcriptomic analysis involves studying the complete set of RNA transcripts in the cell. Since previous studies showed increased expression of transcription factors in chronic pain conditions, researches were motivated to assess, transcription of which genes was affected in specific pain conditions. Early studies used quantitative PCR (q-PCR) to detect up- or down- regulation in the expression of genes by assessing the relative abundance of mRNA transcripts. Okamoto *et al*., utilised this approach to demonstrate the increase in the expression of specific cytokines (IL-b1, IL6, and TNF) at different time points in the sciatic nerve of rats that underwent CCI (Okamoto *et al.*, 2001). Another study also used reverse transcriptase PCR to quantify the relative abundance of specific mRNAs in brain stem after nerve injury and suggested upregulation of NMDA receptors (Miki *et al.*, 2002).

With further developments, qPCR could be conducted and analysed in real-time, and was known as Real time quantitative PCR (qRT-PCR), though some studies refer the real time version also as qPCR. This development circumvented the need to electrophorese the PCR product on agarose gel and quantify the intensity of bands. Instead, cycle threshold (C_t value), which is the number of amplification cycles required by a transcript to generate enough signal to cross the set threshold, was used as a measure to quantify the abundance of the transcript. A more abundant transcript would require lesser number of amplification cycles to generate a signal higher than the threshold, and thus, have a lower Ct value.

Tools to study the expression levels of genes at the level of transcription have evolved at a relatively faster rate than those for studying other levels of gene expression (e.g. proteomics). Development of microarray and particularly advancement in sequencing technologies enabled researchers to analyze absolute and relative changes in levels of mRNA transcripts on a global scale. Wang *et al*., exploited microarray to obtain a global view of gene expression in a model of neuropathic pain (SNL) in rats at day 13 post injury. They confirmed some of their significantly differentially regulated genes (DEGs) by real time quantitative PCR and immunohistochemistry. The significant findings from the study were identification of a subset of genes in DRG which showed immediate upregulation, and that genes involved in ion channels, and signalling molecules

related to excitability of neurons and neuroinflammation markers were also differentially expressed after injury (Wang *et al.*, 2002). Another study with a similar approach compared the transcriptomic profile of lumbar spinal cord and DRGs in neuropathic (CCI induced) and inflammatory (Complete Freund's Adjuvant [CFA] induced) pain (Parkitna *et al.*, 2006). They identified an upregulation of genes associated with immune response and microglia activation, as well as cytoskeleton rearrangement that were associated with neuropathic pain. They also implicated the role of calcitonin gene related peptide (CGRP) expression in the lumbar spinal cord to be involved in the persistence of neuropathic pain.

While the most commonly assessed tissues were DRG and spinal cord, a research group from Korea assessed the differential gene expression in spinal nerves between normal rats, those undergoing neuropathic pain, and rats treated with electroacupuncture (EA) (Ko *et al.*, 2002). They found that genes differentially regulated in rats with neuropathic pain were rescued showing normal expression levels when treated with EA. They also stated that opioid signalling events might be involved in neuropathic pain and analgesic effects of EA. Recently, Cobos *et el*., dissected the cold allodynia from mechanical allodynia experienced after SNI, both in terms of time of onset and the global gene expression pattern. Using microarray and Ingenuity pathway analysis (IPA), they showed that transcripts that were aligned more with the development of cold allodynia were nociceptor-related, whereas those associated with tactile hypersensitivity were "immune cell centric" (Cobos *et al.*, 2018).

Several researchers also analyzed the transcriptomic profiles in cells outside of nervous system tissue. Human-based microarray data was generated by Adıgüzel *et al.* in 2015 to identify differentially expressed genes in peripheral blood mononuclear cells (PBMCs) from human patients suffering from intractable neuropathic pain (He *et al.*, 2017). They identified ornithine decarboxylase 1 (ODC1) and ornithine aminotransferase (OAT) to be regulated by additional transcription factors. Another study assessed the differentially regulated genes in PBMCs in a cohort of knee osteoarthritis (OA) patients vs. a healthy cohort (Attur *et al.*, 2011). They classified certain significantly differentially expressed genes as diagnostic biomarkers for OA. Interestingly, they could sub divide the OA cohort in two groups based on the differential overexpression of inflammatory genes. Group with increased expression of interleukin 1-β (IL1-β) were associated with a greater risk of radiographic progression of OA.

Other preclinical modeled, microarray-based studies led to the discovery of genes that were not thought to be 'pain-associated', for example, Potassium voltage-gated channel subfamily S member 1 (KCNS1) (Costigan *et al.*, 2010), GTP cyclohrdrolase 1 (GCH1) (Tegeder *et al.*, 2006), and neuropeptide VGF nerve growth factor inducible (Moss *et al.*, 2008).

While many studies exploited microarray for transcriptomic analysis in pain conditions, the development of sequencing technologies was being carried out in parallel. The advent of RNA sequencing (RNA-seq) led to significant advancements in transcriptomic analysis of chronic pain.

1.2.2.1. Evolution of transcriptomic studies in pain with the advent of next-generation sequencing technologies

After microarray, the next prominent advancement that led to an increase in studies of global gene expression was affordable next generation sequencing technologies, or high throughput sequencing technologies (e.g. Illumina, SOLiD, Roche). Particularly, RNA-seq was a more informative and reliable alternative, because it does not require any prior knowledge as compared to microarray and quantitative PCR, which require prior information about the organisms and its genes to design primers/probes (Wang *et al.*, 2009; Costa *et al.*, 2013), and thus limit the interrogation to a subset of already known or predicted transcripts. Furthermore, drawbacks like non-specific binding and signal saturation makes it also difficult to spot differential expression of genes that have very low or very high copy numbers. On the other hand, RNA-seq is capable of revealing information about various complexities if mRNA expression such as allele-specific expression, alternative splicing, and even identification of novel transcripts.

Perkins *et al.* in 2014 did a comparison-based study to evaluate the ability of exon microarrays vs. RNA-seq to identify differentially expressed genes after L5 spinal nerve transection model in rat. Even though microarray and RNA-seq data largely agree, based on their comparison of the two platforms for various criteria, they recommended the use of RNA-seq technology for transcriptional profiling. They demonstrated that RNA-seq can identify a greater number of DEGs, owing to its increased sensitivity and dynamic range. Greater sequencing depth also benefits the analysis. Finally, they also appreciated the ability of RNA-seq to detect novel and previously unannotated transcripts (Perkins *et al.*, 2014). Hammer *et al.* exploited this agonistic feature of RNA-seq to unravel more than ten thousand novel exons and discovered new exons of which 21% were differentially regulated in pain (Hammer *et al.*, 2010).

However, most pain researches utilised RNA-seq for gene expression profiling in order to explore differential expression of genes in samples from different conditions such as different treatment, tissue type or time points (Oshlack *et al.*, 2010). Usually researchers aim to compare gene expression in wild-type vs. mutant or knockouts, uninjured vs. preclinical pain models or different time points in the same tissue and pain model. The general pipeline followed for such analysis is extraction of RNA from samples and preparation of cDNA libraries, which are sequenced on a high throughput sequencing platform to give tens of millions of raw reads. These reads are mapped to the reference genome (genome of the organisms from which the initial tissue/cell samples were obtained). Then a summary table is generated which describes the number of reads mapping to coding region, exons, or junctions etc. following which the data is normalized by calculating RPKM. The next step involves statistical analysis to identify differentially expressed genes (DEGs), and their associated p-values and fold change values. Usually a gene ontology search is also performed in the end to find biological insights on the DEGs (Oshlack *et al.*, 2010).

A study aimed at obtaining a comprehensive view of altered gene expression in DRG, post-nerve injury was carried out by Xiao *et al.* in 2002. The group prepared cDNA libraries from the DRG of rats which underwent peripheral axotomy at days 2, 4,7 and 14 post-injury. They found 76 genes belonging to neuropeptides (about 50%), receptors (majorly G-protein coupled receptors [GPCRs]) and ion channels (together less than 10%) to be strongly regulated after injury. They noted that the majority of the differentially expressed genes were 'upregulated' and most of the significant changes were observed starting at day 2 and 7 and were maintained up to day 14. Transcription factor JUN-D and translation initiation factor eukaryotic initiation factor 4E (eIF4E) were identified to be significantly upregulated (Xiao *et al.*, 2002). Similarly, another group investigated the transcriptome in dorsal spinal cord under persistent inflammatory hyperalgesia, using RNA-Seq. They found the expression levels of several transcription factors (c-Fos, the Fos paralog Fra2, and JunB, and 3 paralogs of NGFI-B [nuclear receptor family]) were elevated at 2h and returned to normal by 48h. Increased expression of certain neuropeptide transcripts (prodynorphin, proenkephalin and protachykinin) remained stable. Most DEGs were immune-linked and contribute to the microglial complement system (Sapio *et al.*, 2017). They studied the transcriptome in pain processing tissues, extended from rodent (mouse and rat) models of pain to humans as well. Recently, a group based in The University of Texas at Dallas, performed RNA-seq based transcriptomic analysis on lumbar (L2) DRG from 3 female human organ donors who

had no previous history of chronic pain. They then contrasted the transcriptomic data from human DRGs (hDRGs) with other publicly available data sets from human tissues and orthologous tissues in mouse (Price *et al.*, 2016; Ray *et al.*, 2018).

The quest to identify differentially regulated genes has been carried out in other central nervous system tissues, beyond DRG and spinal cord. In 2013 a group based in McGill University assessed the genome wide transcriptomic changes in the prefrontal cortex of mice sustaining SNI for 6 months. They identified both coding (exonic) and non-coding (interonic and intergenic) regions to be differentially expressed. The differentially expressed genes identified by them included genes involved in brain structure and function (*clca1, syt2, grin1, scn1a, krt20, xlr4b, gfap, lbp and robo3*) in addition to genes involved in neuronal plasticity (*grin1 and gfap*) (Alvarado *et al.*, 2013).

1.2.2.2. Cell specific transcriptomic profiling of pain

Recently, the focus of some researchers has shifted from analyzing whole tissues, to studying specific cell types for example, nociceptors. Berta *et al.* took into consideration the complex composition of DRG and performed a distinct study aimed at characterizing the gene expression profile separately in injured and non-injured nociceptive neurons using microarray and qRT-PCR validation. The cell bodies of injured and non-injured neurons were separated by laser-capture microdissection (LCM). They pointed out that the global transcriptomic signature was very different from that of whole DRG following SNI. They also showed that non-injured neurons exhibited very few changes at the transcriptional level, while several novel transcripts were differentially expressed in the injured neurons, with most of them being related to oxidative stress (Berta *et al.*, 2017).

In another study, Goswami *et al.* exploited the fact that sensory neurons which transduce pain signals generated from heat or inflammation express the transient receptor potential cation channel, subfamily V, member 1 (TRPV1) ion channel. They fluorescently labelled these TRPV1 expressing neurons using bacterial artificial chromosome (BAC)- transgenic, TRPV1-promotor-*Cre* recombinase system expression in mice and sorted the fluorescently labelled cells from non-TRPV1 neurons in the DRG using fluorescence-activated cell sorting (FACS). In contrast, they also deleted TRPV1 expressing neurons in a different set of mice (TRPV1-DTA transgenic mouse line) by expressing diphtheria toxin fragment A (DTA) in TRPV1 positive neurons. They were thus, able to perform transcriptomic profiling selectively in a pool of TRPV1 expressing neurons

and in DRG with TRPV-cells deleted, against a wild type control having mixed population of sensory neurons and glial cells (Goswami *et al.*, 2014). Similarly, Chiu *et al.* sorted labelled neurons (nociceptive, pruriceptive, and proprioceptive) from three different lines of mice using FACS and studied the transcriptome using microarray (Chiu *et al.*, 2014).

Such cell specific approaches greatly benefited from developments in RNA-seq technology. Introduction of single cell RNA-seq (scRNA-seq) was used by several researchers to study the transcriptome of specific neurons (Thakur *et al.*, 2014; Usoskin *et al.*, 2015; Hu *et al.*, 2016; Li *et al.*, 2016). Usoskin *et al.* adopted a '*de-novo*' approach to categorize DRG neurons in mice, based on their transcriptional profile. They randomly picked single cells from lumbar DRGs and plated them in wells of a 96-well plate. Out of the cells picked, 622 were neurons and each of them were individually sequenced on an Illumina platform. Neurons were grouped based on the similarities and differences in the sequenced transcriptome, resulting into 11 types, reiterating the complexity of the somatosensory system (Usoskin *et al.*, 2015). Another study moved ahead with employing higher coverage scRNA-seq to classify somatosensory neurons (Li *et al.*, 2016). They collected different populations of neurons from the lumbar (L5) DRG of 19 mice. Immunostaining was used to differentiate neurons based on the differential markers expressed. Isolectin B4 (IB4) was used as a marker for non-peptidergic small neurons, CGRP was used to label the peptidergic subset and neurofilament 200 (NF200) for large neurons. Hu *et al.* studied neuron specific transcriptomics using scRNA-seq post-sciatic nerve transection (SNT) injury. They found that differential transcriptional regulation of certain subsets of genes was correlated with neuronal subtypes; however, interestingly they found regeneration associated genes (RAGs) to be upregulated in all the studied neuron types (nonpeptidergic nociceptors, peptidergic nociceptors, mechanoreceptors and proprioceptors) (Hu *et al.*, 2016).

1.2.3. Translational control of gene expression and chronic pain

Chronic pain is a debilitating condition affecting more than 20 percent of the population worldwide (Steglitz *et al.*, 2012; de Souza *et al.*, 2017). Chronic pain is most commonly triggered by tissue inflammation or nerve injury, which are caused by metabolic diseases (diabetes), autoimmune diseases, viral infection (herpes zoster), cancer, chemotherapy drugs (e.g. platinums, taxanes, epothilones, and vinca alkaloids), and nerve entrapment or blunt trauma. Chronic pain, however,

can also appear without any recognizable trigger such as in fibromyalgia, migraine, irritable bowel syndrome, and interstitial cystitis.

In most cases, the pain is a result of increased sensitivity of peripheral or central nociceptive circuits to stimulation, causing painful sensation in response to a normally *innocuous* stimulus. The increase in sensitivity, also called sensitization, is mediated by a combination of mechanisms taking place at several levels along the pain pathway including primary sensory neurons, spinal cord, and higher brain areas (Todd, 2010; Yekkirala *et al.*, 2017).

Long-lasting increases in the sensitivity and responsiveness of pain circuits is ultimately accompanied by changes in gene expression, which support biochemical and structural alterations in neuronal and non-neuronal cells involved in pain processing. Gene expression is a multi-step process that is tightly regulated at different levels. Regulation of the rate by which mRNA is translated into a protein is called translational control (Sonenberg & Hinnebusch, 2009; Robichaud *et al.*, 2018). Translational control has a strong impact on the abundance of proteins in the cell, and its dysregulation contributes to many pathologies in the nervous system including developmental abnormalities, metabolic dysregulation, autism spectrum disorder (ASD), and epilepsy (Buffington *et al.*, 2014b; Tahmasebi *et al.*, 2018). Tissue injury, metabolic diseases, and certain drugs (e.g. anticancer and opioids) cause an upregulation of mRNA translation in pain-processing tissues such as dorsal root ganglion (DRG) and dorsal horn of the spinal cord (Melemedjian & Khoutorsky, 2015; Khoutorsky & Price, 2018). Inhibition of translational control signalling in these tissues reduces the sensitization of nociceptive circuits and alleviates pain, demonstrating a central role of translational upregulation in the development of persistent pain (Price *et al.*, 2007; Jimenez-Diaz *et al.*, 2008; Asante *et al.*, 2009; Geranton *et al.*, 2009; Price & Geranton, 2009; Melemedjian *et al.*, 2010; Xu *et al.*, 2011; Bogen *et al.*, 2012; Ferrari *et al.*, 2013; Obara & Hunt, 2014). The rate of mRNA translation is controlled via several mechanisms (Costa-Mattioli *et al.*, 2009; Robichaud *et al.*, 2018). The recruitment of the ribosome to the mRNA is a central step in translation initiation and the major site for regulation. A key mechanism to regulate this process is controlling the activity of the eukaryotic translation initiation factor 4E (eIF4E), which binds a mRNA "cap" structure (a 7-methylguanosine linked to the first nucleotide at the 5' end of all nuclear transcribed eukaryotic mRNAs) and initiates ribosome recruitment (Altmann *et*

al., 1985; Sonenberg & Hinnebusch, 2009). In this review, we focus on the regulation of eIF4E-dependent mRNA translation initiation in nociceptive plasticity, highlighting a central role of this mechanism in the development of chronic pain.

1.2.3.1. Translational control mechanisms

The process of translation can be divided into three phases: initiation, elongation, and termination. Most of the regulation of translation occurs at the initiation step (Sonenberg & Hinnebusch, 2009; Merrick & Pavitt, 2018). The initiation is regulated by a large number of translation initiation factors, which mediate the recruitment of the ribosome to the mRNA, followed by scanning of the 5' untranslated region (5' UTR) of the mRNA for the presence of an AUG start codon. A critical step in this process is the binding of eIF4E to the mRNA cap. Following binding to the cap, eIF4E binds an mRNA helicase eIF4A and a large scaffolding protein eIF4G to form a tri-subunit complex, called eIF4F (Figure 1.4). eIF4F facilitates the recruitment of the 43S preinitiation complex (PIC) to the mRNA. PIC is composed of a small 40S ribosomal subunit, translation factors eIF1, eIF1A, and eIF3, and a ternary complex (eIF2: GTP bound to initiator, Met-$tRNA_i^{Met}$). Recruitment of the PIC is followed by the scanning of the mRNA 5' UTR and joining of a large ribosomal subunit (60 S), upon encountering a start codon, to form an 80 S ribosome that is competent to proceed to the elongation phase of translation. Importantly, the helicase activity of eIF4F (mediated by eIF4A) is required for unwinding the mRNAs 5' UTR secondary structure to allow the scanning process and translation (Parsyan *et al.*, 2011).

Other major mechanisms involved in the regulation of translation initiation include regulation of ternary complex availability (via phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 α [eIF2α] (Trinh & Klann, 2013)); regulation of the length of mRNA poly(A) tail which promotes translation and protects mRNA from degradation (Gray *et al.*, 2000; Kahvejian *et al.*, 2001; Derry *et al.*, 2006); and finally translation initiation via a cap-independent mechanism (mediated by internal ribosome entry site, IRES) (Pelletier & Sonenberg, 1988; Macejak & Sarnow, 1991; Leppek *et al.*, 2018). Since the expression levels of eIF4E are the lowest among all translation initiation factors, the formation of the eIF4F complex and correspondingly, translation initiation are the rate-limiting steps for translation under most circumstances.

1.2.3.2. Regulation of cap-dependent translation

eIF4E activity is tightly regulated via two mechanisms. Translational repressor 4E-binding protein (4E-BP) binds eIF4E and prevents its association with eIF4G, and thus precludes the formation of the eIF4F complex. In mammals, there are three 4E-BP isoforms - 4E-BP1, 4E-BP2, and 4E-BP3, which have similar functions but exhibit differences in tissue distribution. The binding of 4E-BP to eIF4E depends on the 4E-BP phosphorylation state. Upon phosphorylation by the mechanistic target of rapamycin complex 1 (mTORC1), the affinity of 4E-BP to eIF4E is reduced, leading to its dissociation from eIF4E and allowing the formation of eIF4F complex at the mRNA cap. This promotes the recruitment of 43S PIC to the mRNA and stimulation of translation (Figure 1.4). Even though all eukaryotic mRNAs have a cap, not all cellular mRNAs are equally sensitive to this mechanism, and the translation of "eIF4E-sensitive mRNAs" is preferentially stimulated by increased eIF4E activity. For example, housekeeping mRNAs such as *GAPDH* and *β-actin* are less sensitive to eIF4E as compared to mRNAs involved in cell growth, proliferation, and immune responses (e.g., c-MYC, cyclins, BCL-2, MCL1, osteopontin, survivin, vascular endothelial growth factor [VEGF], fibroblast growth factors [FGF], and matrix metalloproteinase 9 [MMP-9]) (Rousseau *et al.*, 1996; Sonenberg & Gingras, 1998; Bhat *et al.*, 2015; Chu & Pelletier, 2018). The mRNA features rendering eIF4E-sensitivity have been typically associated with 5'UTRs enriched with high-complexity secondary structures (Pelletier & Sonenberg, 1985; Sonenberg & Gingras, 1998). It has been demonstrated that a long 5'UTR favours the formation of stable secondary structures, and that the proximity of these structures to the cap obstructs eIF4F complex formation. On the other hand, hairpin structures with a greater free energy, located further away from the cap, restrict 5'UTR scanning (the progression of the PIC toward the start codon) (Kozak, 1989; Pickering & Willis, 2005). However, translation of a subset of mRNAs without long 5'UTR can still be sensitive to eIF4E, indicating that other 5'UTR signatures may also render this sensitivity (Leppek *et al.*, 2018). Potential mechanisms include the presence of 5′ terminal oligopyrimidine tracts (5′*TOPs*) (Thoreen *et al.*, 2012) and *cis*-regulatory elements (Wolfe *et al.*, 2014; Truitt *et al.*, 2015; Hinnebusch *et al.*, 2016; Truitt & Ruggero, 2016; Leppek *et al.*, 2018) at the 5'UTR. For example, a Cytosine-rich 15-nucleotide motif, termed Cytosine Enriched Regulator of Translation (CERT), was shown to be responsible for conferring eIF4E sensitivity under oncogenic transformation and oxidative stress (Truitt *et al.*, 2015).

Although most studies have attributed the elevated translation of mRNAs with highly structured 5'UTRs to the cap-binding ability of eIF4E and it being the limiting component of the eIF4F complex, other studies did not find that the cap-binding ability completely explained eIF4E function and explored further mechanisms of eIF4E-mediated translation regulation. This led to the identification of an additional function of eIF4E – stimulation of eIF4A helicase activity, which is independent of its cap-binding ability (Feoktistova *et al.*, 2013).

In addition to regulation by mTORC1/4E-BP, eIF4E activity is also controlled via phosphorylation of its sole phosphorylation site, Ser 209, by mitogen activated protein kinase [MAPK]-interacting protein kinases (MNKs) 1 and 2, downstream of the extracellular-signal-regulated kinase (ERK) and the p38 MAPK signaling cascades (Figure 1.4) (Pyronnet *et al.*, 1999; Waskiewicz *et al.*, 1999). The phosphorylation of eIF4E is associated with altered translation of a subset of mRNAs, although the mechanisms underlying the effect of this phosphorylation event on translational efficiency and transcript-specificity remain elusive.

Since eIF4E is a downstream effector of both mTORC1 (via 4E-BP-dependent repression) and ERK (via eIF4E phosphorylation), its activity can be modulated by a multitude of external and internal cues that activate these central cellular signaling pathways. Numerous membrane receptors activate mTORC1 and ERK signaling in neurons including tyrosine receptor kinase A (trkA) and trkB, receptors from the insulin receptor family (IR, IGF1R, EGFR), and metabotropic glutamate and NMDA receptors. In addition to the extracellular cues, these pathways integrate intracellular signals conveying information on the status of cellular energy (via AMPK), oxygen levels (via activation of AMPK and REDD1 [Regulated in DNA damage and development 1]), and DNA damage (via the induction of p53 target genes) (Saxton & Sabatini, 2017) (Figure 1.4).

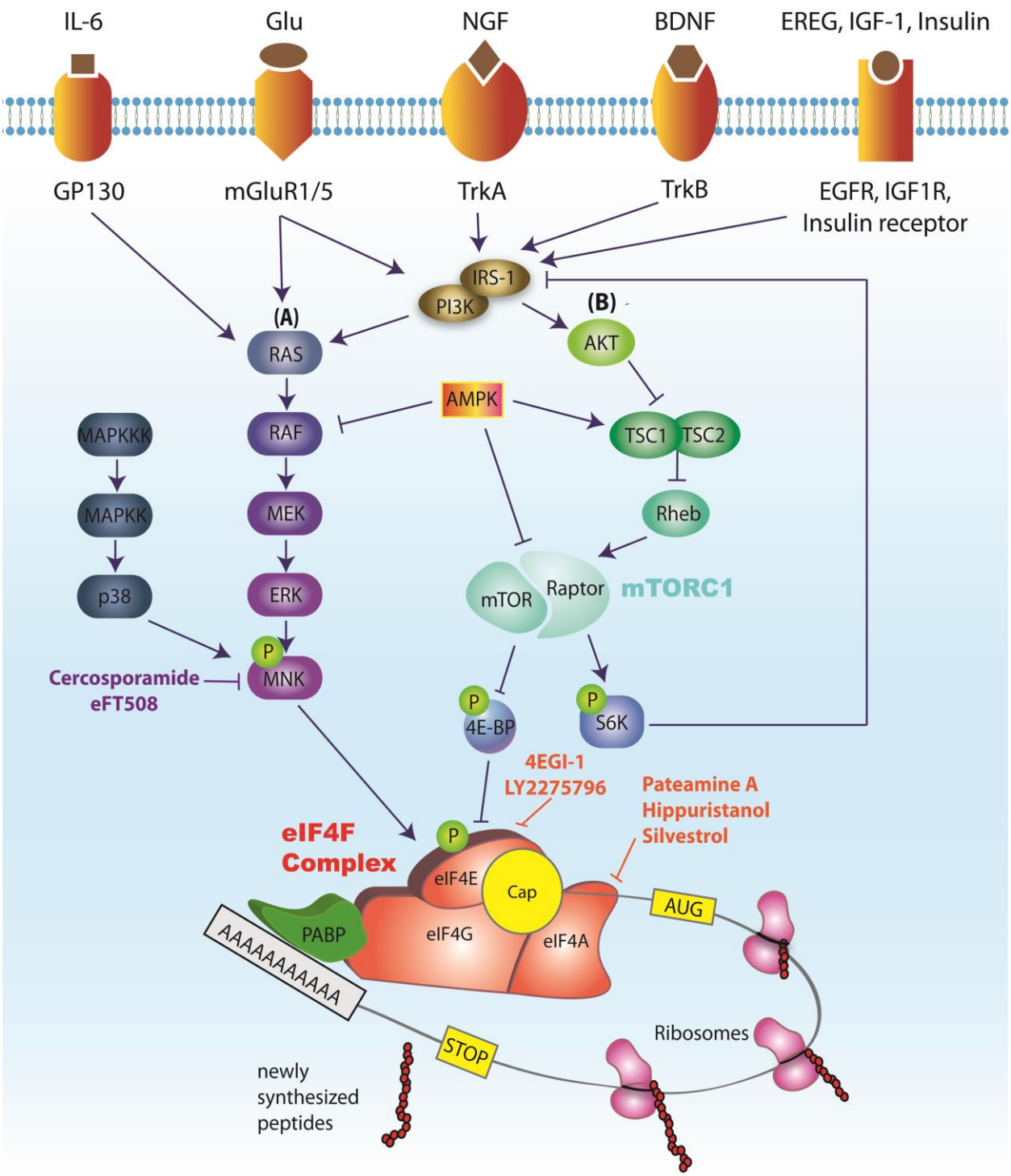

Figure 1.4. Schematic illustration of the major signalling pathways regulating eIF4E activity and translation initiation

Figure 1.4. Schematic illustration of the major signalling pathways regulating eIF4E activity and translation initiation

The cap binding ability of eIF4E makes it a central regulator of translation. A critical step in the translation initiation process is the binding of eIF4E to the mRNA cap. eIF4E mediates the formation of the eIF4F complex on the mRNA cap structure (a 7mGp bound to the first nucleotide). eIF4F complex, in addition to eIF4E, consists of eIF4G (scaffolding protein) and eIF4A (helicase). Successful formation of eIF4F complex on the mRNA cap further promotes the recruitment of the pre-initiation complex (PIC), followed by 5'UTR scanning to reach the start codon AUG and joining of 60S ribosomal subunit. This event marks the completion of translation initiation.

eIF4E is a downstream effector of both mTORC1 (via 4E-BP-dependent repression) and ERK (via eIF4E phosphorylation by MNK 1/2). The activities of mTORC1 and ERK signalling pathways are in turn modulated by a multitude of external signals (tyrosine receptor kinase A (trkA) and trkB, receptors from the insulin receptor family (IR, IGF1R, EGFR), and metabotropic glutamate and NMDA receptors) and internal cues (status of cellular energy (via AMPK), oxygen levels (via activation of AMPK and REDD1 [Regulated in DNA damage and development 1], and DNA damage [via the induction of p53 target genes]). Various inhibitors of cap dependent translation initiation have been identified. 4EGI-1 inhibits eIF4E's interaction with eIF4G, thus inhibiting the formation of eIF4F complex. Cercosporamide blocks MNK phosphorylation, which in turn prevents phosphorylation of eIF4E. Inhibitors of eIF4A have also been identified which function by either blocking its helicase activity (hippuristanol) or by preventing its participation in the eIF4F complex (pateamine A, and silvestrol).

1.2.3.3. eIF4E in regulation of peripheral nociceptive plasticity

Tissue injury induces profound changes in the phenotype of sensory neurons, increasing their excitability and changing the connectivity within the peripheral tissue and spinal cord. These alterations are driven by pro-inflammatory molecules released from injured tissues, such as neurotrophin nerve growth factor (NGF) and cytokine interleukin 6 (IL-6), as well as by neuronal activity evoked by direct injury to the nerve. ERK and mTORC1, two central intracellular pathways, are stimulated by tissue inflammation and nerve injury, diabetes, cancer, and drug-induced neuropathies (Melemedjian & Khoutorsky, 2015; Khoutorsky & Price, 2018). In addition to the phosphorylation-mediated activation of mTOR, downstream of PI3K/AKT pathway, a recent study showed that nerve injury stimulates local axonal *mTOR* mRNA translation (Terenzio *et al.*, 2018). Translation profiling of DRG tissue from mice subjected to nerve injury showed that ERK is a key regulatory hub controlling both transcriptional and translation gene expression networks (Uttam *et al.*, 2018).

Inhibition of ERK and mTORC1 signaling alleviates the development of pain hypersensitivity in a variety of pain models (Ji *et al.*, 2009; Chen *et al.*, 2018; Khoutorsky & Price, 2018). Since ERK and mTORC1 pathways converge on eIF4E to control the rate of cap-dependent translation, it was suggested that eIF4E might play a central role in the sensitization of pain circuits via regulating the translation of specific mRNAs. The physiological significance of eIF4E phosphorylation was studied using mice lacking eIF4E phosphorylation (knock-in mutation of serine^{209} to alanine, *eIF4E*^{S209A}). These mice display greatly reduced mechanical and thermal hypersensitivity in response to intraplantar administration of IL-6, NGF, and carrageenan, as well as diminished hyperalgesic priming (Moy *et al.*, 2017). Moreover, the increase in excitability of *eIF4E*^{S209A} primary sensory neurons in response to IL-6 and NGF was reduced as compared to wild-type (WT) controls. These findings were recapitulated in *MNK1/2* knockout mice, which also lack eIF4E phosphorylation. In the nerve injury model of neuropathic pain, spared nerve injury (SNI), the development of mechanical and cold hypersensitivity was reduced in both *eIF4E*^{S209A} and *MNK1/2* knockout mice. Notably, local intraplantar inhibition of MNK with cercosporamide reduced mechanical hypersensitivity in response to NGF and alleviated hyperalgesic priming (Moy *et al.*, 2017). These findings support the notion that the stimulation of eIF4E phosphorylation is imperative for the phenotypic changes of sensory neurons, promoting the hyperalgesic state and

contributing to the development of chronic pain, and that this likely occurs independently of effects on inflammation (Moy *et al.*, 2018b). Experiments with local administration of cercosporamide also indicate that pro-inflammatory mediators- or tissue injury-induced phosphorylation of eIF4E mediates sensitization of sensory neurons via local mRNA translation.

The advances in translational profiling techniques have provided important insights into the potential mechanisms by which eIF4E phosphorylation regulates neuronal functions. In the brain, eIF4E phosphorylation controls the translation of mRNAs involved in inflammatory responses such as IκBα, a repressor of the transcription factor NF-κB that regulates the expression of the cytokine tumor necrosis factor (TNFα) (Aguilar-Valles *et al.*, 2018). Genome-wide translational profiling of the brain from eIF4E^{S209A} mice revealed that eIF4E phosphorylation controls translation of mRNAs involved in inflammation (IL-2 and TNFα), organization of the extracellular matrix (*Prg2, Mmp9, Adamts16, Acan*), and the serotonin pathway (*Slc6a4*) (Amorim *et al.*, 2018b).

In the DRG, phosphorylation of eIF4E stimulates translation of brain derived neurotropic factor (*Bdnf*) mRNA. eIF4E^{S209A} mice show reduced protein levels of BDNF under baseline conditions and fail to translate *Bdnf* mRNA to protein in response to pro-inflammatory cytokines despite an increase in *Bdnf* mRNA levels (Moy *et al.*, 2018a). BDNF is a key molecule mediating pain plasticity and identification of MNK/eIF4E signaling as a central regulator of *Bdnf* translation has important therapeutic implications. Cell type-specific translational profiling of nociceptors (using translating ribosome affinity purification [TRAP] approach) in a mouse model of chemotherapy-induced neuropathic pain revealed that MNK-eIF4E signaling controls translation of *RagA* mRNA, a key regulator of mTORC1 (Megat *et al.*, 2018b). This finding suggests crosstalk between ERK/MNK/eIF4E and mTORC1 signaling pathways in promoting pain hypersensitivity in chemotherapy-induced neuropathies.

In addition to phosphorylation, eIF4E in primary sensory neurons is also regulated via mTORC1/4E-BP. IL-6 and NGF activate mTORC1, promote 4E-BP1 phosphorylation, and increase eIF4F complex formation and nascent protein synthesis in cultured sensory neurons (Melemedjian *et al.*, 2010). Intraplantar administration of IL-6 or NGF induced mechanical allodynia, which is blocked by subcutaneous administration of the mTORC1 inhibitor rapamycin,

as well as by 4EGI-1, an inhibitor of eIF4F complex formation that disrupts eIF4E and eIF4G interaction. Intraplantar 4EGI-1 also blocked the establishment of the sensitization state in a hyperalgesic priming model in response to IL-6 and NGF injection (Asiedu *et al.*, 2011).

These findings support a model that local activation of mTORC1 stimulates eIF4F complex formation, promoting pain hypersensitivity via axonal mRNA translation. 4E-BP1 is a major isoform involved in regulation of nociception, whereas in the brain 4E-BP2 is the dominant isoform. 4E-BP1 is highly expressed in nociceptors and mice lacking 4E-BP1, but not 4E-BP2, exhibit enhanced mechanical hypersensitivity. Notably, *eif4ebp1* knockout mice show no alterations in thermal sensitivity, suggesting a mechanical-specific effect of eIF4E activation via 4E-BP-dependent mechanisms (Khoutorsky *et al.*, 2015).

A second major downstream effector of mTORC1, p70S6 ribosomal kinase (S6K1 and S6K2) may not play as significant a role in regulation of nociceptive sensitization. Mice lacking S6K1/2 do exhibit increased mechanical pain sensitivity, but normal thermal thresholds, and an inhibitor of S6K1/2 recapitulates this phenotype (Melemedjian *et al.*, 2013). This finding seems paradoxical; however, further analysis revealed that loss of S6K1/2 function engages a feedback loop that stimulates enhanced ERK phosphorylation, driving mechanical sensitization (Melemedjian *et al.*, 2013). Therefore, it is tempting to speculate that most of the pain inhibitory effects of mTORC1 inhibition are mediated via the suppression of 4E-BP1/eIF4E-dependent protein synthesis. The role of other translation-independent outputs of mTORC1, such as regulation of autophagy, lipogenesis, and mitochondrial function, remain unknown.

1.2.3.4. eIF4E in regulation of spinal plasticity

The spinal cord integrates peripheral somatosensory inputs to generate, after processing, an output that is conveyed to the brain where the perception of pain ultimately arises. Peripheral injury, disease, and certain drugs can cause an increase in the gain of spinal nociceptive circuits, resulting in disproportional amplification of somatosensory inputs, and therefore increased pain. These maladaptive plastic changes in the spinal cord, frequently referred to as central sensitization, significantly contribute to the development of pathological pain states. Central sensitization leads to a lowered threshold for the induction of pain (allodynia), an increase in the responsiveness to

noxious stimuli (hyperalgesia), and an enlargement of the receptive field, resulting in pain sensation from non-injured areas (secondary hyperalgesia).

Long-lasting spinal plasticity critically relies on new protein synthesis to allow alterations in the cellular proteome, and consequently, sensitization of the pro-nociceptive circuits. Numerous studies have demonstrated the activation of ERK and mTORC1 signaling in the spinal cord following peripheral tissue injury, cancer, and opioid treatment (Geranton *et al.*, 2009; Ji *et al.*, 2009; Norsted Gregory *et al.*, 2010; Xu *et al.*, 2011; Shih *et al.*, 2012; Jiang *et al.*, 2013; Liang *et al.*, 2013; Zhang *et al.*, 2013; Xu *et al.*, 2014). Intrathecal delivery of pharmacological inhibitors targeting these pathways efficiently alleviates pathological pain without affecting the baseline mechanical and thermal sensitivity (Ji *et al.*, 2009; Melemedjian & Khoutorsky, 2015; Martin *et al.*, 2017). There is evidence that the beneficial effect of mTORC1 inhibition on pain in the spinal cord is largely mediated via mTORC1/4E-BP1-dependent regulation of eIF4E activity. Pain hypersensitivity, produced by intrathecal injection of epiregulin (EREG), an endogenous agonist of the epidermal growth factor receptor (EGFR), upstream of mTORC1, is blocked by intrathecal injection of 4EGI-1 (Martin *et al.*, 2017). Moreover, specific deletion of 4E-BP1 in the dorsal horn of the spinal cord causes mechanical hypersensitivity (Khoutorsky *et al.*, 2015). Mice lacking 4E-BP1 show increased excitatory and inhibitory synaptic transmission in lamina II neurons as well as enhanced potentiation of spinal excitatory field potentials following sciatic nerve stimulation. Taken together, these results indicate that enhanced eIF4F complex formation in the spinal cord promotes spinal plasticity and contributes to the development of central sensitization.

1.2.3.5. Therapeutic approaches to target eIF4E-dependent mechanisms to alleviate pain

Several lines of evidence suggest that targeting eIF4E is a potentially promising therapeutic strategy to inhibit aberrant pain plasticity. First, due to low expression levels, eIF4E's activity is a rate-limiting factor for translation initiation and a central node of regulation. eIF4E integrates signals from two major signaling pathways, ERK and mTORC1, both of which have important functions in the development of pain. Second, eIF4E does not strongly affect general translation, but mainly regulates the translation of a subset of mRNAs involved in cell growth, proliferation, immune responses, and neuronal plasticity. Mice with partial reduction of eIF4E protein levels (e.g. expressing inducible eIF4E short hairpin RNAs or eIF4E heterozygous mice) show no

developmental abnormalities or changes in survival rate or body weight (Lin *et al.*, 2012; Truitt *et al.*, 2015). Third, whereas acute inhibition of mTORC1 is effective in alleviating pain, long-term mTORC1 inhibition leads to the hyperactivation of ERK via a mTORC1-S6K1-IRS1 negative feedback loop (Veilleux *et al.*, 2010; Melemedjian *et al.*, 2013). Since ERK is a well-known sensitizer of neurons involved in pain transmission, both in the periphery and the spinal cord, chronic mTORC1 inhibition leads to mechanical hypersensitivity and pain. Thus, long-term treatment with compounds targeting mTORC1 is unlikely to be clinically applicable. Conversely, chronic inhibition of eIF4E does not activate these compensatory mechanisms. Mice lacking eIF4E phosphorylation do not exhibit alterations in pain sensation at baseline (Furic et al., 2010, Gkogkas et al., 2014; Moy et al., 2017), but show reduced nociceptive plasticity in response to pro-inflammatory and nerve injury stimuli (Moy et al., 2017). Finally, compelling preclinical studies have demonstrated beneficial effects of pharmacologically targeting eIF4E in alleviating persistent pain using 4EGI-1, an inhibitor of eIF4 complex formation or cercosporamide, an inhibitor of MNK. Efforts to develop and test new translation inhibitors are fuelled by their potential use for treatment of cancer (Stumpf & Ruggero, 2011), malaria (Baragana *et al.*, 2015), and bacterial infection (Bhat *et al.*, 2015).

1.2.4. Importance of translation regulation - dichotomy between mRNA and protein levels

Translational control, which regulates the synthesis of proteins from mature mRNA transcripts, is an important level of gene expression regulation, particularly for persistent pain. Translational control enables neurons to rapidly modify their cellular or synaptic proteome in response to noxious stimuli. Conventionally, mRNA abundance was used as a proxy for protein abundance based on the assumption that changes in mRNA abundance is proportional to changes in protein synthesis. As researchers leveraged the advancements in sequencing technologies and generated large amounts of high quality data, they became aware of the dichotomy that exists between mRNA and protein levels. Several systematic studies have investigated the correlation between mRNA and protein levels and have established that the correlation is only partial, pointing out various factors which affect the expression level of protein beyond mRNA abundance (Liu *et al.*, 2016). The foremost factor is ‘translation rate’ – it can significantly change the synthesis of protein from mRNA molecules. Translation rate is in turn influenced by the mRNA sequence in the upstream open reading frames (uORFs) (Wethmar *et al.*, 2010) and presence of internal ribosome entry sites

(IRES). Moreover, the translation rate can be regulated via binding of proteins to regulatory elements on the transcripts, binding of micro-RNAs, or availability of ribosomes. A study using deep sequencing identified more than 200 microRNAs in DRG and in the proximal stump of rats at several time points (0, 1, 4, 7, and 14 days) after spinal nerve transection (Yu *et al.*, 2011). Further, there is a delay between availability of the mature mRNA transcript in the cytoplasm and synthesis of proteins, and changes in mRNA transcript levels can affect protein levels only after a temporal delay. Post-translational regulation also modulates protein abundance. In conclusion, the above-mentioned factors explain the poor correlation of mRNA and protein abundance, underscoring the importance of translation regulation. To overcome the dependency on mRNA levels, several researchers aimed to measure protein levels directly.

To quantify the protein abundance directly, techniques like western-blot and immunohistochemistry have been widely used. But these approaches only enable relative quantification of a few selected proteins at once. Another approach was assessing protein abundance by using whole-proteome mass spectrometry. Advancement in proteomic technologies, particularly feasible mass-spectrometry based proteomics, has enabled profiling of protein abundance on a global scale. Several researchers assessed the differential proteome post-injury involving damage to nervous system tissue, for example, in brain tissue and SNC injury (Moghieb *et al.*, 2013), in spinal cord post-transection injury (Ding *et al.*, 2006) or spinal nerve ligation (Sui *et al.*, 2014). Other researchers used cerebrospinal fluid to look for biomarkers of neuropathic pain (Lind *et al.*, 2016), fibromyalgia and inflammatory pain (Khoonsari *et al.*, 2018). Other studies also extended the analysis to urine (Methadone *et al.*, 2010; Manchikanti *et al.*, 2011) and trapezius muscle (Mathiassen *et al.*, 1995) samples.

While these advancements in proteomic technologies, such as data-independent acquisition-mass spectrometry (DIA-MS) have enabled assessment of global protein abundance (Gomez-Varela & Schmidt, 2016), the depth and coverage of proteomic data lags behind that of genomic studies. Thus, studying translational regulation which controls the synthesis of proteins from mRNAs can better predict protein abundance. Further, translational regulation is the last step of gene expression regulation in which next generation sequencing technologies can be applied, benefiting from its features like high-throughput ability, increased depth, and genome wide coverage.

1.2.4.1. Genome wide translational profiling studies in chronic pain

While techniques to assess transcriptional regulation are well established, techniques for studying translational regulation have lacked high-throughput power. Thus, investigation of genome wide translational control has lagged behind.

However, recent advances in sequencing technologies (particularly the advent of next-generation high throughput sequencing technologies) has benefited translational profiling as well. Starting with 'polysome profiling', it was possible to identify different mRNAs based on the number of bound ribosomes (Zong *et al.*, 1999; Arava *et al.*, 2003). This technique involves fractionating transcripts through a sucrose gradient using velocity sedimentation and then identifying the transcripts using microarray (in earlier years) or recently RNA-seq (Spangenberg *et al.*, 2013; Liang *et al.*, 2018). It gives an estimated mRNA transaltion rates based on the number of ribosomes bound to each mRNA. Differential regulation at the level of translation is reflected as a shift of the absorbance profile of the collected sucrose fraction. While this technique has been very useful in measuring genome wide translation, it suffers various limitations. Imprecise polysome fractionation limits quantitative resolution and accuracy. A fundamental limitation to this technique is the inability to distinguish ribosomes bound to the upstream open reading frames (uORFs). This can lead to overestimation of mRNA translation as uORF bound ribosomes are not yet engaged in translation (Meijer & Thomas, 2002). Recently, there have been advancements in translational profiling techniques which overcome some of the limitations of polysome profiling. The following section describes these advancements and mentions their use in pain research.

1.2.4.2. Neuron specific translational profiling

Since DRG and spinal cord, the two most widely studied nervous system tissues are composed of many other cell types apart from neurons, using whole tissues for gene expression profiling studies makes it difficult to identify the cell types associated with the differentially expressed genes.

Heiman *et al.* identified the diverse and intermixed architecture of nervous system tissues as a barrier in assessing cell type specific gene expression. In order to overcome this, the group developed a method capable of purifying "cell-type-specific" mRNAs undergoing translation. This development enabled researchers to study the complete "translated mRNA complement of any genetically defined cell population". The method indirectly tags mRNA in specific cells by incorporating an affinity tag (e.g. enhanced green fluorescent protein [EGFP]) on the large

ribosomal subunit protein L10a. This has a two-fold advantage – firstly, the localization of these ribosomes can be visualized in the cells/tissue; and secondly, the tagged ribosomes can be affinity purified using EGFP antibodies coupled to magnetic beads. This also gives the name to this procedure – "translating ribosome affinity purification (TRAP)". By stabilizing the ribosomes bound to mRNA during translation, it enables collection of mRNAs undergoing translation, in the affinity purification step. This stabilization process, also known as polysome stabilization can be achieved by treatment of the cells/tissue with cycloheximide and magnesium and controlling RNase contamination (Heiman *et al.*, 2014). The purified product from this procedure can be used as input in various downstream procedures including qPCR and RNA-Seq. Tagging of ribosomes involves cell-type-specific expression of an EGFP-L10a ribosomal transgene in a specific cell population. EGFP tagging of ribosomes was first achieved by cell-type-specific genetic targeting by Bacterial Artificial Chromosome (BAC) vectors for which various BAC-TRAP transgenic mouse lines are available (Heiman *et al.*, 2008). More recently, *Cre* driver lines in conjugation with viral transduction of TRAP construct are being used (Heiman *et al.*, 2014). Similarly, using a different tag for ribosomes has also been performed. Kang *et al.* demonstrated the use of the 'RiboTag translational *Cre*/Lox- based profiling system" for isolation of transcripts specifically from microglial cells (Kang *et al.*, 2018). This procedure employed the tagging the ribosomal protein L22 (RPL22), with a hemagluttanin (HA) epitope tag and affinity purification using anti-HA antibodies coated onto magnetic beads.

Several researchers exploited the TRAP technology to study neuron specific translatome. But most of these studies were performed in brain and had applications in memory and aging (Gray *et al.*, 2018; Kang *et al.*, 2018). Until now there has just been one study specific to nociceptors, which has aimed to identify transcripts undergoing translation (Megat *et al.*, 2018a). The study was designed to characterize changes in mRNA translation, specifically in *Scn10a*-positive nociceptors under chemotherapy-induced peripheral neuropathy (CIPN) condition, generated by intraperitoneal administration of Paclitaxel, a widely used chemotherapy drug. They employed the TRAP model in conjugation with $Nav^{1.8Cre}$ (expressing *Cre* recombinase in Nav1.8 positive neurons) and $ROSA^{26fs\text{-}TRAP}$ (contain a transgene for eGFP-L10a fusion protein, its expression is stopped in absence of *Cre*) mice. These mice were crossed to express eGFP-L10a fusion protein such that the ribosomes are eGFP tagged, specifically in Nav1.8-positive neurons. Using immunoprecipitation, they were successfully able to isolate mRNA from DRG nociceptive

neurons. These isolated mRNAs were validated by qPCR to check for nociceptive markers and were then fed into high throughput sequencing (TRAP-seq). They combined their sequencing results with existing data from single cell RNA sequencing studies of DRG to calculate translation efficiencies (TEs) of translated mRNA specific to Nav1.8 positive neurons. Comparing the TEs for different families of genes, they found that genes from ion channels and GPCRs families had a higher TE as compared to the kinases in Nav1.8 positive nociceptors. Further, they also noted an enrichment of genes involved in cap-dependent translation regulation. To confirm this, they assessed the steady state protein levels of mTOR in CIPN mice. They also found increased expression of p-4E-BP1 and p-eIF4E protein level. Inhibition of eIF4E phosphorylation by administration of MNK inhibitor or genetic disruption (using $eIF4E^{S209A}$ mice) led to decrease in mechanical hypersensitivity and spontaneous pain in CIPN mice. These results were replicated by specifically blocking MNK1/2-mediated eIF4E phosphorylation. They also noticed an increase in raga mRNA in Nav1.8 neurons and increase in (Ras-related GTP-binding protein A) RagA protein levels in DRG on administration of paclitaxel. Interestingly, the expression of RagA was coupled to that of p-eIF4E as confirmed by no effect on RagA levels in DRG of $eIF4E^{S209A}$ mice treated with paclitaxel. Together, their findings supported that translation of RagA mRNA is controlled by MNK1- mediated phosphorylation of eIF4E. Increased p-eIF4E and RagA help in the establishment of neuropathic pain through mTORC1 activation (Megat *et al.*, 2018a).

Nearly all the studies using TRAP based approach to enrich ribosome associated mRNAs use RNA-seq as the following downstream process to identify and quantify the abundance of the mRNAs. While this approach has significant advantages over whole-tissue profiling or other techniques to isolate specific cells (LCM and FACS), it is not able to reveal the precise positions of ribosomes. The following section describes the Ribosome profiling technique, which allows high resolution translational profiling that reveals ribosome position with nucleotide specific resolution. More or with incorporation of mRNA-seq, it allows reliable computation of translation efficiency of all the mRNAs in the cell.

1.2.4.3. Genome wide assessment of translational regulation with Ribosome profiling

The development of 'ribosome profiling' protocol enabled accurate assessment of mRNA abundance as well as mRNA translation rates from the same sample (Ingolia *et al.*, 2009). The developers of this technique explained it to be principally based on the fact that a ribosome bound

to a mRNA template protects a ~30 nucleotide long stretch of the mRNA (referred to as a 'footprint') from nuclease digestion. In addition to this, they integrate the high-throughput ability of deep-sequencing technology to parallelly sequence tens of millions of short DNA sequences. They bridged these two principles by generating 'footprints' from mRNAs being translated and converting them into deep-sequencing amenable cDNA libraries (Ingolia *et al.*, 2012) (Figure 1.5). This sophisticated technique overcomes the limitations of polysome profiling by giving information about the ribosome position with "single-nucleotide resolution" (Ingolia *et al.*, 2009). Further analysis of ribosome profiling data enables the quantification of rate of translation by assessing the translation efficiency over a wide range of mRNAs. Translation efficiency, being a measure of protein synthesis qualifies as a better predictor of protein abundance than measurement of mRNA abundance alone.

The establishment of ribosome profiling protocol has benefited research on translational regulation in many subject areas, particularly cancer. However, only two studies have been published recently which have used this technology in nervous system tissues, both aimed at studying mechanisms of memory formation (Cho *et al.*, 2015; Yordanova *et al.*, 2018) and depression (Amorim *et al.*, 2018a).

Thus, while numerous studies support the crucial role of translation in establishment of abnormal nociceptive plasticity and pain phenotype, there is no previous study that conducted global translational profiling in pain conditions.

One of the major limitations of Ribosome profiling compared to other approaches based on mRNA-sequencing is the requirement of relatively large quantity of sample to obtain enough quantity of ribosome footprints. Since, only a small fraction of mRNA undergoes translation at a given time, the quantity of footprints recoverable from mRNA is very small. Therefore, it is challenging to perform Ribosome profiling on single cell.

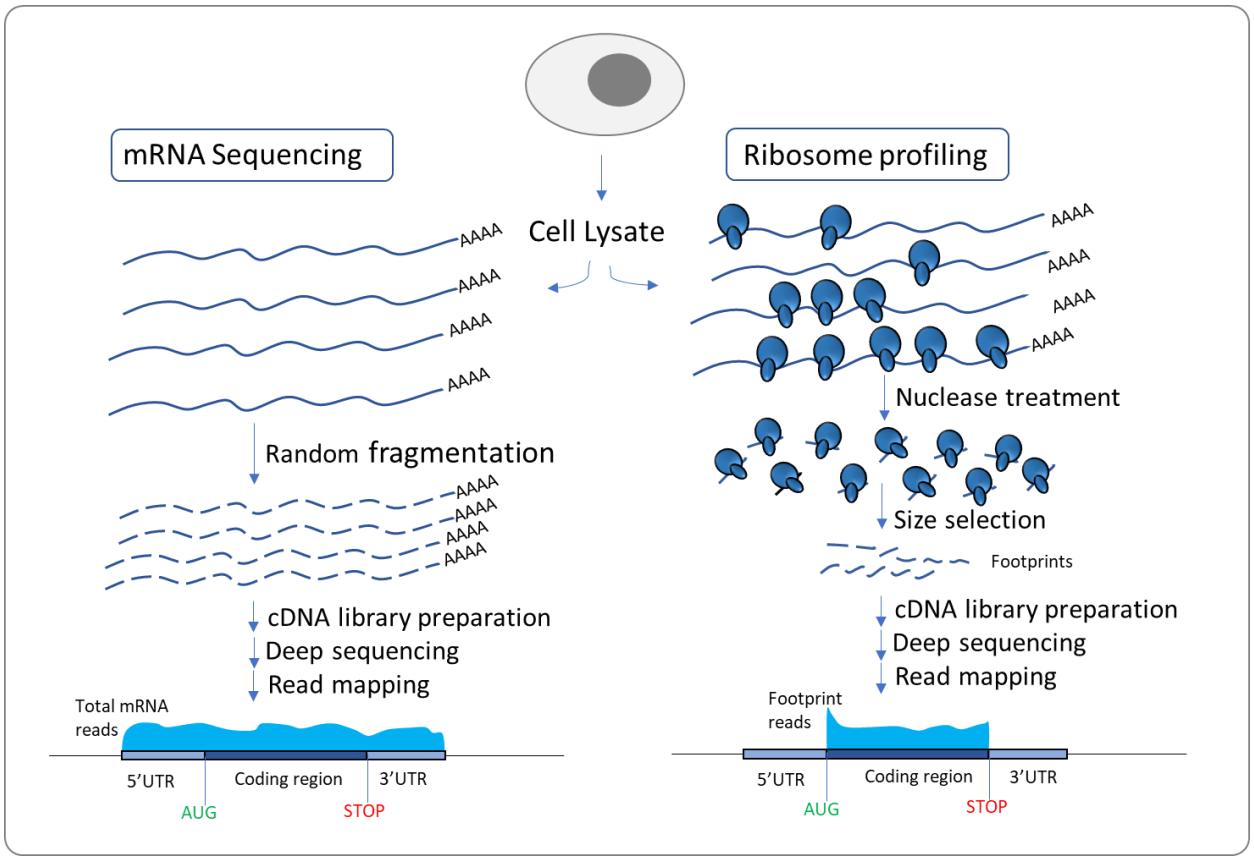

Figure 1.5. Schematic view of Ribosome Profiling vs. mRNA profiling (modified from (Brar & Weissman, 2015))

1.3. Summary

Chronic pain ranks as one of the leading causes of long-term disability and suffering in humans. Affecting nearly 18% of the adult population, it imposes a significant economic burden on both the patient and the society (Toth *et al.*, 2009). A US based study has reported that "the annual cost (health care and productivity) of pain ranks higher than that of heart disease, cancer, or diabetes, and is about 30 percent higher than the combined cost of cancer and diabetes (Gaskin & Richard, 2012). Common chronic pain conditions include headache, lower back pain, cancer pain, arthritis pain, and neuropathic pain (pain resulting from damage to the peripheral nerves or to the central nervous system itself). In addition to its impact on the somatosensory system, chronic pain also negatively affects the emotional and mental health of patients, leading to depression, anxiety, sleep disorders, low self-esteem, as well as impairments in attention and memory (Duenas *et al.*, 2016).

Available treatments have limited efficacy and only 30-40% of chronic pain patients report satisfactory pain relief. For many years, antidepressants, anticonvulsants, and opioids have been widely employed for pain management. While the use of the first two reduce pain to some degree, it does not apply for all patients (Foley, 2003). On the other hand, the use of opioids is not recommended. Apart from posing the risk of side effects, it can cause other problems including the development of tolerance and addiction. The limited treatments available are a consequence of insufficient understanding of the molecular mechanisms of pain development and maintenance.

Chronic pain can be caused by many different insults, such as nerve injury, inflammation, viral infection, autoimmune diseases, cancer, metabolic disorders, and in some cases, appears without any identifiable aetiology, such as fibromyalgia, irritable bowel syndrome, and interstitial cystitis. Sensitization of the peripheral and central nociceptive circuits is the main underlying cause of pain hypersensitivity in chronic pain conditions. The pathophysiology of sensitization is not well understood and is the subject of intensive research. Alterations in nociceptors, spinal neurons, and non-neuronal cells contribute to this sensitization and more importantly, rely on new gene expression to support biochemical and structural reorganization of the pain pathway.

1.3.1. Rationale and Objectives

Gene expression can be modulated at different levels, including transcription, RNA splicing, mRNA translation, and post-translational modification of proteins. Most of the studies to date have focused on transcriptional control of gene expression in chronic pain. Despite these results, these studies have not provided a significant contribution to our understanding of the pathophysiology of chronic pain nor led to the development of new treatments. There is strong evidence suggesting that the cellular abundance of proteins is predominantly controlled at the level of protein synthesis (also referred to as "mRNA translation") (Schwanhäusser *et al.*, 2011). Translational control allows changes in protein levels by regulating the efficiency by which mRNA is translated into proteins. In neurons, it supports rapid modifications of the axonal/dendritic proteome by local translation of pre-existing mRNAs. Translational control of gene expression plays key roles in different forms of nervous system plasticity such as long-term potentiation (LTP) and long-term depression (LTD). Previous studies have shown that translational control mechanisms are dysregulated in sensory and dorsal horn neurons in animal models of chronic pain, and inhibitors of mRNA translation (rapamycin, CCI-779, 4EGI-1) effectively ameliorate this hypersensitivity.

In this project, we aim to measure the translation efficiency of mRNAs in the mammalian nervous system under chronic pain conditions. By employing ribosome profiling for genome-wide quantitative analysis of mRNA translation *in vivo*, we have analyzed DRG and spinal cord dorsal horn lysates from a mouse model of neuropathic pain, 30 days post-nerve injury. In parallel with translational profiling, we performed RNAseq to measure mRNA levels.

Chapter 2: Translational profiling of dorsal root ganglia and spinal cord in a mouse model of neuropathic pain

2.1. Abstract

Acute pain serves as a protective mechanism, guiding the organism away from actual or potential tissue injury. In contrast, chronic pain is a debilitating condition without any obvious physiological function. The transition to, and the maintenance of chronic pain require new gene expression to support biochemical and structural changes within the pain pathway. The regulation of gene expression at the level of mRNA translation has emerged as an important step in the control of protein expression in the cell. Recent studies show that signaling pathways upstream of mRNA translation, such as mTORC1 and ERK, are upregulated in chronic pain conditions, and their inhibition effectively alleviates pain in several animal models. Despite this progress, mRNAs whose translation is altered in chronic pain conditions remain largely unknown. Here, we performed genome-wide translational profiling of dorsal root ganglion (DRG) and spinal cord dorsal horn tissues in a mouse model of neuropathic pain, spared nerve injury (SNI), using the ribosome profiling technique. We identified distinct subsets of mRNAs that are differentially translated in response to nerve injury in both tissues. We discovered key converging upstream regulators and pathways linked to mRNA translational control and neuropathic pain. Our data are crucial for the understanding of mechanisms by which mRNA translation promotes persistent hypersensitivity after nerve injury.

2.2. Highlights

1) Translational landscape in DRG and spinal cord in SNI assay of neuropathic pain was established.

2) ERK is a central hub of both transcriptionally and translationally controlled genes.

3) Changes in translation efficiency and mRNA levels occur in the opposite direction for multiple mRNAs.

2.3. Introduction

Chronic pain debilitates over twenty percent of the population worldwide, and is the leading cause of long-term disability in humans (Souza *et al.*, 2017). The most common chronic pain conditions include headache, low back pain, cancer pain, arthritis pain, and neuropathic pain, which can result from damage to peripheral nerves or to the central nervous system itself. In addition to dysfunction of the somatosensory system, chronic pain has multi-dimensional effects on the emotional and mental health of patients that can lead to depression, anxiety, sleep disorders, low self-esteem, and impairments in attention and memory (Duenas *et al.*, 2016). Pain management depends largely on antidepressants, anticonvulsants, and opioids; however, pain relief is incomplete under most circumstances and is achieved only in a fraction of patients (Foley, 2003; Kalso *et al.*, 2004; Højsted & Sjøgren, 2007; Moulin *et al.*, 2007; Ballantyne & Shin, 2008).

The inadequate management of chronic pain is a consequence of our incomplete understanding of the mechanisms underlying the induction and maintenance of pain states, leading to treatments that only target symptomatology without addressing the etiology of the disease. Sensitization of nociceptive circuits, both in the central and peripheral nervous systems, leads to mechanical hypersensitivity (allodynia), which is a hallmark of many chronic pain conditions. This sensitization is supported by the expression of new genes, which are required for the biochemical and structural reorganization of the pain pathway. With advancements in microarray and sequencing technologies, transcriptional changes associated with chronic pain have been extensively studied, providing important insights into the transcriptional landscape and identification of a subset of genes with differential expression in various chronic pain conditions (LaCroix-Fralish *et al.*, 2011; Hu *et al.*, 2016; Ray *et al.*, 2017).

Cellular abundance of proteins is highly controlled at the level of mRNA translation (Schwanhausser *et al.*, 2011). Translational control is a powerful modulator of protein levels by regulating the efficiency by which mRNA is converted to proteins.

Translation control involves a variety of mechanisms, including regulation of the vast translational machinery and modulation of the signaling pathways upstream of translation. The extracellular signal-regulated kinase (ERK) pathway and mechanistic target of rapamycin complex 1 (mTORC1) kinase and its downstream effectors have been extensively studied to understand the

contribution of translation in the development of hypersensitivity (Khoutorsky & Price, 2017). Suppressing translation by inhibition of mTORC1 reduces mechanical hypersensitivity associated with inflammation (Price *et al.*, 2007; Gregory *et al.*, 2010; Ferrari *et al.*, 2013) and neuropathic pain (Geranton *et al.*, 2009; Zhang *et al.*, 2013). A recent study described an important role for eukaryotic translation initiation factor 2 (eIF2) in inflammation-induced pain, and identified that phosphorylation of the α subunit of eIF2 (eIF2α) is a key step in controlling noxious heat sensitivity (Khoutorsky *et al.*, 2016). Other studies have established a key role for local translation from pre-existing mRNAs in the modification of axonal/dendritic proteomes to promote the excitability of sensory neurons and induce pain hypersensitivity (Melemedjian *et al.*, 2010; Khoutorsky & Price, 2017; Moy *et al.*, 2017b). Altogether, these studies support an emerging role for translational regulation in the establishment and maintenance of chronic pain.

Neuropathic pain accounts for ~20% of chronic pain cases (Lisi *et al.*, 2015), and arises from damage to the nervous system. This damage can result either from a direct injury to peripheral nerves, spinal cord, or the brain, or be caused by a disorder affecting the somatosensory system such as metabolic stress, autoimmunity, degenerative or chronic inflammation, or from idiopathic origin (Guha & Shamji, 2016). Various rodent assays, mostly involving surgical injury, have been developed to study neuropathic pain (Mogil, 2009). Spared nerve injury (SNI) is a model of sympathetic-independent neuropathic pain with long-term chronicity (Decosterd & Woolf, 2000). SNI typically involves a lesion of the tibial and common peroneal branches of the sciatic nerve, while leaving the sural branch intact (Figure 2.1A). This procedure causes severe and persistent (at least 6 months) neuropathic pain in the animal, manifested in the sural territory of the ipsilateral paw as mechanical and cold hypersensitivity (Decosterd & Woolf, 2000).

In this study, we have adopted a genome-wide approach to identify mRNAs that are either significantly up- or down-regulated at the level of translation after SNI. For this purpose, we implemented a high throughput RNA sequencing-based methodology, called ribosome profiling, in parallel with measurements of mRNA levels. We analyzed lysates from DRGs and spinal cord (SC) dorsal horn tissues from mice subjected to SNI and mapped the translational and transcriptional landscapes. In addition, we carried out meta-gene analysis by Ingenuity Pathway Analysis (IPA) and identified commonly affected pathways.

2.4. Results

To understand the global pattern of translational control, and identify which mRNAs are differentially regulated following nerve injury, we performed genome-wide translational profiling of DRG and dorsal horn of the spinal cord in the SNI assay of neuropathic pain. For the analysis, we collected L3 to L5 DRG and the corresponding lumbar segment of the spinal cord (Rigaud *et al.*, 2008) 30 days post-SNI. The dorsal half of the spinal cord was dissected and used for the analysis as sensory processing is restricted to this area (illustrated in a schematic diagram in Figure 2.1A). We confirmed that mechanical thresholds, as measured by the von Frey test, were significantly reduced at 30 days after the nerve injury (Figure 2.1B). Thus, we reasoned that the 30 day time point was appropriate for tissue collection in order to study the chronic phase of neuropathic pain.

To quantitatively measure *in-vivo* genome-wide translational efficiency of mRNAs in DRG and spinal cord, we adopted the ribosome profiling methodology (Ingolia *et al.*, 2012). Ribosome/RNA complexes were isolated from cell lysates and digested with an endoribonuclease (RNase I), which degrades all RNAs that are not protected by bound ribosomes (Figure 2.1C). This generated ~30 nucleotide long fragments of ribosome-protected mRNAs, or "footprints". These footprints were reverse-transcribed and cloned into a cDNA library for RNA sequencing (RNA-seq) (Figure 2.1D). Libraries were then sequenced to measure the number of footprints per mRNA for the entire genome. Supplementary Table 1 shows the total number of sequenced reads and number of filtered reads (reads uniquely mapped to non-ribosomal region of reference genome DNA) for each sample. In parallel, transcriptome analysis (mRNA-seq) was performed in parallel to account for changes in mRNA abundance. Thus, using the number of footprints (as a proxy for translation) for a given mRNA, normalized to its abundance (as a proxy for transcription), we can calculate translational efficiency (TE) for each mRNA, which has been previously shown to be a strong predictor of protein abundance (Ingolia *et al.*, 2009).

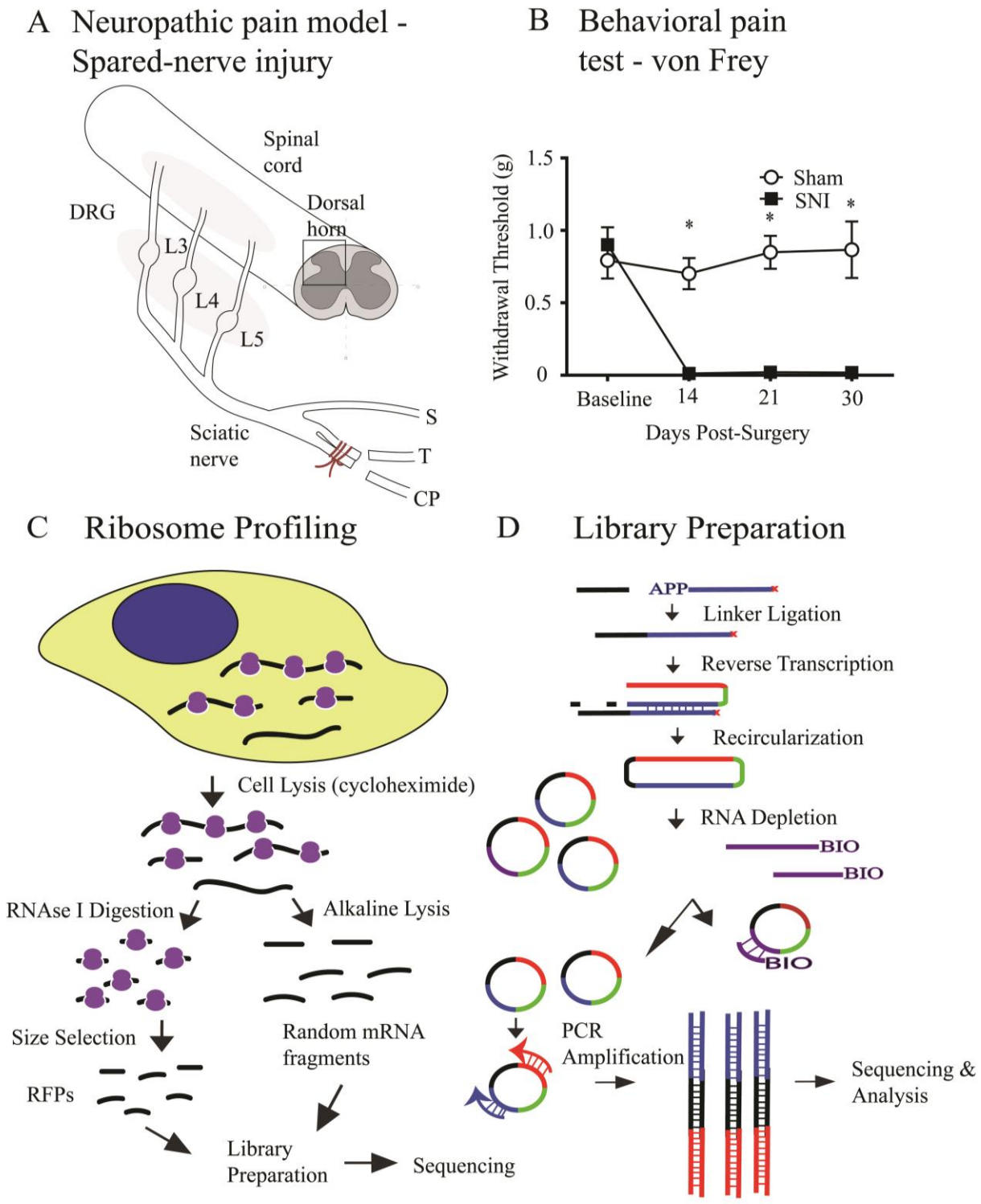

Figure 2.1. Analysis of gene-expression in the mouse model of neuropathic pain using ribosome profiling and RNA sequencing

Figure 2.1. Analysis of gene-expression in the mouse model of neuropathic pain using ribosome profiling and RNA sequencing.

A. A schematic illustration of the SNI assay of neuropathic pain. L3, L4, L5: Lumbar 3,4 and5 level DRG, respectively; S: Sural branch, T: Tibial branch and CP: Common peroneal branch. **B.** Paw-withdrawal threshold (g) measured for SNI and sham-operated animals at baseline and 14, 21 and 30 days post-surgery. Symbols represent mean ± SEM; n=8/condition. *p<0.05 compared to other condition. **C.** Experimental flowchart of ribosome profiling technique. **D.** Library generation steps of ribosome profiling.

Footprints had a narrow size distribution, with a peak corresponding to 28–32 nucleotides, whereas the length of sequencing reads from randomly lysed mRNA fragments as a result of alkaline fragmentation had a broad size distribution ranging from 28–45 nucleotides (Figure 2.2A) (Ingolia *et al.*, 2009). mRNA-Seq reads were equally distributed between the three possible frames for the start codon, whereas footprint reads displayed a bias for the canonical Frame 1 (Figure 2.2B). Likewise, because the size of the protected ribosomal footprint is ~28 nt (Figure 2.2C), extending from -12 to +15 (0 being the start codon at the P site of the ribosome), reads around the start codon, stop codon and within the coding sequence follow the periodicity of mRNA codons (3 nucleotides) (Ingolia *et al.*, 2009) (Figure 2.2C). As expected, the footprints were largely restricted to the coding sequence (CDS), while the mRNA fragment reads were evenly distributed throughout the 5′untranslated region (5′ UTR), CDS and the 3 UTR (Figure 2.2D). The three-nucleotide periodicity of the ribosome footprints (RFPs) (Figure 2.2D), as well as the significantly higher number of RFP reads within the coding region, as compared to UTRs, demonstrates the specificity of the recovered ribosome footprints.

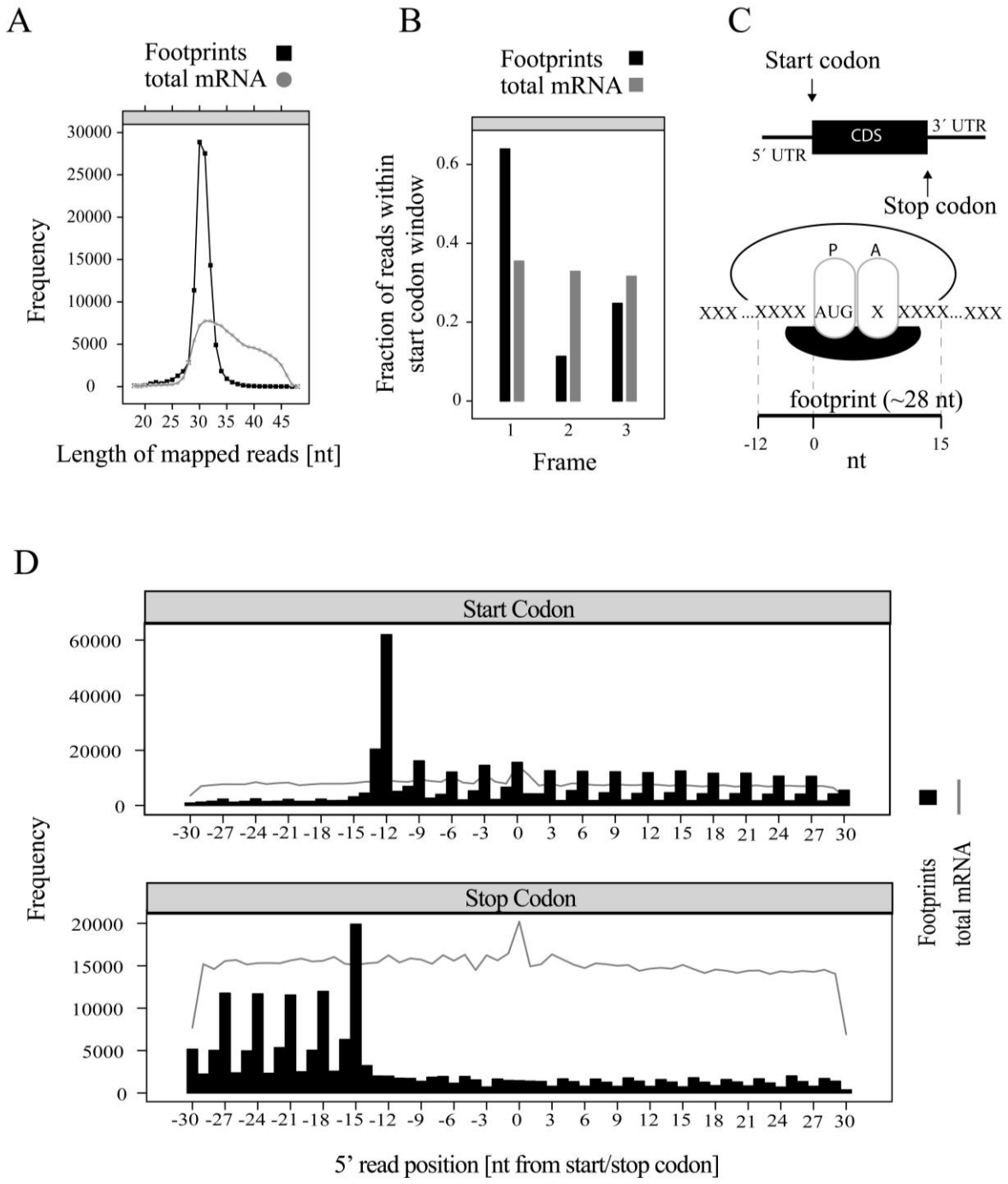

Figure 2.2. Quality control of ribosome profiling

Figure 2.2. Quality control of ribosome profiling.

A. Frequency of mapped reads from RNA-seq data corresponding to ribosomal footprints (~28–32 nt) or total RNA fragments following alkaline fragmentation (~28–45 nt). **B.** Fraction of reads within start codon window for each one of the three possible frames for footprints and total mRNA. **C.** Top: Depiction of a eukaryotic mRNA with 5′ and 3′ UTRs, CDS (coding sequence) and start and stop codons. Bottom: Depiction of the P and A sites on a translating ribosome showing the size and orientation of, and the area occupied by, a typical eukaryotic ribosomal footprint. The start codon AUG is shown; X: any three nucleotides corresponding to a codon. **D.** Frequency of footprints and mRNA reads with respect to position from the start (top) and stop (bottom) codons.

Footprints and mRNA densities were computed in units of reads per kb per million (RPKM) to normalize for gene length and total reads per sequencing run. All conditions demonstrated a strong correlation between biological replicates (Figures 2.3A and 2.4A – R^2; Pearson Correlation). Based on changes in translational efficiency, 74 mRNAs were upregulated (fold change>1.5, $p<0.05$) in the DRG of SNI mice as compared to sham animals, while translation was downregulated (0.5>fold change, $p<0.05$) for 31 mRNAs (Figure 2.3B left, for the complete list of genes see Supplementary Table 2). mRNA-seq analysis revealed that 144 mRNA were transcriptionally upregulated and 33 were downregulated in DRG after SNI (Figure 2.4B right, for the complete list of genes see Supplementary Table 2). In the spinal cord, 103 mRNAs were translationally upregulated and 27 were downregulated (Figure 2.4B left, for the complete list of genes see Supplementary Table 2), whereas 25 mRNAs were transcriptionally upregulated and 7 were downregulated after SNI (Figure 3B right, for the complete list of genes see Supplementary Table 2).

Ingenuity Pathway Analysis (IPA) of differentially regulated genes (both translationally and transcriptionally) in SNI revealed top cellular functions and subcellular localizations in the DRG (Figure 2.3C) and spinal cord (Figure 2.4C). We also used the IPA network analysis of differentially regulated genes to generate a node graph of potential regulatory networks based on the ribosome profiling data for DRG (Figure 2.5) and spinal cord (Supplementary Figure 1).

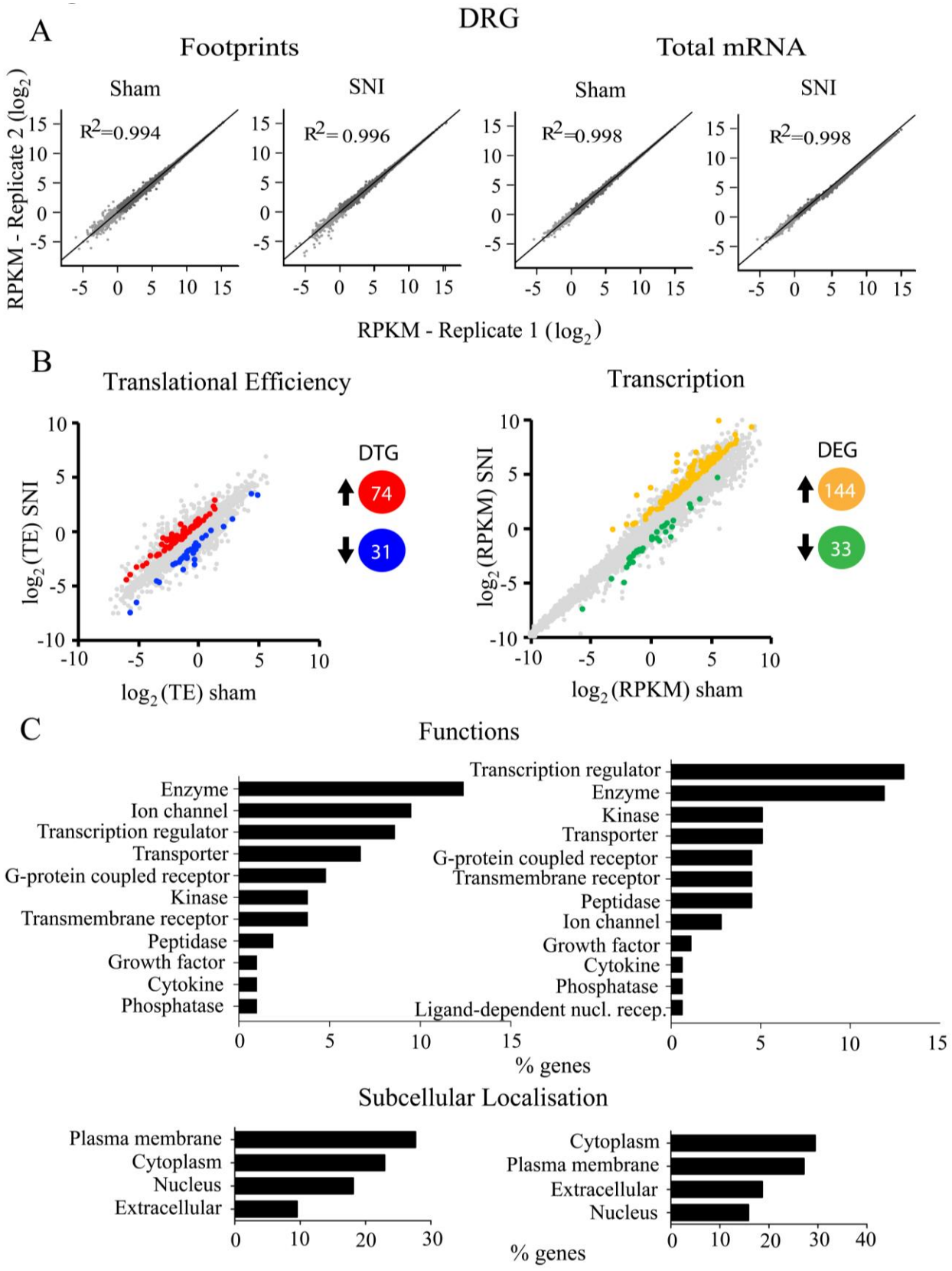

Figure 2.3. The DRG translational and transcriptional landscape after SNI.

Figure 2.3. The DRG translational and transcriptional landscape after SNI.

A. Correlation between replicates for footprint (left) and total mRNA (right) are shown for sham or SNI groups in DRG. **B.** Changes (log_2) in translational efficiency (left) and transcription (right) and differentially translated or transcribed genes (upregulated and downregulated; $p<0.05$ and 0.5>fold change>1.5) between sham- and SNI-treated mice are depicted from ribosome profiling analysis in tissue from DRG**.** The number of differentially translated genes (DTG) or differentially expressed genes (DEG) is depicted in different colours (red/blue, orange/green). Spearman's rank correlation coefficient (R^2) is shown for log_2 comparisons. **C.** Representative functional analysis characteristics using IPA of differentially regulated genes at the level of translation (left) and transcription (right) in DRG, 30 days post-SNI.

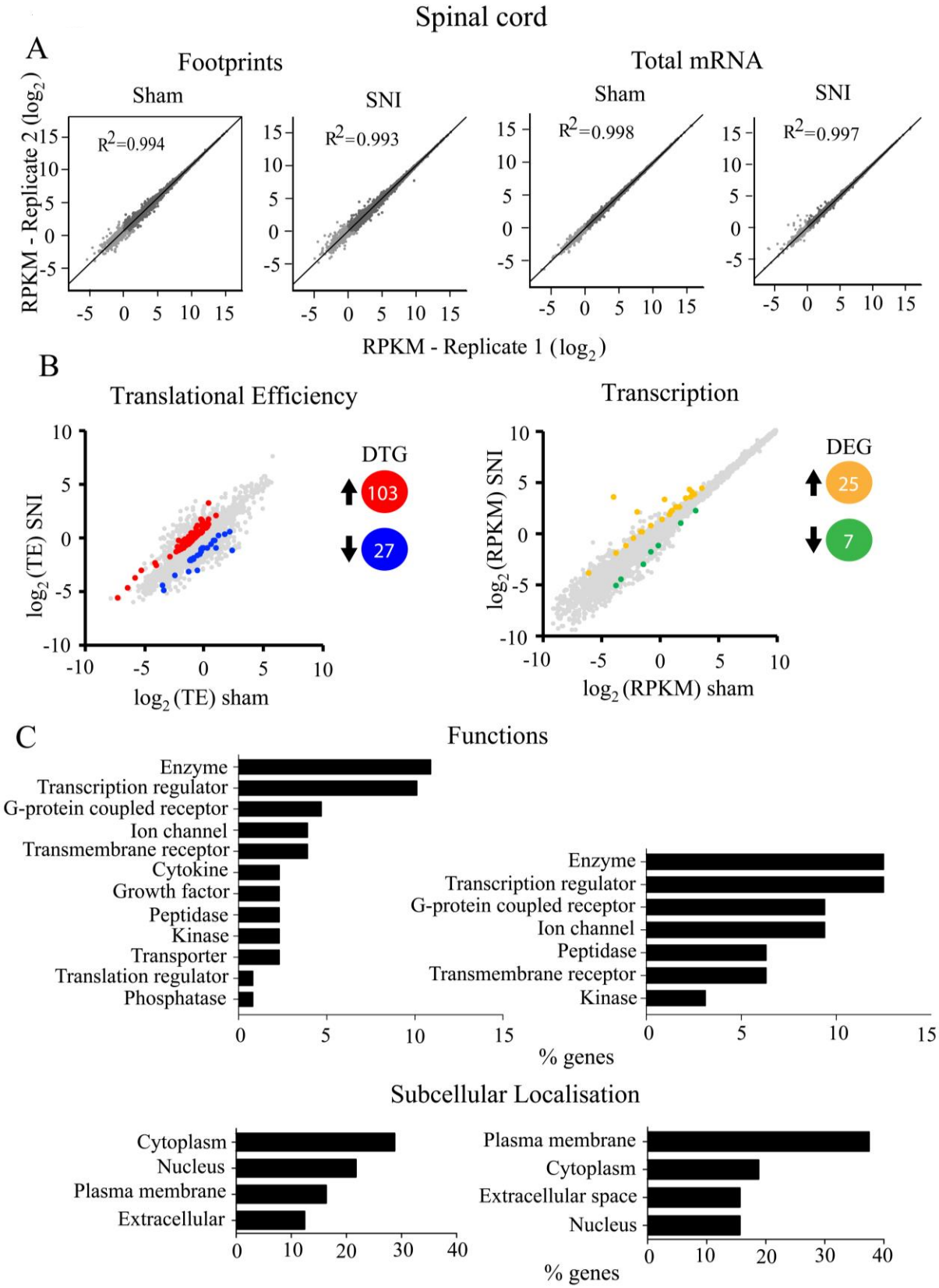

Figure 2.4. The dorsal horn of the spinal cord translational and transcriptional landscape after SNI.

Figure 2.4. The dorsal horn of the spinal cord translational and transcriptional landscape after SNI.

A. Correlation between replicates for footprint (left) and total mRNA (right) are shown for sham or SNI groups for spinal cord. Spearman's rank correlation coefficient **(**R^2**)** is shown for log_2 comparisons. **B.** Changes (log_2) in translational efficiency (left) and transcription (right) and differentially translated or transcribed genes (upregulated and downregulated; $p<0.05$ and $0.5>$fold change>1.5) between sham and SNI treated animals are depicted from ribosome profiling analysis in spinal cord**.** The number of differentially translated genes (DTG) or differentially expressed genes (DEG) is depicted with different colors (red/blue, orange/green). **C.** Representative functional analysis characteristics using IPA of differentially regulated genes at the level of translation (left) and transcription (right) are shown for sham or SNI groups in spinal cord, 30 days post-SNI.

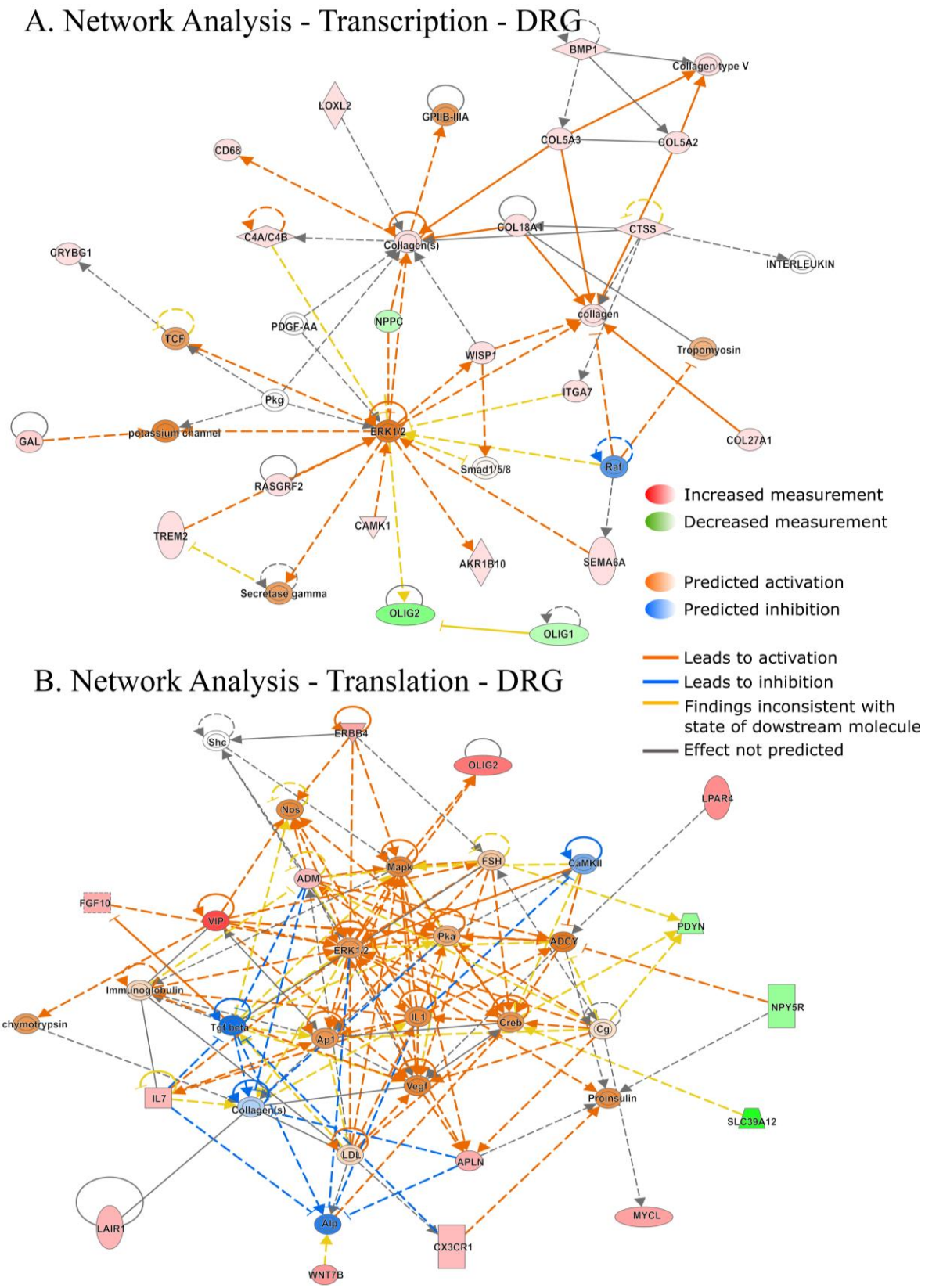

Figure 2.5. Network analysis generated by IPA of differentially transcribed and translated mRNAs in DRG 30 days post-SNI.

Figure 2.5. Network analysis generated by IPA of differentially transcribed and translated mRNAs in DRG 30 days post-SNI.

Red: increased measurement; green: decreased measurement; orange: predicted activation; blue: predicted inhibition; yellow: findings inconsistent with state of downstream molecule; grey: effect not predicted; solid line: direct interaction; dashed line: indirect interaction.

2.5. Discussion

Translational control of gene expression has emerged as a prominent mechanism in the regulation of gene expression in pathological pain states (Price & Geranton, 2009; Melemedjian & Khoutorsky, 2015; Khoutorsky & Price, 2018). Indeed, signaling upstream to the translation machinery is upregulated in several chronic pain conditions (Price *et al.*, 2007; Jimenez-Diaz *et al.*, 2008; Geranton *et al.*, 2009; Ji *et al.*, 2009; Khoutorsky & Price, 2017). Moreover, an inhibition of mRNA translation has been shown to effectively alleviate pain in several preclinical assays (Geranton *et al.*, 2009; Asante *et al.*, 2010; Obara *et al.*, 2011). Despite this progress, the repertoire of mRNAs showing altered translation in pain conditions remains largely unknown. Our study provides the first genome-wide translational profiling of DRG and spinal cord tissues in a mouse model of neuropathic pain. We identified 74 mRNAs in DRG and 103 mRNAs in the spinal cord whose translation is increased 30 days following SNI, and 31 mRNAs in DRG and 27 mRNAs in the spinal cord with decreased translation. The higher number of upregulated versus downregulated mRNAs in DRG after SNI is consistent with previous studies showing increased signaling upstream of translation following nerve injury in DRG (Obata *et al.*, 2004; Price *et al.*, 2007; Khoutorsky *et al.*, 2016; Moy *et al.*, 2017a) and increased rates of translation in sensory neurons in response to pronociceptive stimulation (Melemedjian *et al.*, 2010). The parallel analysis of changes in mRNA levels and their translational efficiency demonstrates that changes in these processes occur in the opposite direction for multiple mRNAs, suggesting translational buffering (Laurent *et al.*, 2010; McManus *et al.*, 2014) (see Supplementary Table 3). For example, in the DRG, seven genes (*Myh* 7, *Mobp*, 1500009C09Rik, *Sall1*, *Grin2b*, *Olig2* and 3110035E14Rik) are transcriptionally down regulated but translationally upregulated. In the spinal cord, four genes (*Scn4a*, *Htr3b*, *Sprr1a* and *Rtn4rl2*) are transcriptionally upregulated but translationally down regulated, whereas *Tmem54* is transcriptionally downregulated but translationally upregulated. Several genes that have been previously studied in relation to pain show opposite changes in mRNA levels and their translation efficiency (spinal cord: *Scn4a*, *Htr3b*, *Sprr1a*, *Rtn4rl2*, *Tmem54*; DRG: *Myh7*, *Mobp*, 1500009C09Rik, *Sall1*, *Grin2b*, *Olig2* and 3110035E14Rik). For example, *Scn4a* gene codes for the alpha subunit of the voltage-dependent sodium channel, and mutations in this gene have been associated with sodium channel myotonia (Orstavik *et al.*, 2015). *Htr3b* codes for the serotonin-3B receptor. *Htr3b* rs1176744 polymorphisms are proposed to influence and predict the development of chronic pain disorders like chronic myalgia (Louca

Jounger *et al.*, 2016). In a transcriptomic analysis of human DRG, *Sprr1a* (small proline-rich protein 1a) was identified as a signature gene associated with pain experienced in sickle cell disease (Paul *et al.*, 2017). Additionally, *Sprr1a* is involved in regeneration (Jing *et al.*, 2012) and its protein levels are elevated in DRG following peripheral nerve injury (Starkey *et al.*, 2009).

We predict that genes showing changes in the same direction in their mRNA levels and TE, such as *Pkd2l1*, *Unc45b*, *Tmem88b* and *Trhr*, will exhibit robust changes in the corresponding protein levels. Polycystic kidney disease protein 2-like 1 (PKD2L1) is a member of the transient receptor potential superfamily which is known to be involved in a number of sensory functions, ranging from detection of light, force, osmolality, temperature, odour, taste, and pain (Hussein *et al.*, 2015). A study identified *Tmem88b* in DRG to be transcriptionally upregulated following burn injury (Yin *et al.*, 2016). However, the physiological role of *Tmem88b* in sensory neurons and pain remains poorly defined.

To better understand the biological context of the identified genes, we analysed our datasets using the IPA platform. IPA analysis has categorized the differentially regulated genes in DRG and spinal cord, post-SNI, into functional and subcellular localization categories, identifying several overlapping functions between transcriptionally and translationally regulated genes (Figures 2.3C and 4C), including enzyme, transcription regulator, ion channel, and G protein-coupled receptors. Interestingly, the network analysis identified ERK as a central hub of both transcriptionally and translationally controlled genes, depicted by the large number of edges converging and diverging from the node corresponding to ERK (Figure 2.5). This finding is in accordance with previous studies establishing the central role of ERK pathway in the development of hypersensitivity associated with both inflammatory and neuropathic pain (Ji *et al.*, 2002; Zhuang *et al.*, 2005). Indeed, in DRG, several vital transcriptional and growth factors, cytokines, and other signalling molecules (i.e., CREB and MAPK) participate in the network by either activating or inhibiting ERK. In response to noxious stimulation, ERK phosphorylates and activates CREB, thus facilitating transcription of CREB-dependent genes, many of which are implicated in pain (Ji *et al.*, 1999). In addition, activation of ERK promotes mRNA translation via mitogen-activated protein kinase interacting kinase (MNK1/2)-dependent phosphorylation of eukaryotic initiation factor 4E (eIF4E), the cap binding protein, which is critical for ribosome recruitment to the mRNA (Waskiewicz *et al.*, 1997; Moy *et al.*, 2017a). This phosphorylation event promotes the excitability

of DRG neurons (Moy *et al.*, 2017a) and leads to the enhanced translation of brain-derived neurotrophic factor mRNA in DRG neurons (Moy *et al.*, 2018) which in turn induce translation and transcription of pain-relevant genes. Together, our network analysis provides further evidence for the involvement of ERK in both transcriptional and translational gene networks, supporting the model of feed-forward loops between transcriptional and translational control mechanisms in which the ERK pathway is serving as a central regulatory mechanism.

Changes in transcriptional and translational regulation in the spinal cord could be underrepresented in our analysis, considering that we extracted tissue from the entire dorsal half of the spinal cord, whereas most of the sensory processing is restricted to the dorsal horn area. Since we analyzed lysates prepared form spinal cord and DRG tissues, we most likely detect changes in both neuronal and non-neuronal cellular populations, including infiltrated immune cells. It is also important to note that this study is based on female mice. Since pain-processing mechanisms might differ between sexes (Sorge *et al.*, 2015), similar studies in males, as well in other species, are ultimately required.

In summary, we performed the first translational profiling study of DRG and spinal cord tissues after nerve injury, and identified mRNAs whose translational efficiency is altered in the SNI animal model of neuropathic pain. The IPA analysis revealed altered cellular pathways, including identification of ERK as a key regulator of both translational and transcriptional networks. This information is instrumental for furthering our understanding of the molecular underpinnings of chronic pain.

2.6. Acknowledgements

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2.7. Materials and Methods

2.7.1. Neuropathic pain

All procedures involving mice were carried out in compliance with the Canadian Council on Animal Care guidelines and were pre-approved by the McGill University Animal Care Committee. C57BL/6J female mice, at 8 weeks of age, underwent the bilateral SNI surgical procedure as described previously (Decosterd & Woolf, 2000; Shields *et al.*, 2003) to induce neuropathic pain. Briefly, under 2% isoflurane anesthesia, the lateral surface skin of the thigh was shaved and

incised. The biceps femoris muscle was incised to expose the sciatic nerve just below its branching point. The tibial and common peroneal branches were tightly ligated using 7-0 silicone coated silk (Covidien, S-1768K) and a 3–4 mm portion of each of the ligated branches was sectioned and removed distal to the ligation point. Finally, the muscle and the skin incisions were closed using 6-0 Vicryl suture (Ethicon, J489G). During the entire process, great care was taken to leave the sural branch unharmed. The mouse was returned to its home-cage for recovery. Sham animals were used as controls, where the surgical procedure was carried out identically, but all three branches of the sciatic nerve were left untouched and unharmed. The animals were sacrificed 30 days post-surgery, and DRG and dorsal horn of the spinal cord samples were extracted. Tissues from 10 animals were pooled per sample and 2 independent replicates were made for each of the four conditions.

2.7.2. Harvesting of DRG and dorsal horn of spinal cord

To collect DRG and dorsal horn of the spinal cord, animals were sacrificed by brief isoflurane anesthesia followed by decapitation. The animal was secured on a bed of dry ice and the spinal cord was exposed and doused with RNA*later* stabilization solution (Ambion, AM7020). Lumbar DRG (level L3–L5) were excised for all animals. Next, the lumbar region of the spinal cord at which the L3–L5 DRG branch into was excised and placed on a bed of dry ice/metal plate and allowed to freeze after which it was cut along the frontal plane to separate the dorsal horn section. The DRG and dorsal horn were collected in non-stick, RNase free microcentrifuge tubes (Ambion, AM12450), immediately snap-frozen in liquid nitrogen, and stored at -80 °C until further processing.

2.7.3. Ribosomal profiling

2.7.3.1. Tissue homogenization and cell lysis

Flash frozen DRG and dorsal horn tissue was lysed in ice-cold cell lysis buffer (1% Polysome buffer (20 mM TrisCl (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT and 100 µg/ml cyclohexamide, 8% glycerol), 1% Triton X-100 and 25 U/ml Turbo DNase I) in a glass homogenizer system. The total lysate was divided into two fractions. A fraction containing at least 150 µg of total RNA was reserved for ribosome footprinting (RFP fraction) and the remaining (at least 100 µg) was processed for mRNA-Seq.

2.7.3.2. Obtaining ribosome footprints (RFPs)

Ribosome footprinting was carried out as previously described (Ingolia *et al.*, 2012) with minor modifications. Briefly the RFP fraction was subjected to RNase I treatment (Ambion, AM2295) at a concentration of 2 U/µg of RNA, at 4 °C for 45 min with end over end mixing and quenched for 5 min by adding 4U SUPERaseIn (Ambion, AM2696) for every 5 U of RNase I. Monosomes were pelleted by ultracentrifugation (Beckman Coulter, Optima MAX-UP)) through a 34% sucrose cushion (in polysome buffer) at 70,000 RPM for 3h at 4°C. The resulting RNA pellet was resuspended in 600 µl Tris Cl (pH 7) and RNA was extracted by double acid Phenol and one Chloroform extraction, precipitated by 1 volume Isopropanol and 1/9 volume 3 M NaOAc (pH %.5) and 2 µl Glycoblue (15mg/mg stock, Invitrogen, AM9515) at -80 °C overnight followed by centrifugation at 20,000 *g* at 4 °C for 30 min. Purified RNA was resolved on a 15% polyacrylamide urea gel (Invitrogen, EC6885BOX) and bands corresponding to 28–32 nucleotides, containing the desired ribosome footprints (RFPs), was excised and extracted for RNA using Costar Spin-X column (Sigma, CLS8160).

2.7.3.3. Random RNA fragmentation of cytoplasmic RNA

Poly (A)+ mRNAs were purified from 100 µg of cytoplasmic RNA, using magnetic oligo-dT DynaBeads. The purified RNA was then subjected to alkaline fragmentation by treating it with an equal volume of 2X alkaline fragmentation solution (2 mM EDTA, 10 mM Na2CO3, 90 mM NaHCO3, pH 9.2) for 20 min at 95 °C. The reaction was stopped by addition of the precipitation solution (300 mM NaOAc pH 5.5 and 2 µl GlycoBlue), followed by Isopropanol. Fragmented mRNAs were size-selected on a denaturing 10% polyacrylamide-urea gel and the bands corresponding to 30–50 nucleotides were excised, eluted, and precipitated with Isopropanol.

2.7.3.4. Library preparation for sequencing

Fragmented mRNA and RFPs were subjected to PNK dephosphorylation and 10 pmol of the dephosphorylated RNA fragments were used for ligation to a pre-adenylated and 3′-blocked linker, followed by separation on a 10% polyacrylamide urea gel. Linker ligated bands were excised and extracted for RNA, which was reverse transcribed using oNTI223 adapter (Illumina) and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instruction manual. The resulting cDNA was purified by size selection on a 10% polyacrylamide Tris/Borate/EDTA-urea (TBE-urea) gel. The cDNA was then circularized using CircLigase

(Epicentre, CL4111K). Products arising from ribosomal sequences were depleted using biotinylated rDNA complementary oligos (Ingolia *et al.*, 2012) and MyOne Streptavidin C1 dynabeads. The remaining products were amplified by PCR (11 cycles) using indexed primers, size-selected on a 8% polyacrylamide gel and purified. At these intermediate steps, bands in the gels that were very close to the fragment size + adapter were excised and purified. The resulting cDNA library samples were analyzed on an Agilent Bioanalyzer High Sensitivity DNA chip to confirm the size and concentration and then sequenced using the non Strand–Specific, single-read 50 (SR50) on the Illumina HiSeq 2500 Sequencing platform according to the manufacturer's instructions, with sequencing primer oNTI202 (5CGACAGGTTCAGAGTTCTACAGTCCGACGATC).

2.7.4. Bioinformatics analysis of ribosomal footprinting data

Raw sequencing data were de-multiplexed by the sequencing facility (Genome Quebec). Sequences were analyzed using a custom developed bioinformatics pipeline adapted from (Ingolia *et al.*, 2012) as described in (Silva Amorim *et al.*, 2018). In brief, reads were adapter-trimmed, contaminant sequences (rRNA, tRNA) were removed using bowtie with optimised parameters for ribosome profiling as per (Ingolia *et al.*, 2009) and reads were aligned to a reference mouse genome (GRCm38.p5). Since the RNA-seq and ribosome footprint assays were paired for each sample of the four conditions (DRG_SNI; spinal cord_SNI; DRG_Sham and spinal cord_Sham), the RNA-seq data were used to normalize the footprint numbers to derive the Translation efficiency (TE).

Reads Per Kilobase of transcript per Million mapped reads (RPKM) was calculated using an in-house R-script described in (Ingolia *et al.*, 2009) for each transcript. TE for each transcript was calculated by dividing RPKM values of the RFP libraries by RPKM values of the total mRNA libraries for each of the two sample condition replicates and then averaged. Z-score, P-values and FDR were calculated for all transcripts as in (Silva Amorim *et al.*, 2018). Genes with <128 reads were discarded. A Supplementary Table 4 includes RPKM abundances for all genes for all experiments. Raw RNA-seq data is available upon request.

2.7.5. IPA

Pathway Analysis was performed using the Ingenuity Pathway Analysis Software (IPA; Qiagen; version 42012434). Datasets previously filtered to include only differentially expressed and differentially translated genes were submitted to IPA. Location and Type information were

obtained from the IPA annotated datasets to determine the % of genes from each dataset belonging to individual subcellular localisation and molecular type/function categories. Data was plotted as % of genes in each category, with category "other" not shown. IPA annotated datasets were submitted to Core Analysis with analysis parameters set to include "Direct and indirect interactions" and "Experimentally observed data only". Network data was obtained for all datasets and a Molecular Activity Predictor (MAP) analysis was applied based on the differentially regulated genes belonging to each individual network.

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Chapter 3: General Discussion

The importance of translational control in regulating protein levels has been well established by previous studies. In the field of pain, it has been demonstrated that new gene expression is required for the development and maintenance of hypersensitivity, the hallmark of chronic pain. Inhibition of transcription and translation via blocking upstream signalling pathways prevents the establishment of abnormal plasticity and hypersensitization.

Systematic studies comparing the regulation of gene expression at both the transcriptional and the translational level with protein abundance have identified translational control to be a better predictor of protein levels (Schwanhäusser *et al.*, 2011). This is evident in situations where translation leads to changes in gene expression in the absence of new transcription as seen in embryogenesis (Curtis *et al.*, 1995) or when translation of pre-existing mRNAs is used to rapidly modify the proteome in stressful conditions (Spriggs *et al.*, 2010). Furthermore, translation can be localized in the cell (Buffington *et al.*, 2014a), a phenomena particularly observed in neurons. In the pain state, localized translation in the axonal compartment of neurons has been implicated to play a key role in modulating the physiology of nociceptors and leading to the development of abnormal plasticity (Price & Géranton, 2009; Terenzio *et al.*, 2018).

While the importance of assessing gene expression at the translational level has been advocated since at least the last decade, the tools to comprehensively assess global translation regulation have emerged only recently. However, even in the absence of contemporary translational profiling techniques, researchers have extensively assessed the expression of proteins involved in signalling pathways that regulate translation. These studies have underscored the role of translation regulation but could not identify translationally regulated genes on a genome-wide scale. Proteomic studies have been used to assess protein abundance, but the depth and coverage of investigation drastically lagged behind that of nucleotide sequencing technologies. Since studying translation regulation involves mRNA, it is the last step of gene expression regulation that can allow to leverage the advancements in sequencing technologies. Early techniques developed to study translation regulation like polysome profiling (Arava *et al.*, 2003), did provide insight regarding which mRNA transcripts are more associated with ribosomes but suffered certain critical

limitations, particularly because they failed to provide information about the precise location of ribosome on the transcript. Ribosome profiling, developed in 2009, takes into account these limitations and allows quantitative genome-wide measurement of translation in high resolution (Ingolia *et al.*, 2009). In the present study, we have aimed to identify differentially translated mRNAs in a mouse model of neuropathic chronic pain, in parallel we also performed RNA-seq to measure genome-wide total mRNA abundance.

3.1. Library construction, sequencing and bioinformatic analysis

Footprints and total mRNA extracted from DRG and dorsal horn spinal cord tissues from mice having SNI (or sham surgery) for 30 days were processed to prepare cDNA libraries amenable to Illumina HiSeq sequencing. In total, 16 libraries were sequenced, out of which 8 were footprint libraries and the remaining half were prepared from randomly fragmented total mRNA. We were able to obtain more than 56 to 96 million reads per library (supplementary table 1) for both types of libraries, with an average of 74 million reads/library. This sequencing depth is on par with previous studies which have employed RNA-seq to study gene expression in pain.

Since ribosomal RNA (rRNA) is the most abundant form of RNA in the cell, it is essential to remove rRNA contamination both while preparing the library and during *in silico* analysis. After filtering out the reads which mapped to ribosomal DNA (rDNA) sequences, 56% of the total mRNA reads and 25% of footprint reads were obtained and considered for further analysis. This difference arises from the use of different methods to remove rDNA sequences while preparing the libraries. In the case of footprints, biotin labelled rDNA oligos and streptavidin beads were used to subtract rDNA fragments whereas, in the case of total mRNA library preparation, mRNAs were pulled out using polydT beads. Out of the non-rDNA mapped reads, 66% of footprint reads and 81% of total mRNA reads were uniquely mapped. Recently, several groups have tried to refine the ribosome profiling protocol and have suggested using the Ribo-zero kit (Ambion) (McGlincy & Ingolia, 2017) or performing sequence-independent rRNA depletion using duplex-specific nuclease (DSN) or using a combination of depletion strategies (Chung *et al.*, 2015) to subtract rDNA during the footprint library preparation. However, these alternative rDNA depletion strategies have not been tested in nervous system tissue. Therefore, it would be worthy to compare

the efficiency of different rRNA depletion methods for preparing footprint libraries from nervous tissues.

It should be noted that interpretation of Ribosome profiling data requires careful consideration of certain aspects in order to avoid over interpretation of the datasets. Ribosome profiling data provides average ribosome density along the mRNA, this information along with abundance of mRNA molecules is used to infer translation efficiency and rate of synthesis. The accuracy of this approach is based on the premise that all bound ribosomes complete translation of the mRNA and that, on average, different mRNA species in a cell have similar ribosome elongation rates. The use of cycloheximide is known be associated with stalling of ribosomes at the 5' end of mRNA (Ingolia *et al.*, 2009), posing a challenge for proper data analysis. Various analysis pipelines have become available which allow implementation of rigorous quality check parameters and allows extension of the analysis by providing tools to inspect 'footprint periodicity', perform 'upstream Open Reading Frame (uORF) search', detect different 'translation initiation sites', analyze 'codon usage' and search for 'translation pauses', to name a few (Eastman *et al.*, 2018). In our study we have employed an analysis pipeline described by (Amorim *et al.*, 2018a) which is based on calculating Translational efficiency (TE) for each transcript by dividing the RPKM values of the footprint libraries by the RPKM values of the total RNA libraries. Further, changes in TE and transcription (mRNA RPKM) values were analyzed for predefined pairwise comparisons between experimental groups (DRG_SNI vs DRG_Sham and spinal cord_SNI vs spinal cord_Sham). A z-score and ap-value derived from the z-score were calculated for each gene.

Another aspect to consider is that binding of other RNA-binding proteins (RBPs) as well as certain secondary structure can arguably protect mRNA from nuclease digestion and thus contaminate the resulting footprint pool. In order to avoid such discrepancies, we assessed the length of the sequenced footprints and inspected footprint periodicity. We observed the footprints to correspond to a length of 28 to 32 nucleotide long fragments, displaying a 3-nucleotide periodicity reassuring that the sequenced footprints have been obtained from ribosome protected mRNA fragments. Moreover, we see an enrichment of footprints in the coding sequence of the gene. False-footprints fragments resulting from mRNA protection by other RBPs would not display these characteristics.

3.2. Transcriptional and translational control

Integrated analysis of both mRNA-seq data and ribosome profiling data was used to distinguish between transcriptional and translational regulation. In the following paragraphs, differentially regulated genes refer to only the significantly regulated genes, with $p<0.05$ and $0.5>fold\ change>1.5$.

In the significantly differentially regulated genes, a greater subset of these genes was upregulated than downregulated at both the transcriptional and the translational levels. This trend has also been noted by a previous study which found a greater number of upregulated than downregulated genes among genes differently expressed at the transcriptional level (Xiao *et al.*, 2002). We show that this trend is also visible in the genes differentially regulated at the translational level. This is in accordance with upregulation of transcription and translation factors observed in neuropathic pain conditions. On comparing the total number of differentially regulated genes, a tissue specific pattern was observed. In DRG, a greater subset (177) of genes were transcriptionally regulated compared to the number of translationally regulated genes (105). In the spinal cord, this pattern was the exact opposite, with 130 genes translationally regulated and only 32 genes transcriptionally regulated. This indicates that transcription might play a greater role in controlling gene expression in DRG, while translational regulation might be more prevalent in the dorsal horn of the spinal cord. The DRG comprise of neuron cell bodies, where transcription takes place. However, all transcribed mRNAs are not necessarily translated in the DRG, which is acknowledged by several studies and known as local translation (Terenzio *et al.*, 2018). mRNA transcribed in DRG neurons might be transported to other sites in the neuron like axons where they are ultimately translated. On the other hand, local dorsal horn neurons comprise the majority of the dorsal horn neurons. This could explain greater transcriptional changes observed in DRG as compared to translational changes. However, this observation needs further validation under standardized analysis.

Several genes were differentially regulated both at the transcriptional and translational levels. Interestingly, the direction of regulation (up- or down-) was not always the same for both levels of gene expression. While some genes (Pkd2l1, Unc45b, Tmem88b, and Trhr in the DRG) had the

same direction of regulation at both levels, a subset of genes had oppositely regulated transcription and translation (Myhy7, Mobp, 1500009C09Rik, Sall1, Grin2b, Olig2 and 3110035E14Rik in the DRG and Tmem54, Scn4a, Htr3b, Sprr1a and Rtn4rl2 in the spinal cord). The latter scenario reiterates the notion that transcript abundance alone is not a reliable predictor of protein abundance, since translation control can intervene to regulate the expression in the opposite direction and undo or reverse the differential regulation caused at the transcriptional level. This is a demonstration of translational buffering, wherein the mRNA abundance and translation efficiency (TE) are negatively correlated (McManus *et al.*, 2013; Jeong *et al.*, 2016).

Furthermore, it is interesting to consider the genes which were not differentially regulated at the transcriptional level but were upregulated only at the translational level. While these genes remain unidentified in a mRNA abundance only based study, they represent an example where translation regulation plays a more significant role in their expression. This can also be thought as an example of "translation on demand" wherein a protein can be rapidly available without the need of increasing mRNA abundance. This phenomenon is also referred to as "post-transcriptional regulation" and has been observed in previous studies in yeast (Beyer *et al.*, 2004) and dendritic cells (Jovanovic *et al.*, 2015).

3.3. ERK as a central hub of both transcriptionally and translationally regulated genes

ERK is a mitogen activated protein kinase, whose role in the development and establishment of neuropathic pain has been supported by many studies. In the present study, we have identified ERK as a central regulatory hub, upstream of the transcriptionally and translationally regulated genes in neuropathic pain. ERK regulates transcription and translation by a variety of signalling pathways. As described in section 1.2.3.3., phosphorylation of eIF4E at Ser 209 by MNK1/2 is crucial for the activation of eIF4E. Blocking eIF4E phosphorylation by using either pharmacological or genetic approaches, a reduction is hypersensitivity is seen. The activation of MNK1/2 is dependent upon ERK and p53 mediated phosphorylation (Pyronnet *et al.*, 1999; Waskiewicz *et al.*, 1999). Taken together, this study highlights the role of ERK in development of neuropathic pain and presents the ERK/MNK/eIF4E axis of translational regulation as a direction worth further investigation.

In 2005, Zhuang *et al.* found the expression of ERK in DRG and dorsal horn of spinal cord to be sequential. More precisely, they found the activated form of ERK (phospho-ERK [p-ERK]) to be first increased in DRG and dorsal horn spinal cord neurons immediately after nerve injury. However, this early increase in activation of ERK lasted only a few hours before shifting to other cell types, both in the DRG and the spinal cord. In the dorsal horn of the spinal cord, the increased activation of ERK was seen to be concentrated in microglia at 2 days after injury. Gradually, from day 10 to day 21, the presence of p-ERK decreased in the microglia and was seen in astrocytes. On the other hand, in the DRG, p-ERK was not seen until after 6h post injury and was seen to moderately increase in satellite cells and Schwan cells after 6 h (Zhuang *et al.*, 2005). In the present study, we have assessed the transcriptional and translational profile 30 days after injury in whole DRG and dorsal horn of spinal cord tissue. These tissues are composed of neurons imbedded in a complex of non-neuronal cells including Schwann cells, fibroblasts, satellite glial cells and astrocytes. Hence it is possible, that differentially regulated genes which fall under the control of ERK come mostly from the non-neuronal cells and not the neurons. Cell type specific translational profiling is required to confirm this, while our study highlights the role of involvement of ERK in maintenance of neuropathic pain at both transcriptional and translational levels of gene expression.

3.4. Future directions

The most immediate future directions to the study presented here would be to select and validate a few targets genes based on their differential regulation status and their known involvement in development and maintenance of neuropathic pain. Preliminary validation studies can be performed using qPCR to judge the mRNA abundance and western blot to analyze the protein levels. However, it should be noted that several forms of regulation are in effect after translation, and hence all the genes significantly regulated at the translational level, based on TE, might not show a corresponding pattern in western blot analysis. For the target genes which do show differential expression by western blot analysis, their localization to cell types can be assessed using immunohistochemistry on DRG or spinal cord slices. The next step after preliminary validation would be to characterize the function of the top validated hits using transgenic mouse models, viral vector-mediated gene silencing, as well as pharmacological and molecular tools. The genes *Scn4a*, *Htr3b*, *Sprr1a*, *Rtn4rl2*, *Tmem54* in the spinal cord and *Myh7*, *Mobp*,

1500009C09Rik, *Sall1*, *Grin2b*, *Olig2* and 3110035E14Rik in the DRG can be interesting targets to start with. Most of them have also been mentioned in previous pain studies. Furthermore, this study provides a dataset which can be referred to when assessing the expression of any gene in the DRG and dorsal horn of the spinal cord. Comparison of our dataset with previous transcriptome datasets and future translatome datasets in the field of pain would also be an interesting avenue to explore.

Now that we have demonstrated the feasibility of ribosome profiling in DRG and spinal cord tissue in mice, another direction to the future studies would be to incorporate cell specificity along with genome-wide translational profiling. Performing Ribosome profiling in cell type-specific manner is challenging as obtaining enough quantities of footprints, amenable to library preparation requires large quantities of total RNA, which would call for a large number of specific cell types and even grater quantities of tissue. This becomes particularly difficult for tissues like DRG and spinal cord, where the number of specific cells of interest (e.g. nociceptors) is relatively small. Moreover, methods to isolate distinct cell types from tissues such as FACS or LCM, increase processing steps and time before footprints can be harvested. This in turn poses a risk of altering the local distribution of ribosomes on mRNA. Among the various techniques used by researchers to perform cell-type specific gene-expression profiling, using TRAP is advantageous as it minimizes the processing times to obtain specific cells, such as neurons after tissue harvest, avoids perfusing tissue and other stresses caused during cell isolation, all of which can lead to loss of tissue intrinsic signal (Heiman *et al.*, 2014). While most of the studies employing TRAP use RNA-seq as a downstream process, combining TRAP with ribosome profiling will enable neuron specific translational profiling at the global scale.

As reviewed in chapter 1, previous studies have highlighted the central role of eIF4E in regulating cap-dependent translation and importance of eIF4E-dependent translational control in the development and maintenance of chronic pain. Various pharmacological inhibitors of eIF4E are available in addition to knockout mice (for example, MNK1/2 knockout mice) for proteins upstream of eIF4E as well as transgenic mice with mutant eIF4E (*eIF4E*^{S209A}). Therefore, it would be valuable to employ translational profiling in combination with inhibition of eIF4E activity, to identify translation of which mRNAs is hampered.

Since the establishment of chronic pain can be dissected into development and maintenance phases, a time course analysis of translational profiling would suggest if the contribution of transcription and translation change over time. We hypothesize that translational regulation would play a major role in the initial stages as translation of pre-existing mRNAs can rapidly modify the cellular proteome as a result of stress responses. Expression involving upregulation of mRNA abundance might follow in the later time points. Furthermore, studies have shown altered translation to be localized in the axonal compartment of the neurons post-injury, thus it would be imperative to extend the comprehensive gene expression profiling further from DRG and spinal cord to nerve segments, containing axonal compartments of the injured neurons.

Chapter 4: Conclusion and Summary

Both transcription and translation regulation are crucial for bringing into effect the phenotypic changes in the nociceptors in response to injury and establishing hypersensitivity. Gene expression is tightly regulated at various steps, including during the synthesis of RNA and its further translation into protein. Chapter 1 extensively reviews the studies which have investigated the transcriptional or translational profile with respect to pain. Several studies on individual genes have shown that regulation of gene expression is important for development and maintenance of pain, and genome wide transcriptomic studies have revealed the changes in mRNA abundances, but the relative contribution of transcriptional and translational regulation remains largely unknown. On the other hand, few studies have employed proteomics to study changes in the proteome under painful conditions; however, these studies still fail to answer the question. It is well accepted that comparison of steady state mRNA and protein levels even from the same sample pose considerable discrepancies.

Studies of translational control mechanisms in peripheral nerve injury (PNI) have revealed profound changes in the peripheral and central nervous systems of rodents with neuropathic pain. PNI increases the activity of kinases associated with translation control, such as AKT, mTOR, S6K, and ERK. Moreover, it has been established that injury activates translational machinery (eIF4E, eIF4A, eIF2α) and RNA processing and binding proteins. These results demonstrate that PNI induces a fundamental reorganization of translational signaling pathways and machinery in the sciatic nerve and in spinal neurons by modulating the translation of a subset of mRNAs. Notably, it is highly important to identify mRNAs whose translation is altered under conditions of chronic pain and whose functions remain elusive.

Up until now, only one other study, published a few months after our study, has attempted to identify genes undergoing translation in a mouse model of chemotherapy induced neuropathic pain (CINP). In this study Megat *et al.* have sequenced mRNAs associated with ribosomes from DRG neurons. With this approach, while they have obtained tissue specificity, their assessment of translation efficiency is based on comparison of RNA-seq results from two different studies and does not provide information about the reading frame being translated.

Ribosome profiling enables quantitative translational profiling at high resolution. The present study presents the first attempt to employ this technique in mouse DRG and spinal cord. Furthermore, it provides the first genome-wide translational and transcriptional landscape in a mouse model of neuropathic pain. The higher number of upregulated versus downregulated mRNAs is consistent with the known increase in signaling upstream of translation following nerve injury in DRG. Several interesting phenomena are observed by parallel analysis of translation efficiency and mRNA abundance. For example, opposing changes in mRNA levels and their translational efficiency is demonstrated for multiple mRNA, suggesting translational buffering. We predict that genes showing changes in the same direction at their mRNA levels and TE will exhibit robust changes in the corresponding protein levels. Additionally, IPA network analysis revealed altered cellular pathways, including identification of ERK as a key regulator of both translational and transcriptional networks.

In conclusion, we identified distinct subsets of mRNAs that are differentially translated in response to nerve injury in DRG and dorsal horn of spinal cord tissue. We also discovered key pathways linked to mRNA translational control and neuropathic pain. Our study underlines the importance of considering translation regulation in pain by showing that a large subset of genes is translationally regulated at 30 days post-SNI. Finally, the data generated are crucial for the understanding of mechanisms by which mRNA translation promotes persistent hypersensitivity after nerve injury.

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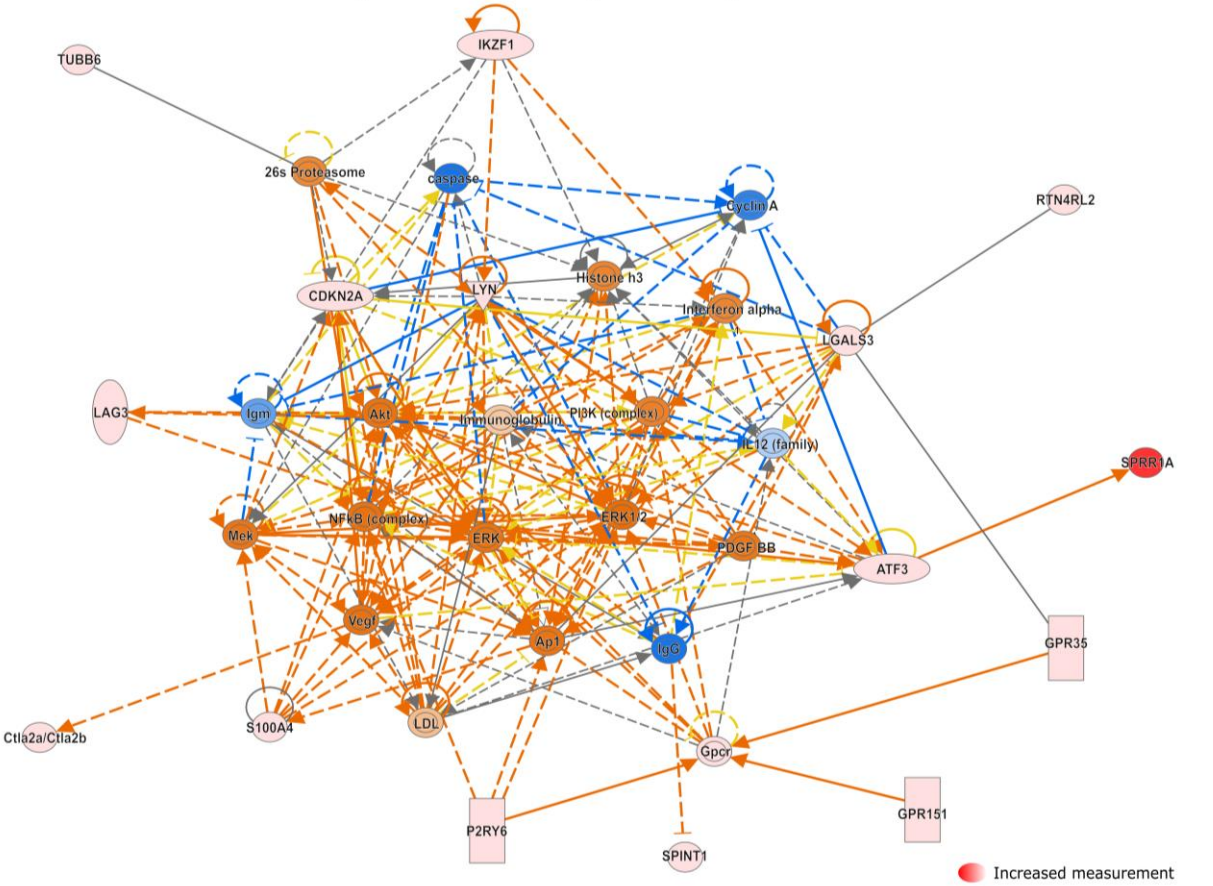
Appendix A: Supplementary figure and tables

Supplementary Figure 1. Network analysis of differentially transcribed and translated mRNAs in spinal cord, 30 days post-SNI

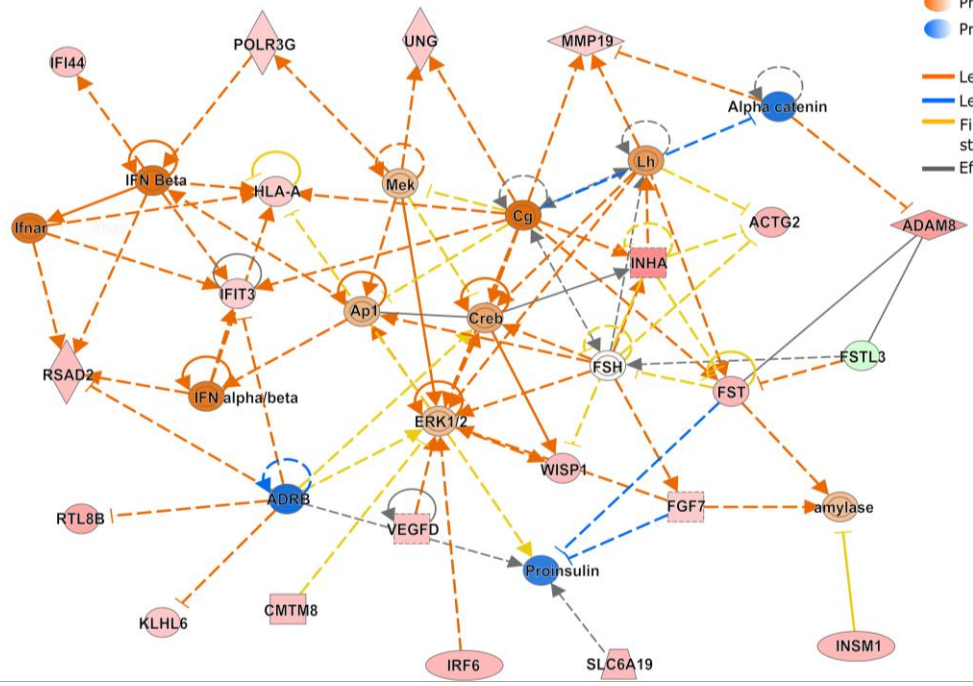
Red: increased measurement; green: decreased measurement; orange: predicted activation; blue: predicted inhibition; yellow: findings inconsistent with state of downstream molecule; grey: effect not predicted; solid line: direct interaction; dashed line: indirect interaction.

Supplementary Figure 1

A. Network Analysis - Transcription - Spinal Cord



B. Network Analysis - Translation - Spinal Cord



Supplementary Table 1. Number of total sequenced reads, reads mapped to non- rDNA region, un-mapped reads and uniquely mapped reads and their proportion in each sample.

Sample Description	Total number of reads	Not maped to rDNA	Not maped to rDNA%	un-mapped	non-unique	uniquely mapped	uniquely mapped%
SC_Sham_mRNA_1	82,647,804	48,133,287	59.60%	1,385,689	6,320,900	40,426,698	84.00%
SC_Sham_mRNA_2	71,069,918	40,358,380	58.60%	1,075,515	5,252,953	34,029,912	84.30%
SC_SNI_mRNA_1	72,644,137	40,765,720	57.50%	1,129,918	5,351,964	34,283,838	84.10%
SC_SNI_mRNA_2	67,808,722	38,399,069	58.20%	1,352,019	5,106,134	31,940,916	83.20%
DRG_Sham_mRNA_1	70,956,972	35,225,963	51.40%	1,527,306	5,485,505	28,213,152	80.10%
DRG_Sham_mRNA_2	64,839,345	32,744,922	52.30%	1,325,527	5,081,988	26,337,407	80.40%
DRG_SNI_mRNA_1	96,934,224	51,588,734	55.30%	3,559,183	7,659,026	40,370,525	78.30%
DRG_SNI_mRNA_2	71,849,595	39,488,769	57.00%	2,788,037	5,899,622	30,801,110	78.00%
SC_Sham_RFP_1	56,261,200	11,622,385	21.20%	1,285,619	2,653,768	7,682,998	66.10%
SC_Sham_RFP_2	75,547,500	17,138,148	23.10%	1,933,036	3,737,484	11,467,628	66.90%
SC_SNI_RFP_1	70,945,007	12,676,586	18.50%	1,774,180	2,812,381	8,090,025	63.80%
SC_SNI_RFP_2	61,891,605	11,135,691	18.40%	1,455,077	2,348,430	7,332,184	65.80%
DRG_Sham_RFP_1	61,282,781	11,632,656	19.40%	1,775,552	2,111,146	7,745,958	66.60%
DRG_Sham_RFP_2	77,732,363	26,537,796	35.30%	3,531,342	4,872,850	18,133,604	68.30%
DRG_SNI_RFP_1	82,695,879	19,033,784	23.40%	2,830,429	3,417,423	12,785,932	67.20%
DRG_SNI_RFP_2	83,803,887	32,839,010	40.50%	4,442,524	6,091,439	22,305,047	67.90%

Supplementary table 1: Number of total sequenced reads, reads mapped to non- rDNA region, un-mapped reads and uniquely mapped reads and their proportion in each sample

The first column describes the samples used in the experiment, in the format 'Tissue type_Procedure_Type of read_Replicate number'. The procedure is either spared nerve injury (SNI) or Sham surgery (sham). Two replicates (# 1 and # 2) were used for each condition. Spinal cord (SC); dorsal root ganglion (DRG).

Supplementary Table 2. List of differentially translated or transcribed genes (upregulated and downregulated; $p<0.05$ and $0.5>$fold change>1.5) between sham and SNI-treated animals in spinal cord and DRG.

Not included due to size restrictions. Available online at:
https://www.sciencedirect.com/science/article/pii/S2452073X18300072#s0075

Supplementary Table 3. List of genes showing changes in different directions in TE and mRNA levels.

	Gene symbol	Gene name	Transcription	Translation
DRG	Pkd2l1	polycystic kidney disease 2-like 1	UP	UP
	Unc45b	unc-45 myosin chaperone B		
	Tmem88b	transmembrane protein 88B		
	Trhr	thyrotropin releasing hormone receptor	DOWN	DOWN
	Myh7	myosin, heavy polypeptide 7, cardiac muscle, beta	DOWN	UP
	Mobp	myelin-associated oligodendrocytic basic protein		
	1500009C09Rik	RIKEN cDNA 1500009C09 gene		
	Sall1	sal-like 1 (Drosophila)		
	Grin2b	glutamate receptor, ionotropic, NMDA2B (epsilon 2)		
	Olig2	oligodendrocyte transcription factor 2		
	3110035E14Rik	RIKEN cDNA 3110035E14 gene		
Spinal cord	Tmem54	transmembrane protein 54	DOWN	UP
	Scn4a	sodium channel, voltage-gated, type IV, alpha	UP	DOWN
	Htr3b	5-hydroxytryptamine (serotonin) receptor 3B		
	Sprr1a	small proline-rich protein 1A		
	Rtn4rl2	reticulon 4 receptor-like 2		
Supplementary Table 3 - List of genes showing changes in different directions in TE and mRNA levels.				

Supplementary Table 4. The table includes RPKM abundances for all genes for all experiments

Not included due to size restrictions. Available online at:
https://www.sciencedirect.com/science/article/pii/S2452073X18300072#s0075

Appendix B

Manuscript 1: eIF4E-dependent Translational Control: A Central Mechanism for Regulation of Pain Plasticity

eIF4E-dependent Translational Control: A Central Mechanism for Regulation of Pain Plasticity

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eIF4E, mRNA translation, Pain -, sensitization, Treatment

Abstract

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Translational control of gene expression has emerged as a key mechanism in regulating different forms of long-lasting neuronal plasticity. Maladaptive plastic reorganization of peripheral and spinal nociceptive circuits underlies many chronic pain states and relies on new gene expression. Accordingly, downregulation of mRNA translation in primary afferents and spinal cord neurons inhibits tissue injury-induced sensitization of nociceptive pathways, supporting a central role for translation dysregulation in the development of persistent pain. Translation is primarily regulated at the initiation stage via the coordinated activity of translation initiation factors. The mRNA cap-binding protein, eIF4E, is involved in the recruitment of the ribosome to the mRNA cap structure, playing a central role in the regulation of translation initiation. eIF4E integrates inputs from the mTOR and ERK signaling pathways, both of which are activated in numerous painful conditions to regulate the translation of a subset of mRNAs. Many of these mRNAs are involved in the control of cell growth, proliferation, and neuroplasticity. However, the full repertoire of eIF4E-dependent mRNAs in the nervous system and their translation regulatory mechanisms remain largely unknown. In this review, we summarize the current evidence for the role of eIF4E-dependent translational control in the sensitization of pain circuits and present pharmacological approaches to target these mechanisms. The understanding eIF4E-dependent translational control mechanisms and their roles in aberrant plasticity of nociceptive circuits might reveal novel therapeutic targets to treat persistent pain states.

eIF4E-dependent Translational Control: A Central Mechanism for Regulation of Pain Plasticity

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Abstract

Translational control of gene expression has emerged as a key mechanism in regulating different forms of long-lasting neuronal plasticity. Maladaptive plastic reorganization of peripheral and spinal nociceptive circuits underlies many chronic pain states and relies on new gene expression. Accordingly, downregulation of mRNA translation in primary afferents and spinal cord neurons inhibits tissue injury-induced sensitization of nociceptive pathways, supporting a central role for translation dysregulation in the development of persistent pain. Translation is primarily regulated at the initiation stage via the coordinated activity of translation initiation factors. The mRNA cap-binding protein, eIF4E, is involved in the recruitment of the ribosome to the mRNA cap structure, playing a central role in the regulation of translation initiation. eIF4E integrates inputs from the mTOR and ERK signaling pathways, both of which are activated in numerous painful conditions to regulate the translation of a subset of mRNAs. Many of these mRNAs are involved in the control of cell growth, proliferation, and neuroplasticity. However, the full repertoire of eIF4E-dependent mRNAs in the nervous system and their translation regulatory mechanisms remain largely unknown. In this review, we summarize the current evidence for the role of eIF4E-dependent translational control in the sensitization of pain circuits and present pharmacological approaches to target these mechanisms. The understanding eIF4E-dependent translational control mechanisms and their roles in aberrant plasticity of nociceptive circuits might reveal novel therapeutic targets to treat persistent pain states.

Keywords: eIF4E, mRNA translation, persistent pain, sensitization, treatment

Introduction

Chronic pain is a debilitating condition affecting more than 20 percent of the population worldwide (de Souza et al., 2017; Steglitz et al., 2012). Chronic pain is most commonly triggered by tissue inflammation or nerve injury, which are caused by metabolic diseases (diabetes), autoimmune diseases, viral infection (herpes zoster), cancer, chemotherapy drugs (e.g. platinums, taxanes, epothilones, and vinca alkaloids), and nerve entrapment or blunt trauma. Chronic pain, however, can also appear without any recognizable trigger such as in fibromyalgia, migraine, irritable bowel syndrome, and interstitial cystitis.

In most cases, the pain is a result of increased sensitivity of peripheral or central nociceptive circuits to stimulation, causing painful sensation in response to a normally innocuous stimulus. The increase in sensitivity, also called sensitization, is mediated by a combination of mechanisms taking place at several levels along the pain pathway including primary sensory neurons, spinal cord, and higher brain areas (Todd, 2010; Yekkirala et al., 2017).

Long-lasting increases in the sensitivity and responsiveness of pain circuits is ultimately accompanied by changes in gene expression, which support biochemical and structural alterations in neuronal and non-neuronal cells involved in pain processing. Gene expression is a multi-step process that is tightly regulated at different levels. Regulation of the rate by which mRNA is translated into a protein is called translational control (Robichaud et al., 2018; Sonenberg and Hinnebusch, 2009). Translational control has a strong impact on the abundance of proteins in the cell, and its dysregulation contributes to many pathologies in the nervous system including developmental abnormalities, metabolic dysregulation, autism spectrum disorder (ASD), and epilepsy (Buffington et al., 2014; Tahmasebi et al., 2018). Tissue injury, metabolic diseases, and certain drugs (e.g. anticancer and opioids) cause an upregulation of mRNA translation in pain-processing tissues such as dorsal root ganglion (DRG) and dorsal horn of the spinal cord (Khoutorsky and Price, 2018; Melemedjian and Khoutorsky, 2015). Inhibition of translational control signalling in these tissues reduces the sensitization of nociceptive circuits and alleviates pain, demonstrating a central role of translational upregulation in the development of persistent pain (Asante et al., 2009; Bogen et al., 2012; Ferrari et al., 2013; Geranton et al., 2009; Jimenez-Diaz et al., 2008; Melemedjian et al., 2010; Obara and Hunt, 2014; Price and Geranton, 2009; Price et al., 2007; Xu et al., 2011). The rate of mRNA translation is controlled via several mechanisms (Costa-Mattioli et al., 2009; Robichaud et al., 2018). The

recruitment of the ribosome to the mRNA is a central step in translation initiation and the major site for regulation. A key mechanism to regulate this process is controlling the activity of the eukaryotic translation initiation factor 4E (eIF4E), which binds a mRNA "cap" structure (a 7-methylguanosine linked to the first nucleotide at the 5' end of all nuclear transcribed eukaryotic mRNAs) and initiates ribosome recruitment (Altmann et al., 1985; Sonenberg and Hinnebusch, 2009). In this review, we focus on the regulation of eIF4E-dependent mRNA translation initiation in nociceptive plasticity, highlighting a central role of this mechanism in the development of chronic pain.

Translational control mechanisms

The process of translation can be divided into three phases: initiation, elongation, and termination. Most of the regulation of translation occurs at the initiation step (Merrick and Pavitt, 2018; Sonenberg and Hinnebusch, 2009). The initiation is regulated by a large number of translation initiation factors, which mediate the recruitment of the ribosome to the mRNA, followed by scanning of the 5' untranslated region (5' UTR) of the mRNA for the presence of an AUG start codon. A critical step in this process is the binding of eIF4E to the mRNA cap. Following binding to the cap, eIF4E binds an mRNA helicase eIF4A and a large scaffolding protein eIF4G to form a tri-subunit complex, called eIF4F (Figure 1). eIF4F facilitates the recruitment of the 43S preinitiation complex (PIC) to the mRNA. PIC is composed of a small 40S ribosomal subunit, translation factors eIF1, eIF1A, and eIF3, and a ternary complex (eIF2: GTP bound to initiator, Met-tRNA$_i^{Met}$). Recruitment of the PIC is followed by the scanning of the mRNA 5' UTR and joining of a large ribosomal subunit (60S), upon encountering a start codon, to form an 80S ribosome that is competent to proceed to the elongation phase of translation. Importantly, the helicase activity of eIF4F (mediated by eIF4A) is required for unwinding the mRNAs 5' UTR secondary structure to allow the scanning process and translation (Parsyan et al., 2011).

Other major mechanisms involved in the regulation of translation initiation include regulation of ternary complex availability (via phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 [eIF2α]) (Trinh and Klann, 2013); regulation of the length of mRNA poly(A) tail which promotes translation and protects mRNA from degradation (Derry et al., 2006; Gray et al., 2000; Kahvejian et al., 2001); and finally translation initiation via a cap-independent mechanism (mediated by internal ribosome entry site, IRES) (Leppek et al., 2018; Macejak and Sarnow, 1991; Pelletier and Sonenberg, 1988). Since the expression levels of eIF4E are the lowest among all translation initiation

factors, the formation of the eIF4F complex and correspondingly, translation initiation are the rate-limiting steps for translation under most circumstances.

eIF4E is a central regulator of cap-dependent translation

eIF4E activity is tightly regulated via two mechanisms. Translational repressor 4E-binding protein (4E-BP) binds eIF4E and prevents its association with eIF4G, and thus precludes the formation of the eIF4F complex. In mammals, there are three 4E-BP isoforms - 4E-BP1, 4E-BP2, and 4E-BP3, which have similar functions but exhibit differences in tissue distribution. The binding of 4E-BP to eIF4E depends on the 4E-BP phosphorylation state. Upon phosphorylation by the mechanistic target of rapamycin complex 1 (mTORC1), the affinity of 4E-BP to eIF4E is reduced, leading to its dissociation from eIF4E and allowing the formation of eIF4F complex at the mRNA cap. This promotes the recruitment of 43S PIC to the mRNA and stimulation of translation (Figure 1). Even though all eukaryotic mRNAs have a cap, not all cellular mRNAs are equally sensitive to this mechanism, and the translation of "eIF4E-sensitive mRNAs" is preferentially stimulated by increased eIF4E activity. For example, housekeeping mRNAs such as *GAPDH* and *β-actin* are less sensitive to eIF4E as compared to mRNAs involved in cell growth, proliferation, and immune responses (e.g., c-MYC, cyclins, BCL-2, MCL1, osteopontin, survivin, vascular endothelial growth factor [VEGF], fibroblast growth factors [FGF], and matrix metalloproteinase 9 [MMP-9]) (Bhat et al., 2015; Chu and Pelletier, 2018; Rousseau et al., 1996; Sonenberg and Gingras, 1998). The mRNA features rendering eIF4E-sensitivity have been typically associated with 5'UTRs enriched with high-complexity secondary structures (Pelletier and Sonenberg, 1985; Sonenberg and Gingras, 1998). It has been demonstrated that a long 5'UTR favours the formation of stable secondary structures, and that the proximity of these structures to the cap obstructs eIF4F complex formation. On the other hand, hairpin structures with a greater free energy, located further away from the cap, restrict 5'UTR scanning (the progression of the PIC toward the start codon) (Kozak, 1989; Pickering and Willis, 2005). However, translation of a subset of mRNAs without long 5'UTR can still be sensitive to eIF4E, indicating that other 5'UTR signatures may also render this sensitivity (Leppek et al., 2018). Potential mechanisms include the presence of 5′ terminal oligopyrimidine tracts (5′*TOPs*) (Thoreen et al., 2012) and *cis*-regulatory elements (Hinnebusch et al., 2016; Leppek et al., 2018; Truitt et al., 2015; Truitt and Ruggero, 2016; Wolfe et al., 2014) at the 5'UTR. For example, a Cytosine-rich 15-nucleotide motif, termed Cytosine Enriched Regulator of Translation (CERT), was shown to be responsible for conferring eIF4E sensitivity under oncogenic transformation and oxidative stress (Truitt et al., 2015).

Although most studies have attributed the elevated translation of mRNAs with highly structured 5'UTRs to the cap-binding ability of eIF4E and it being the limiting component of the eIF4F complex, other studies did not find that the cap-binding ability completely explained eIF4E function and explored further mechanisms of eIF4E-mediated translation regulation. This led to the identification of an additional function of eIF4E – stimulation of eIF4A helicase activity, which is independent of its cap-binding ability (Feoktistova et al., 2013).

In addition to regulation by mTORC1/4E-BP, eIF4E activity is also controlled via phosphorylation of its sole phosphorylation site, Ser 209, by mitogen activated protein kinase [MAPK]-interacting protein kinases (MNKs) 1 and 2, downstream of the extracellular-signal-regulated kinase (ERK) and the p38 MAPK signaling cascades (Figure 1) (Pyronnet et al., 1999; Waskiewicz et al., 1999). The phosphorylation of eIF4E is associated with altered translation of a subset of mRNAs, although the mechanisms underlying the effect of this phosphorylation event on translational efficiency and transcript-specificity remain elusive.

Since eIF4E is a downstream effector of both mTORC1 (via 4E-BP-dependent repression) and ERK (via eIF4E phosphorylation), its activity can be modulated by a multitude of external and internal cues that activate these central cellular signaling pathways. Numerous membrane receptors activate mTORC1 and ERK signaling in neurons including tyrosine receptor kinase A (trkA) and trkB, receptors from the insulin receptor family (IR, IGF1R, EGFR), and metabotropic glutamate and NMDA receptors. In addition to the extracellular cues, these pathways integrate intracellular signals conveying information on the status of cellular energy (via AMPK), oxygen levels (via activation of AMPK and REDD1 [Regulated in DNA damage and development 1]), and DNA damage (via the induction of p53 target genes) (Saxton and Sabatini, 2017) (Figure 1).

eIF4E in regulation of peripheral nociceptive plasticity

Tissue injury induces profound changes in the phenotype of sensory neurons, increasing their excitability and changing the connectivity within the peripheral tissue and spinal cord. These alterations are driven by pro-inflammatory molecules released from injured tissues, such as neurotrophin nerve growth factor (NGF) and cytokine interleukin 6 (IL-6), as well as by neuronal activity evoked by direct injury to the nerve. ERK and mTORC1, two central intracellular pathways, are stimulated at by tissue inflammation and nerve injury, diabetes, cancer, and drug-induced neuropathies (Khoutorsky and Price, 2018; Melemedjian and Khoutorsky, 2015). In addition to the phosphorylation-mediated activation of mTOR, downstream of PI3K/AKT pathway, a recent study

showed that nerve injury stimulates local axonal *mTOR* mRNA translation (Terenzio et al., 2018). Translation profiling of DRG tissue from mice subjected to nerve injury showed that ERK is a key regulatory hub controlling both transcriptional and translation gene expression networks (Uttam et al., 2018).

Inhibition of ERK and mTORC1 signaling alleviates the development of pain hypersensitivity in a variety of pain models (Chen et al., 2018; Ji et al., 2009; Khoutorsky and Price, 2018). Since ERK and mTORC1 pathways converge on eIF4E to control the rate of cap-dependent translation, it was suggested that eIF4E might play a central role in the sensitization of pain circuits via regulating the translation of specific mRNAs. The physiological significance of eIF4E phosphorylation was studied using mice lacking eIF4E phosphorylation (knock-in mutation of serine^{209} to alanine, *eIF4E*^{S209A}). These mice display greatly reduced mechanical and thermal hypersensitivity in response to intraplantar administration of IL-6, NGF, and carrageenan, as well as diminished hyperalgesic priming (Moy et al., 2017). Moreover, the increase in excitability of *eIF4E*^{S209A} primary sensory neurons in response to IL-6 and NGF was reduced as compared to wild-type (WT) controls. These findings were recapitulated in *MNK1/2* knockout mice, which also lack eIF4E phosphorylation. In the nerve injury model of neuropathic pain, spared nerve injury (SNI), the development of mechanical and cold hypersensitivity was reduced in both *eIF4E*^{S209A} and *MNK1/2* knockout mice. Notably, local intraplantar inhibition of MNK with cercosporamide reduced mechanical hypersensitivity in response to NGF and alleviated hyperalgesic priming (Moy et al., 2017). These findings support the notion that the stimulation of eIF4E phosphorylation is imperative for the phenotypic changes of sensory neurons, promoting the hyperalgesic state and contributing to the development of chronic pain, and that this likely occurs independently of effects on inflammation (Moy et al., 2018b). Experiments with local administration of cercosporamide also indicate that pro-inflammatory mediators- or tissue injury-induced phosphorylation of eIF4E mediates sensitization of sensory neurons via local mRNA translation.

The advances in translational profiling techniques have provided important insights into the potential mechanisms by which eIF4E phosphorylation regulates neuronal functions. In the brain, eIF4E phosphorylation controls the translation of mRNAs involved in inflammatory responses such as IκBα, a repressor of the transcription factor NF-κB that regulates the expression of the cytokine tumor necrosis factor (TNFα) (Aguilar-Valles et al., 2018). Genome-wide translational profiling of the brain from eIF4E^{S209A} mice revealed that eIF4E phosphorylation controls translation of mRNAs

involved in inflammation (IL-2 and TNFα), organization of the extracellular matrix (*Prg2, Mmp9, Adamts16, Acan*), and the serotonin pathway (*Slc6a4*) (Amorim et al., 2018).

In the DRG, phosphorylation of eIF4E stimulates translation of brain derived neurotropic factor (*Bdnf*) mRNA. eIF4E^{S209A} mice show reduced protein levels of BDNF under baseline conditions and fail to translate *Bdnf* mRNA to protein in response to pro-inflammatory cytokines despite an increase in *Bdnf* mRNA levels (Moy et al., 2018a). BDNF is a key molecule mediating pain plasticity and identification of MNK/eIF4E signaling as a central regulator of *Bdnf* translation has important therapeutic implications. Cell-type specific translational profiling of nociceptors (using translating ribosome affinity purification (TRAP) approach) (Heiman et al., 2014) in a mouse model of chemotherapy-induced neuropathic pain revealed that MNK-eIF4E signaling controls translation of *RagA* mRNA, a key regulator of mTORC1 (Megat et al., 2018). This finding suggests crosstalk between ERK/MNK/eIF4E and mTORC1 signaling pathways in promoting pain hypersensitivity in chemotherapy-induced neuropathies.

In addition to phosphorylation, eIF4E in primary sensory neurons is also regulated via mTORC1/4E-BP. IL-6 and NGF activate mTORC1, promote 4E-BP1 phosphorylation, and increase eIF4F complex formation and nascent protein synthesis in cultured sensory neurons (Melemedjian et al., 2010). Intraplantar administration of IL-6 or NGF induced mechanical allodynia, which is blocked by subcutaneous administration of the mTORC1 inhibitor rapamycin, as well as by 4EGI-1, an inhibitor of eIF4F complex formation that disrupts eIF4E and eIF4G interaction. Intraplantar 4EGI-1 also blocked the establishment of the sensitization state in a hyperalgesic priming model in response to IL-6 and NGF injection (Asiedu et al., 2011).

These findings support a model that local activation of mTORC1 stimulates eIF4F complex formation, promoting pain hypersensitivity via axonal mRNA translation. 4E-BP1 is a major isoform involved in regulation of nociception, whereas in the brain 4E-BP2 is the dominant isoform. 4E-BP1 is highly expressed in nociceptors and mice lacking 4E-BP1, but not 4E-BP2, exhibit enhanced mechanical hypersensitivity. Notably, *eif4ebp1* knockout mice show no alterations in thermal sensitivity, suggesting a mechanical-specific effect of eIF4E activation via 4E-BP-dependent mechanisms (Khoutorsky et al., 2015).

A second major downstream effector of mTORC1, p70S6 ribosomal kinase (S6K1 and S6K2) may not play as significant a role in regulation of nociceptive sensitization. Mice lacking S6K1/2 do exhibit increased mechanical pain sensitivity, but normal thermal thresholds, and an inhibitor of S6K1/2 recapitulates this phenotype (Melemedjian et al., 2013). This finding seems paradoxical;

however, further analysis revealed that loss of S6K1/2 function engages a feedback loop that stimulates enhanced ERK phosphorylation, driving mechanical sensitization (Melemedjian et al., 2013). Therefore, it is tempting to speculate that most of the pain inhibitory effects of mTORC1 inhibition are mediated via the suppression of 4E-BP1/eIF4E-dependent protein synthesis. The role of other translation-independent outputs of mTORC1, such as regulation of autophagy, lipogenesis, and mitochondrial function, remain unknown.

eIF4E in regulation of spinal plasticity

The spinal cord integrates peripheral somatosensory inputs to generate, after processing, an output that is conveyed to the brain where the perception of pain ultimately arises. Peripheral injury, disease, and certain drugs can cause an increase in the gain of spinal nociceptive circuits, resulting in disproportional amplification of somatosensory inputs, and therefore increased pain. These maladaptive plastic changes in the spinal cord, frequently referred to as central sensitization, significantly contribute to the development of pathological pain states. Central sensitization leads to a lowered threshold for the induction of pain (allodynia), an increase in the responsiveness to noxious stimuli (hyperalgesia), and an enlargement of the receptive field, resulting in pain sensation from non-injured areas (secondary hyperalgesia).

Long-lasting spinal plasticity critically relies on new protein synthesis to allow alterations in the cellular proteome, and consequently, sensitization of the pro-nociceptive circuits. Numerous studies have demonstrated the activation of ERK and mTORC1 signaling in the spinal cord following peripheral tissue injury, cancer, and opioid treatment (Geranton et al., 2009; Ji et al., 2009; Jiang et al., 2013; Liang et al., 2013; Norsted Gregory et al., 2010; Shih et al., 2012; Xu et al., 2014; Xu et al., 2011; Zhang et al., 2013). Intrathecal delivery of pharmacological inhibitors targeting these pathways efficiently alleviates pathological pain without affecting the baseline mechanical and thermal sensitivity (Ji et al., 2009; Martin et al., 2017; Melemedjian and Khoutorsky, 2015). There is evidence that the beneficial effect of mTORC1 inhibition on pain in the spinal cord is largely mediated via mTORC1/4E-BP1-dependent regulation of eIF4E activity. Pain hypersensitivity, produced by intrathecal injection of epiregulin (EREG), an endogenous agonist of the epidermal growth factor receptor (EGFR), upstream of mTORC1, is blocked by intrathecal injection of 4EGI-1 (Martin et al., 2017). Moreover, specific deletion of 4E-BP1 in the dorsal horn of the spinal cord causes mechanical hypersensitivity (Khoutorsky et al., 2015). Mice lacking 4E-BP1 show increased excitatory and inhibitory synaptic transmission in lamina II neurons as well as enhanced potentiation of spinal

excitatory field potentials following sciatic nerve stimulation. Taken together, these results indicate that enhanced eIF4F complex formation in the spinal cord promotes spinal plasticity and contributes to the development of central sensitization.

Therapeutic approaches to target eIF4E-dependent mechanisms to alleviate pain

Several lines of evidence suggest that targeting eIF4E is a potentially promising therapeutic strategy to inhibit aberrant pain plasticity. First, due to low expression levels, eIF4E's activity is a rate-limiting factor for translation initiation and a central node of regulation. eIF4E integrates signals from two major signaling pathways, ERK and mTORC1, both of which have important functions in the development of pain. Second, eIF4E does not strongly affect general translation, but mainly regulates the translation of a subset of mRNAs involved in cell growth, proliferation, immune responses, and neuronal plasticity. Mice with partial reduction of eIF4E protein levels (e.g. expressing inducible eIF4E short hairpin RNAs or eIF4E heterozygous mice) show no developmental abnormalities or changes in survival rate or body weight (Lin et al., 2012; Truitt et al., 2015). Third, whereas acute inhibition of mTORC1 is effective in alleviating pain, long-term mTORC1 inhibition leads to the hyperactivation of ERK via a mTORC1-S6K1-IRS1 negative feedback loop (Melemedjian et al., 2013; Veilleux et al., 2010). Since ERK is a well-known sensitizer of neurons involved in pain transmission, both in the periphery and the spinal cord, chronic mTORC1 inhibition leads to mechanical hypersensitivity and pain. Thus, long-term treatment with compounds targeting mTORC1 is unlikely to be clinically applicable. Conversely, chronic inhibition of eIF4E does not activate these compensatory mechanisms. Mice lacking eIF4E phosphorylation do not exhibit alterations in pain sensation at baseline (Furic et al., 2010, Gkogkas et al., 2014; Moy et al., 2017), but show reduced nociceptive plasticity in response to pro-inflammatory and nerve injury stimuli (Moy et al., 2017). Finally, compelling preclinical studies have demonstrated beneficial effects of pharmacologically targeting eIF4E in alleviating persistent pain using 4EGI-1, an inhibitor of eIF4 complex formation or cercosporamide, an inhibitor of MNK. Efforts to develop and test new translation inhibitors are fuelled by their potential use for treatment of cancer (Stumpf and Ruggero, 2011), malaria (Baragana et al., 2015), and bacterial infection (Bhat et al., 2015). Here, we overview the existing and newly developed pharmacological approaches to target eIF4E-dependent translation.

MNK inhibitors

CGP57380 and cercosporamide are two small molecule inhibitors targeting MNK1 and MNK2 (Bhat et al., 2015). Cercosporamide, extracted from the fungus *Cercosporidium henningsii,* is an antifungal agent and a phytotoxin. It has antiproliferative and proapoptotic activities in cancer cells in preclinical animal models of lung and colon carcinomas (Konicek et al., 2011). It readily crosses the blood-brain barrier (BBB) and efficiently reduces p-eIF4E in the brain after peripheral administration (Gkogkas et al., 2013). However, both CGP57380 and cercosporamide have been shown to exhibit off-target effects (Bain et al., 2007; Bhat et al., 2015). More specific MNK inhibitors have been recently developed. eFT508 is a new generation Mnk1/2 inhibitor, which is potent, selective and orally bioavailable (Dreas et al., 2017). Its efficacy has been assessed in preclinical models of diffuse large B-cell lymphoma, and it causes a dose dependent decrease in eIF4E-phosphorylation (Reich et al., 2018). eFT508 is now in phase II clinical trial for the treatment of colorectal cancer. Recent study showed that eFT508 efficiently reduces eIF4E phosphorylation in DRG without affecting other major signaling pathways (ERK, 4E-BP and AKT) and general translation (Megat et al., 2018). eFT508 also alleviated paclitaxel-induced mechanical and thermal sensitivity, supporting its further testing in other chronic pain conditions. BAY 1143269 is another potent, and selective orally administered MNK1 inhibitor (Santag et al., 2017). Additional MNK inhibitors include: 5-(2-(phenylamino)pyrimidin-4-yl)thiazol-2(3*H*)-one derivatives (Diab et al., 2014), resorcylic acid lactone analogues (Xu et al., 2013), and retinoic acid metabolism blocking agents (RAMBAs) (Ramalingam et al., 2014). These compounds need to be better characterized in both *in vitro* and *in vivo* studies.

Inhibitors of eIF4F complex

Three inhibitors disrupting eIF4G:eIF4E interaction have been described: 4EGI-1 (Moerke et al., 2007), 4E1RCat, and 4E2RCat (Cencic et al., 2011). 4EGI-1 is a small molecule, which binds eIF4E at the site distal to the eIF4G-binding epitope, causing localized conformational changes and dissociation of eIF4G from eIF4E (Papadopoulos et al., 2014). 4EGI-1 also impairs mitochondrial functions (Yang et al., 2015). 4EGI-1 has been used in studies examining the role of eIF4F complex in memory (Hoeffer et al., 2011) and autism (Gkogkas et al., 2013; Santini et al., 2013), where it was delivered directly to the brain (intracerebroventricular injection) as it does not readily penetrate the BBB. Rigidified analogues of 4EGI-1 have been developed, showing improved potency in inhibition of eIF4E/eIF4G interaction (Mahalingam et al., 2014)

4E1RCat, and 4E2RCat block the interaction of eIF4E with both eIF4G and 4E-BP1, and thereby prevent the eIF4F complex formation (Cencic et al., 2011). These compounds have not been used yet in the nervous system *in vivo*. Antisense oligonucleotide (ASO) targeting eIF4E (LY2275796) with improved tissue stability and nuclease resistance has been developed (Graff et al., 2007). Since eIF4E is overexpressed in many human cancers (by ~3–10-fold) (Bhat et al., 2015), LY2275796 has been tested as an anti-cancer treatment. Administration of LY2275796 to patients resulted in a reduction of eIF4E mRNA and protein levels in tumor cells but caused dose-dependent toxicity (Hong et al., 2011). The antiviral drug ribavirin has been proposed to mimic the mRNA "cap" to inhibit eIF4E/mRNA interaction (Kentsis et al., 2004). This notion was later disputed, and ribavirin's biological effects were attributed to translation-independent activities (Westman et al., 2005; Yan et al., 2005).

eIF4A inhibitors

eIF4A helicase activity is critically required for the eIF4F complex formation and unwinding of the 5' UTR to allow scanning process. Therefore, targeting eIF4A might be an additional approach to target eIF4F-dependent translation initiation, particularly for mRNAs with highly structured 5'UTRs. Pateamine A, hippuristanol, and silvestrol are the currently known inhibitors of eIF4A, out of which only pateamine A is known to cause irreversible inhibition (Pelletier et al., 2015). Hippuristanol is a member of polyoxygenated steroids family, and it blocks the helicase activity of eIF4A by binding to the C-terminal of eIF4A and imposing allosteric hindrance, thus preventing eIF4A to bind RNA (Sun et al., 2014). On the other hand, pateamine A and silvestrol increase the ATPase, RNA-binding and helicase activity of eIF4A, but in a non-sequence specific manner, thus preventing eIF4A from participating in the formation of eIF4 complex (Bordeleau et al., 2006; Cencic et al., 2009). Out of these three eIF4A inhibitors, silvestrol has been most widely assessed in *in vivo* preclinical cancer models, owing to its high potency, bioavailability, and relatively low toxicity (Raynaud et al., 2007). The role of eIF4A inhibitors in pain has yet to be examined.

Conclusions

A central role of eIF4E-dependent translational control in mediating maladaptive nociceptive plasticity provides an opportunity to develop new therapeutics to prevent the development of the hypersensitivity state or even reverse established pain states by weakening ongoing activity-dependent plasticity. Existing compounds targeting eIF4E (cercosporamide and 4EGI-1) lack

specificity and have poor solubility and BBB permeability (4EGI-1). Therefore, validation of other existing inhibitors for *in vivo* applications and development of more specific and efficacious inhibitors are required. Another important research direction is uncovering cell type-specific translational landscapes (for example using TRAP) in different pain conditions. This work might reveal mRNAs whose aberrant translation drives the pain phenotype and allow targeting these transcripts or the encoded proteins to reverse the hypersensitivity. It is, however, conceivable that a complex pattern of translation drives the hypersensitivity, involving a combinatory effect of several translationally activated and repressed mRNAs. In this scenario, targeting the upstream regulatory mechanisms, such as formation of eIF4F complex, might be a more feasible therapeutic approach. Combination of diverse inhibition strategies could be beneficial to achieve long-lasting effects on pain without triggering compensatory mechanisms.

In summary, a growing recognition of the importance of the eIF4E-dependent translational control in regulation of cellular functions in general and neuronal plasticity in particular, have substantially accelerated studies in the field of pain and advanced our knowledge of how eIF4E-dependent translational dysregulation causes maladaptive plasticity and contributes to the sensitization of the pain pathway. Identification of new molecular targets and pharmacological compounds to target these mechanisms might constitute a basis for next-generation pain therapeutics.

Figure 1. Schematic illustration of the major signaling pathways regulating eIF4E activity and translation initiation

The cap binding ability of eIF4E makes it a central regulator of translation. A critical step in the translation initiation process is the binding of eIF4E to the mRNA cap. eIF4E mediates the formation of the eIF4F complex on the mRNA cap structure (a 7mGp bound to the first nucleotide). eIF4F complex, in addition to eIF4E, consists of eIF4G (scaffolding protein) and eIF4A (helicase). Successful formation of eIF4F complex on the mRNA cap further promotes the recruitment of the pre-initiation complex (PIC), followed by 5'UTR scanning to reach the start codon AUG and joining of 60S ribosomal subunit. This event marks the completion of translation initiation.

eIF4E is a downstream effector of both mTORC1 (via 4E-BP-dependent repression) and ERK (via eIF4E phosphorylation by MNK 1/2). The activities of mTORC1 and ERK signalling pathways are in turn modulated by a multitude of external

(tyrosine receptor kinase A (trkA) and trkB, receptors from the insulin receptor family (IR, IGF1R, EGFR), and metabotropic glutamate and NMDA receptors) and internal cues (status of

cellular energy (via AMPK), oxygen levels (via activation of AMPK and REDD1 [Regulated in DNA damage and development 1], and DNA damage [via the induction of p53 target genes]). Various inhibitors of cap dependent translation initiation have been identified. 4EGI-1 inhibits eIF4E's interaction with eIF4G, thus inhibiting the formation of eIF4F complex. Cercosporamide blocks MNK phosphorylation, which in turn prevents phosphorylation of eIF4E. Inhibitors of eIF4A have also been identified which function by either blocking its helicase activity (hippuristanol) or by preventing its participation in the eIF4F complex (pateamine A, and silvestrol).

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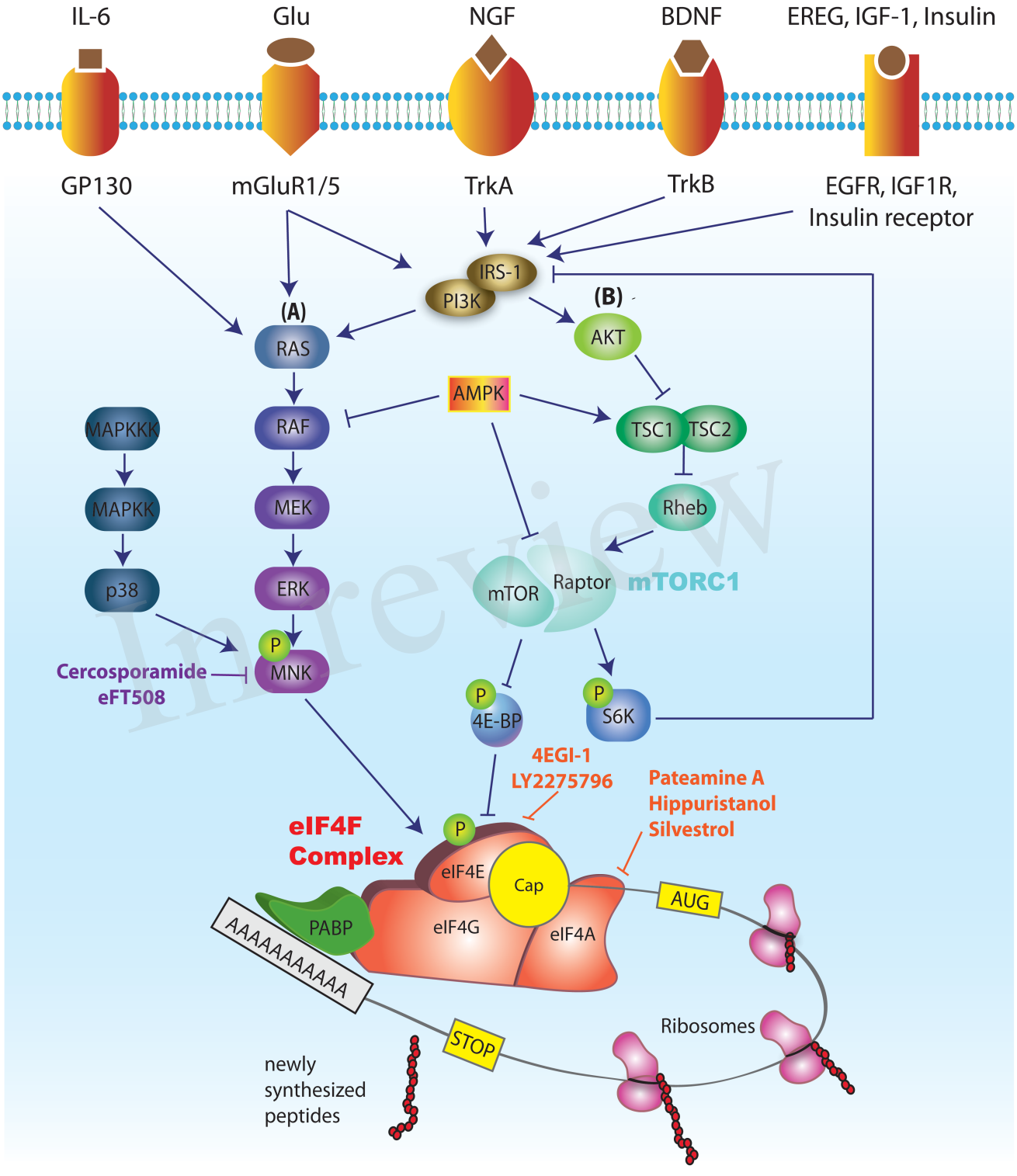
Figure 1.JPEG

Figure 1. Overview of mechanisms of translation initiation



Manuscript 2: Translational profiling of dorsal root ganglia and spinal cord in a mouse model of neuropathic pain

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Original Research

Translational profiling of dorsal root ganglia and spinal cord in a mouse model of neuropathic pain

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A B S T R A C T

Acute pain serves as a protective mechanism, guiding the organism away from actual or potential tissue injury. In contrast, chronic pain is a debilitating condition without any obvious physiological function. The transition to, and the maintenance of chronic pain require new gene expression to support biochemical and structural changes within the pain pathway. The regulation of gene expression at the level of mRNA translation has emerged as an important step in the control of protein expression in the cell. Recent studies show that signaling pathways upstream of mRNA translation, such as mTORC1 and ERK, are upregulated in chronic pain conditions, and their inhibition effectively alleviates pain in several animal models. Despite this progress, mRNAs whose translation is altered in chronic pain conditions remain largely unknown. Here, we performed genome-wide translational profiling of dorsal root ganglion (DRG) and spinal cord dorsal horn tissues in a mouse model of neuropathic pain, spared nerve injury (SNI), using the ribosome profiling technique. We identified distinct subsets of mRNAs that are differentially translated in response to nerve injury in both tissues. We discovered key converging upstream regulators and pathways linked to mRNA translational control and neuropathic pain. Our data are crucial for the understanding of mechanisms by which mRNA translation promotes persistent hypersensitivity after nerve injury.

Introduction

Chronic pain debilitates over twenty percent of the population worldwide, and is the leading cause of long-term disability in humans (Souza et al., 2017). The most common chronic pain conditions include headache, low back pain, cancer pain, arthritis pain, and neuropathic pain, which can result from damage to peripheral nerves or to the central nervous system itself. In addition to dysfunction of the somatosensory system, chronic pain has multi-dimensional effects on the emotional and mental health of patients that can lead to depression, anxiety, sleep disorders, low self-esteem, and impairments in attention and memory (Duenas et al., 2016). Pain management depends largely on antidepressants, anticonvulsants, and opioids; however, pain relief is incomplete under most circumstances and is achieved only in a fraction of patients (Foley, 2003; Kalso et al., 2004; Højsted and Sjøgren, 2007; Moulin et al., 2007; Ballantyne and Shin, 2008).

The inadequate management of chronic pain is a consequence of our incomplete understanding of the mechanisms underlying the induction and maintenance of pain states, leading to treatments that only target symptomatology without addressing the etiology of the disease. Sensitization of nociceptive circuits, both in the central and peripheral nervous systems, leads to mechanical hypersensitivity (allodynia), which is a hallmark of many chronic pain conditions. This sensitization is supported by the expression of new genes, which are required for the biochemical and structural reorganization of the pain pathway. With advancements in microarray and sequencing technologies,

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transcriptional changes associated with chronic pain have been extensively studied, providing important insights into the transcriptional landscape and identification of a subset of genes with differential expression in various chronic pain conditions (LaCroix-Fralish et al., 2011; Hu et al., 2016; Ray et al., 2017).

Cellular abundance of proteins is highly controlled at the level of mRNA translation (Schwanhausser et al., 2011). Translational control is a powerful modulator of protein levels by regulating the efficiency by which mRNA is converted to proteins.

Translation control involves a variety of mechanisms, including regulation of the vast translational machinery and modulation of the signaling pathways upstream of translation. The extracellular signal-regulated kinase (ERK) pathway and mechanistic target of rapamycin complex 1 (mTORC1) kinase and its downstream effectors have been extensively studied to understand the contribution of translation in the development of hypersensitivity (Khoutorsky and Price, 2017). Suppressing translation by inhibition of mTORC1 reduces mechanical hypersensitivity associated with inflammation (Price et al., 2007; Gregory et al., 2010; Ferrari et al., 2013) and neuropathic pain (Geranton et al., 2009; Zhang et al., 2013). A recent study described an important role for eukaryotic translation initiation factor 2 (eIF2) in inflammation-induced pain, and identified that phosphorylation of the α subunit of eIF2 (eIF2α) is a key step in controlling noxious heat sensitivity (Khoutorsky et al., 2016). Other studies have established a key role for local translation from pre-existing mRNAs in the modification of axonal/dendritic proteomes to promote the excitability of sensory neurons and induce pain hypersensitivity (Melemedjian et al., 2010; Khoutorsky and Price, 2017; Moy et al., 2017b). Altogether, these studies support an emerging role for translational regulation in the establishment and maintenance of chronic pain.

Neuropathic pain accounts for ~20% of chronic pain cases (Lisi et al., 2015), and arises from damage to the nervous system. This damage can result either from a direct injury to peripheral nerves, spinal cord, or the brain, or be caused by a disorder affecting the somatosensory system such as metabolic stress, autoimmunity, degenerative or chronic inflammation, or from idiopathic origin (Guha and Shamji, 2016). Various rodent assays, mostly involving surgical injury, have been developed to study neuropathic pain (Mogil, 2009). Spared nerve injury (SNI) is a model of sympathetic-independent neuropathic pain with long-term chronicity (Decosterd and Woolf, 2000). SNI typically involves a lesion of the tibial and common peroneal branches of the sciatic nerve, while leaving the sural branch intact (Fig. 1A). This procedure causes severe and persistent (at least 6 months) neuropathic pain in the animal, manifested in the sural territory of the ipsilateral paw as mechanical and cold hypersensitivity (Decosterd and Woolf, 2000).

In this study, we have adopted a genome-wide approach to identify mRNAs that are either significantly up- or down-regulated at the level of translation after SNI. For this purpose, we implemented a high throughput RNA sequencing-based methodology, called ribosome profiling, in parallel with measurements of mRNA levels. We analyzed lysates from DRGs and spinal cord (SC) dorsal horn tissues from mice subjected to SNI and mapped the translational and transcriptional landscapes. In addition, we carried out meta-gene analysis by Ingenuity Pathway Analysis (IPA) and identified commonly affected pathways.

Results

To understand the global pattern of translational control, and identify which mRNAs are differentially regulated following nerve injury, we performed genome-wide translational profiling of DRG and dorsal horn of the spinal cord in the SNI assay of neuropathic pain. For the analysis, we collected L3 to L5 DRG and the corresponding lumbar segment of the spinal cord (Rigaud et al., 2008) 30 days post-SNI. The dorsal half of the spinal cord was dissected and used for the analysis as sensory processing is restricted to this area (illustrated in a schematic diagram in Fig. 1A). We confirmed that mechanical thresholds, as measured by the von Frey test, were significantly reduced at 30 days after the nerve injury (Fig. 1B). Thus, we reasoned that the 30 day time point was appropriate for tissue collection in order to study the chronic phase of neuropathic pain.

To quantitatively measure *in vivo* genome-wide translational efficiency of mRNAs in DRG and spinal cord, we adopted the ribosome profiling methodology (Ingolia et al., 2012). Ribosome/RNA complexes were isolated from cell lysates and digested with an endoribonuclease (RNase I), which degrades all RNAs that are not protected by bound ribosomes (Fig. 1C). This generated ~30 nucleotide long fragments of ribosome-protected mRNAs, or “footprints”. These footprints were reverse-transcribed and cloned into a cDNA library for RNA sequencing (RNA-seq) (Fig. 1D). Libraries were then sequenced to measure the number of footprints per mRNA for the entire genome. Supplementary Table 1 shows the total number of sequenced reads and number of filtered reads (reads uniquely mapped to non-ribosomal region of reference genome DNA) for each sample. In parallel, transcriptome analysis (mRNA-seq) was performed in parallel to account for changes in mRNA abundance. Thus, using the number of footprints (as a proxy for translation) for a given mRNA, normalized to its abundance (as a proxy for transcription), we can calculate translational efficiency (TE) for each mRNA, which has been previously shown to be a strong predictor of protein abundance (Ingolia et al., 2009).

Footprints had a narrow size distribution, with a peak corresponding to 28–32 nucleotides, whereas the length of sequencing reads from randomly lysed mRNA fragments as a result of alkaline fragmentation had a broad size distribution ranging from 28 to 45 nucleotides (Fig. 2A) (Ingolia et al., 2009). mRNA-Seq reads were equally distributed between the three possible frames for the start codon, whereas footprint reads displayed a bias for the canonical Frame 1 (Fig. 2B). Likewise, because the size of the protected ribosomal footprint is ~28 nt (Fig. 2C), extending from −12 to +15 (0 being the start codon at the P site of the ribosome), reads around the start codon, stop codon and within the coding sequence follow the periodicity of mRNA codons (3 nucleotides) (Ingolia et al., 2009) (Fig. 2C). As expected, the footprints were largely restricted to the coding sequence (CDS), while the mRNA fragment reads were evenly distributed throughout the 5′ untranslated region (5′ UTR), CDS and the 3 UTR (Fig. 2D). The three-nucleotide periodicity of the ribosome footprints (RFPs) (Fig. 2D), as well as the significantly higher number of RFP reads within the coding region, as compared to UTRs, demonstrates the specificity of the recovered ribosome footprints.

Footprints and mRNA densities were computed in units of reads per kb per million (RPKM) to normalize for gene length and total reads per sequencing run. All conditions demonstrated a strong correlation between biological replicates (Figs. 3A and 4A – R2; Pearson Correlation). Based on changes in translational efficiency, 74 mRNAs were upregulated (fold change > 1.5, $p < 0.05$) in the DRG of SNI mice as compared to sham animals, while translation was downregulated ($0.5 >$ fold change, $p < 0.05$) for 31 mRNAs (Fig. 3B left, for the complete list of genes see Supplementary Table 2). mRNA-seq analysis revealed that 144 mRNA were transcriptionally upregulated and 33 were downregulated in DRG after SNI (Fig. 4B right, for the complete list of genes see Supplementary Table 2). In the spinal cord, 103 mRNAs were translationally upregulated and 27 were downregulated (Fig. 4B left, for the complete list of genes see Supplementary Table 2), whereas 25 mRNAs were transcriptionally upregulated and 7 were downregulated after SNI (Fig. 3B right, for the complete list of genes see Supplementary Table 2).

Ingenuity Pathway Analysis (IPA) of differentially regulated genes (both translationally and transcriptionally) in SNI revealed top cellular functions and subcellular localizations in the DRG (Fig. 3C) and spinal cord (Fig. 4C). We also used the IPA network analysis of differentially regulated genes to generate a node graph of potential regulatory networks based on the ribosome profiling data for DRG (Fig. 5) and spinal

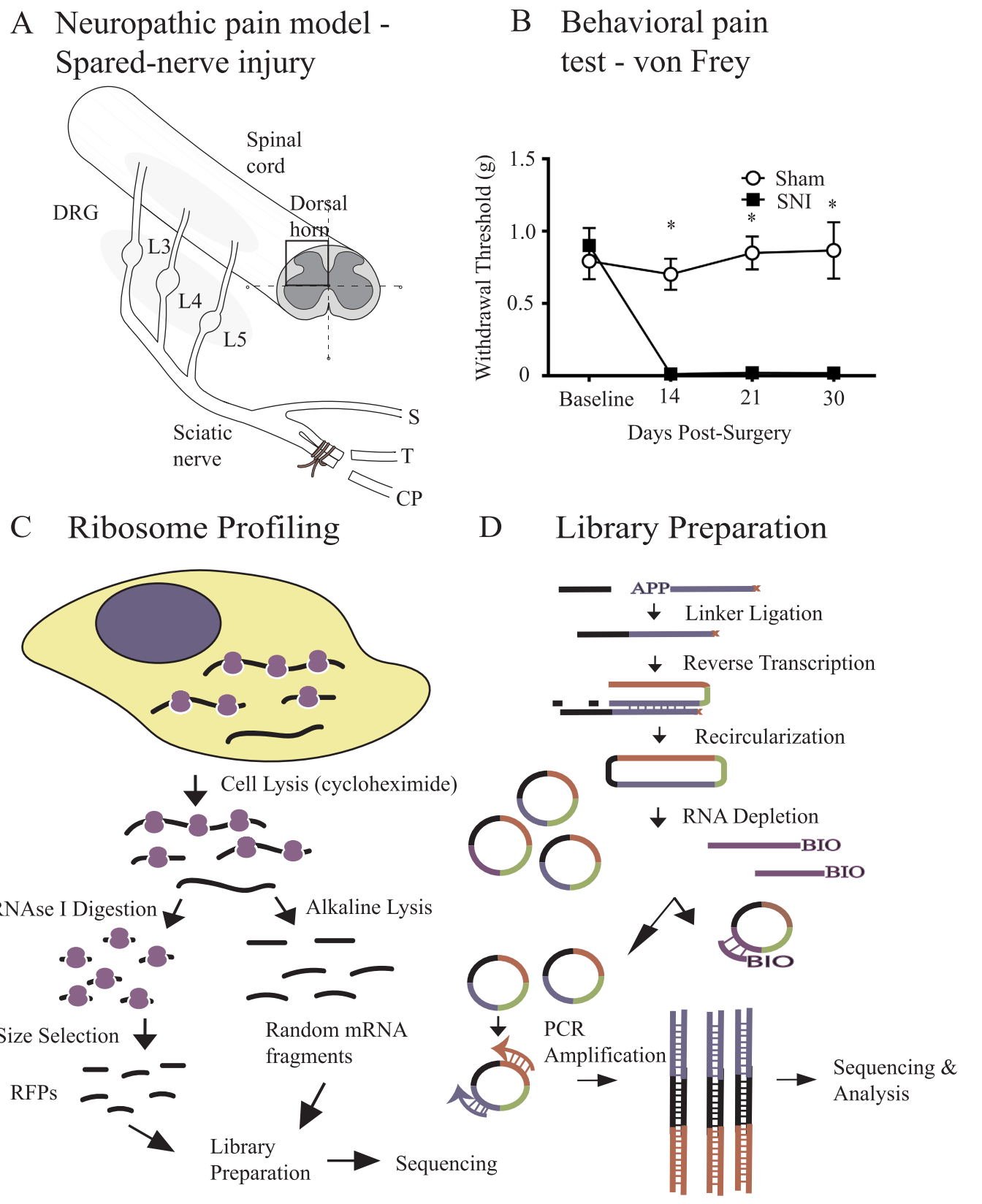

Fig. 1. Analysis of gene-expression in the mouse model of neuropathic pain using ribosome profiling and RNA sequencing. (A) A schematic illustration of the SNI assay of neuropathic pain. L3, L4, L5: Lumbar 3,4 and5 level DRG, respectively; S: Sural branch, T: Tibial branch and CP: Common peroneal branch. (B) Paw-withdrawal threshold (g) measured for SNI and sham-operated animals at baseline and 14, 21 and 30 days post-surgery. Symbols represent mean ± SEM; $n = 8$/condition. $^{*}p < 0.05$ compared to other condition. (C) Experimental flowchart of ribosome profiling technique. (D) Library generation steps of ribosome profiling.

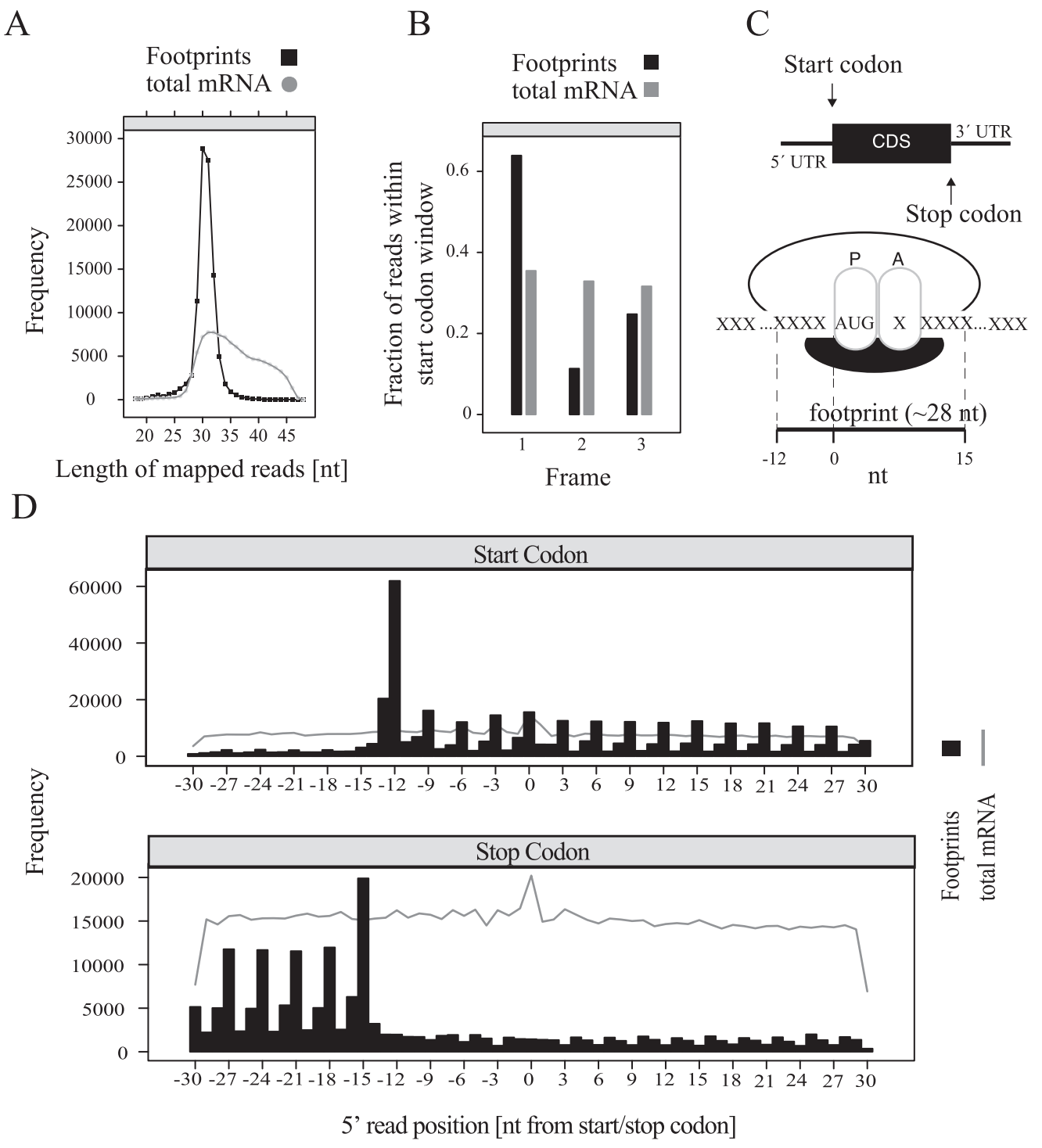

Fig. 2. Quality control of ribosome profiling. (A) Frequency of mapped reads from RNA–seq data corresponding to ribosomal footprints (~28–32 nt) or total RNA fragments following alkaline fragmentation (~28–45 nt). (B) Fraction of reads within start codon window for each one of the three possible frames for footprints and total mRNA. (C) Top: Depiction of a eukaryotic mRNA with 5′ and 3′ UTRs, CDS (coding sequence) and start and stop codons. Bottom: Depiction of the P and A sites on a translating ribosome showing the size and orientation of, and the area occupied by, a typical eukaryotic ribosomal footprint. The start codon AUG is shown; X: any three nucleotides corresponding to a codon. (D) Frequency of footprints and mRNA reads with respect to position from the start (top) and stop (bottom) codons.

cord (Supplementary Fig. 1).

Discussion

Translational control of gene expression has emerged as a prominent mechanism in the regulation of gene expression in pathological pain states (Price and Geranton, 2009; Melemedjian and Khoutorsky, 2015; Khoutorsky and Price, 2018). Indeed, signaling upstream to the translation machinery is upregulated in several chronic pain conditions (Price et al., 2007; Jimenez-Diaz et al., 2008; Geranton et al., 2009; Ji et al., 2009; Khoutorsky and Price, 2017). Moreover, an inhibition of mRNA translation has been shown to effectively alleviate pain in several preclinical assays (Geranton et al., 2009; Asante et al., 2010; Obara et al., 2011). Despite this progress, the repertoire of mRNAs showing

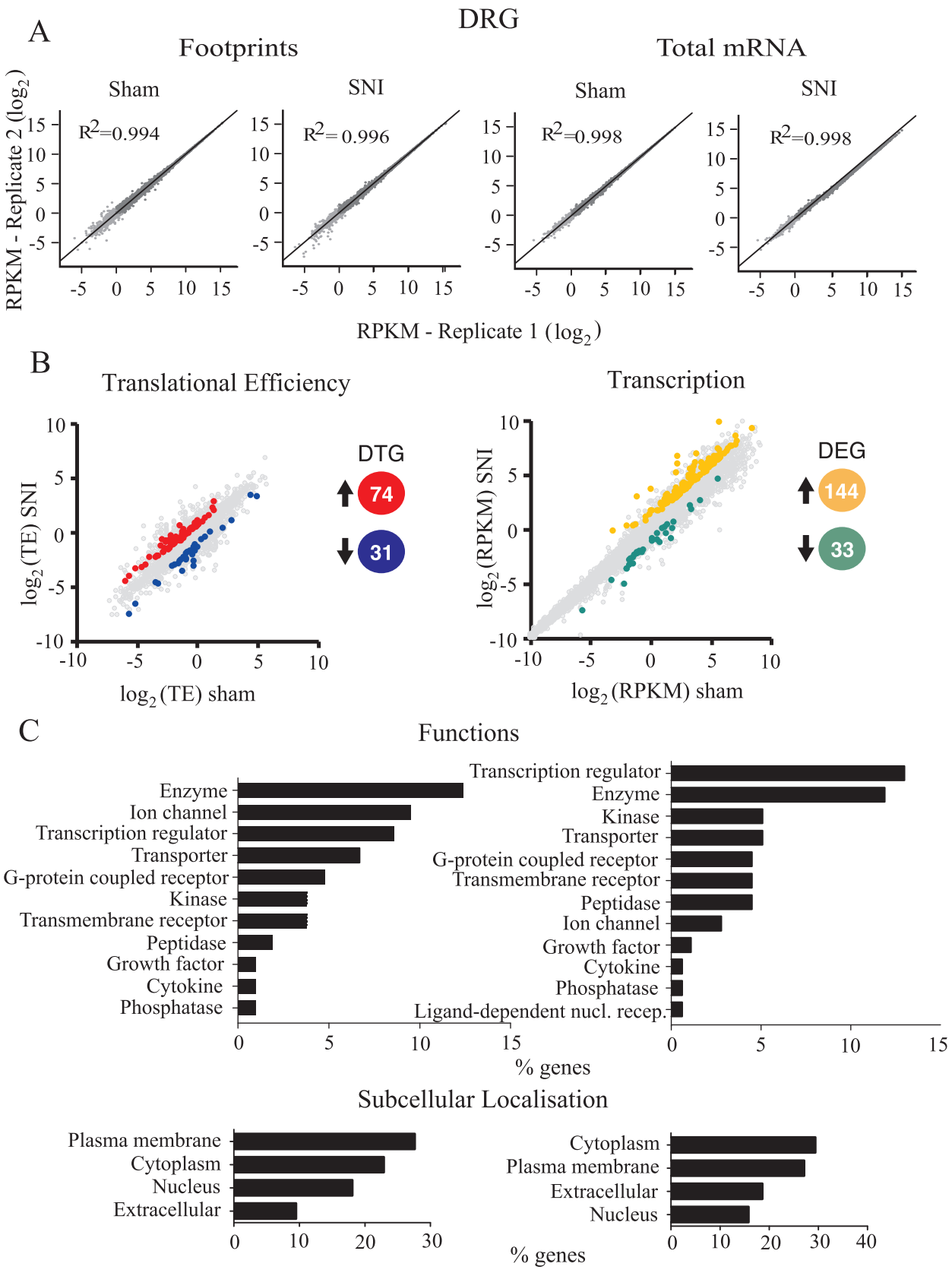

Fig. 3. The DRG translational and transcriptional landscape after SNI. (A) Correlation between replicates for footprint (left) and total mRNA (right) are shown for sham or SNI groups in DRG. (B) Changes (log_2) in translational efficiency (left) and transcription (right) and differentially translated or transcribed genes (upregulated and downregulated; $p < 0.05$ and $0.5 >$ fold change > 1.5) between sham- and SNI-treated mice are depicted from ribosome profiling analysis in tissue from DRG. The number of differentially translated genes (DTG) or differentially expressed genes (DEG) is depicted in different colours (red/blue, orange/green). Spearman's rank correlation coefficient (R^2) is shown for log_2 comparisons. (C) Representative functional analysis characteristics using IPA of differentially regulated genes at the level of translation (left) and transcription (right) in DRG, 30 days post-SNI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

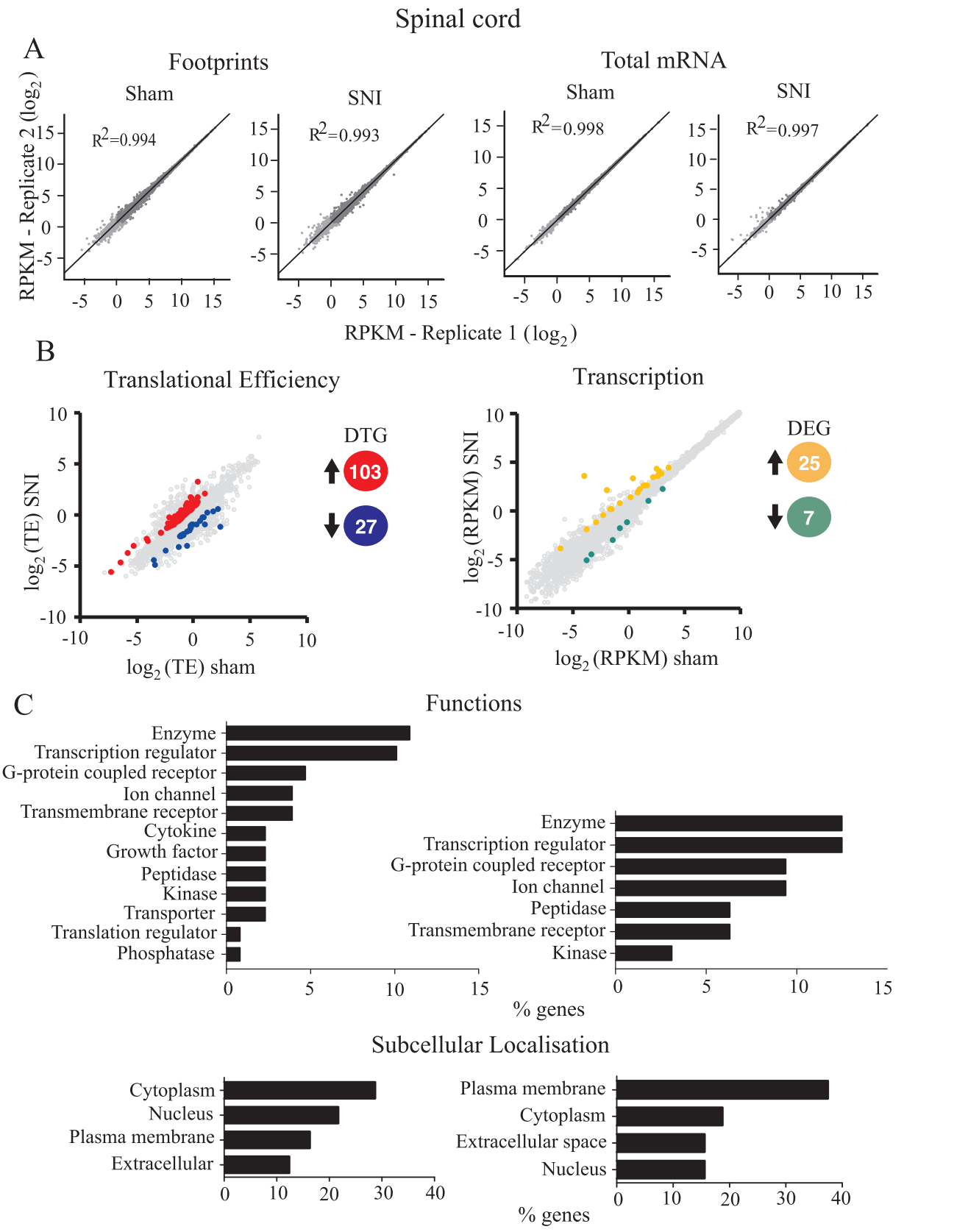

Fig. 4. The dorsal horn of the spinal cord translational and transcriptional landscape after SNI. (A) Correlation between replicates for footprint (left) and total mRNA (right) are shown for sham or SNI groups for spinal cord. Spearman's rank correlation coefficient (R^2) is shown for $\log_2$ comparisons. (B) Changes ($\log_2$) in translational efficiency (left) and transcription (right) and differentially translated or transcribed genes (upregulated and downregulated; $p < 0.05$ and $0.5 >$ fold change > 1.5) between sham and SNI treated animals are depicted from ribosome profiling analysis in spinal cord. The number of differentially translated genes (DTG) or differentially expressed genes (DEG) is depicted with different colors (red/blue, orange/green). (C) Representative functional analysis characteristics using IPA of differentially regulated genes at the level of translation (left) and transcription (right) are shown for sham or SNI groups in spinal cord, 30 days post-SNI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

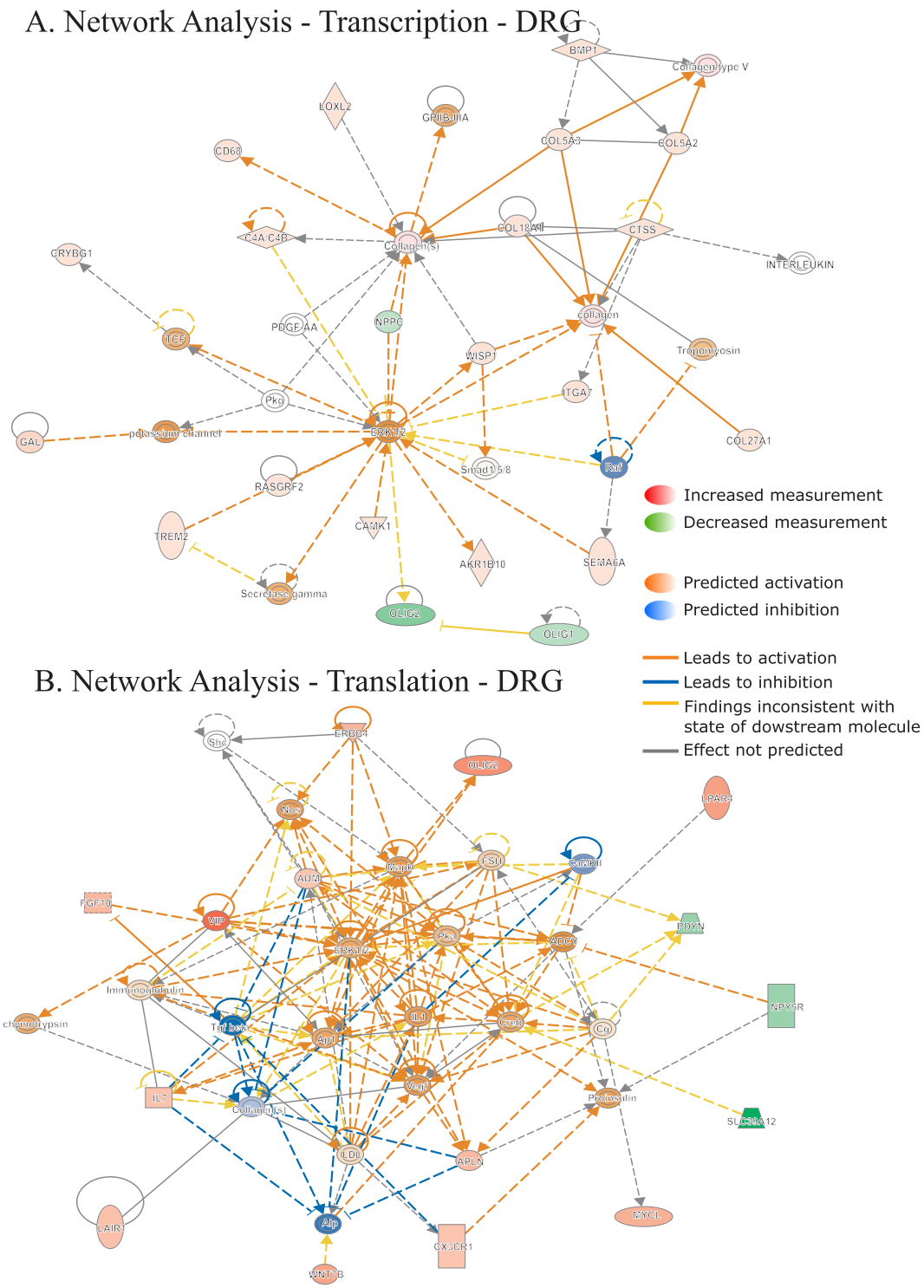

Fig. 5. Network analysis generated by IPA of differentially transcribed and translated mRNAs in DRG 30 days post-SNI. Red: increased measurement; green: decreased measurement; orange: predicted activation; blue: predicted inhibition; yellow: findings inconsistent with state of downstream molecule; grey: effect not predicted; solid line: direct interaction; dashed line: indirect interaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

altered translation in pain conditions remains largely unknown. Our study provides the first genome-wide translational profiling of DRG and spinal cord tissues in a mouse model of neuropathic pain. We identified 74 mRNAs in DRG and 103 mRNAs in the spinal cord whose translation is increased 30 days following SNI, and 31 mRNAs in DRG and 27 mRNAs in the spinal cord with decreased translation. The higher number of upregulated versus downregulated mRNAs in DRG after SNI is consistent with previous studies showing increased signaling upstream of translation following nerve injury in DRG (Obata et al., 2004; Price et al., 2007; Khoutorsky et al., 2016; Moy et al., 2017a) and increased rates of translation in sensory neurons in response to pronociceptive stimulation (Melemedjian et al., 2010). The parallel analysis of changes in mRNA levels and their translational efficiency demonstrates that changes in these processes occur in the opposite direction for

multiple mRNAs, suggesting translational buffering (Laurent et al., 2010; McManus et al., 2014) (see Supplementary Table 3). For example, in the DRG, seven genes (*Myh* 7, *Mobp*, 1500009C09Rik, *Sall1*, *Grin2b*, *Olig2* and 3110035E14Rik) are transcriptionally down regulated but translationally upregulated. In the spinal cord, four genes (*Scn4a*, *Htr3b*, *Sprr1a* and *Rtn4rl2*) are transcriptionally upregulated but translationally down regulated, whereas *Tmem54* is transcriptionally downregulated but translationally upregulated. Several genes that have been previously studied in relation to pain show opposite changes in mRNA levels and their translation efficiency (spinal cord: *Scn4a*, *Htr3b*, *Sprr1a*, *Rtn4rl2*, *Tmem54*; DRG: *Myh7*, *Mobp*, 1500009C09Rik, *Sall1*, *Grin2b*, *Olig2* and 3110035E14Rik). For example, *Scn4a* gene codes for the alpha subunit of the voltage-dependent sodium channel, and mutations in this gene have been associated with sodium channel myotonia (Orstavik et al., 2015). *Htr3b* codes for the serotonin-3B receptor. *Htr3b* rs1176744 polymorphisms are proposed to influence and predict the development of chronic pain disorders like chronic myalgia (Louca Jounger et al., 2016). In a transcriptomic analysis of human DRG, *Sprr1a* (small proline-rich protein 1a) was identified as a signature gene associated with pain experienced in sickle cell disease (Paul et al., 2017). Additionally, *Sprr1a* is involved in regeneration (Jing et al., 2012) and its protein levels are elevated in DRG following peripheral nerve injury (Starkey et al., 2009).

We predict that genes showing changes in the same direction in their mRNA levels and TE, such as *Pkd2l1*, *Unc45b*, *Tmem88b* and *Trhr*, will exhibit robust changes in the corresponding protein levels. Polycystic kidney disease protein 2-like 1 (PKD2L1) is a member of the transient receptor potential superfamily which is known to be involved in a number of sensory functions, ranging from detection of light, force, osmolality, temperature, odour, taste, and pain (Hussein et al., 2015). A study identified *Tmem88b* in DRG to be transcriptionally up-regulated following burn injury (Yin et al., 2016). However, the physiological role of *Tmem88b* in sensory neurons and pain remains poorly defined.

To better understand the biological context of the identified genes, we analyzed our datasets using the IPA platform. IPA analysis has categorized the differentially regulated genes in DRG and spinal cord, post-SNI, into functional and subcellular localization categories, identifying several overlapping functions between transcriptionally and translationally regulated genes (Figs. 3C and 4C), including enzyme, transcription regulator, ion channel, and G protein-coupled receptors. Interestingly, the network analysis identified ERK as a central hub of both transcriptionally and translationally controlled genes, depicted by the large number of edges converging and diverging from the node corresponding to ERK (Fig. 5). This finding is in accordance with previous studies establishing the central role of ERK pathway in the development of hypersensitivity associated with both inflammatory and neuropathic pain (Ji et al., 2002; Zhuang et al., 2005). Indeed, in DRG, several vital transcriptional and growth factors, cytokines, and other signaling molecules (i.e., CREB and MAPK) participate in the network by either activating or inhibiting ERK. In response to noxious stimulation, ERK phosphorylates and activates CREB, thus facilitating transcription of CREB-dependent genes, many of which are implicated in pain (Ji et al., 1999). In addition, activation of ERK promotes mRNA translation via mitogen-activated protein kinase interacting kinase (MNK1/2)-dependent phosphorylation of eukaryotic initiation factor 4E (eIF4E), the cap binding protein, which is critical for ribosome recruitment to the mRNA (Waskiewicz et al., 1997; Moy et al., 2017a). This phosphorylation event promotes the excitability of DRG neurons (Moy et al., 2017a) and leads to the enhanced translation of brain-derived neurotrophic factor mRNA in DRG neurons (Moy et al., 2018) which in turn induce translation and transcription of pain-relevant genes. Together, our network analysis provides further evidence for the involvement of ERK in both transcriptional and translational gene networks, supporting the model of feed-forward loops between transcriptional and translational control mechanisms in which the ERK pathway is serving as a central regulatory mechanism.

Changes in transcriptional and translational regulation in the spinal cord could be underrepresented in our analysis, considering that we extracted tissue from the entire dorsal half of the spinal cord, whereas most of the sensory processing is restricted to the dorsal horn area. Since we analyzed lysates prepared form spinal cord and DRG tissues, we most likely detect changes in both neuronal and non-neuronal cellular populations, including infiltrated immune cells. It is also important to note that this study is based on female mice. Since pain-processing mechanisms might differ between sexes (Sorge et al., 2015), similar studies in males, as well in other species, are ultimately required.

In summary, we performed the first translational profiling study of DRG and spinal cord tissues after nerve injury, and identified mRNAs whose translational efficiency is altered in the SNI animal model of neuropathic pain. The IPA analysis revealed altered cellular pathways, including identification of ERK as a key regulator of both translational and transcriptional networks. This information is instrumental for furthering our understanding of the molecular underpinnings of chronic pain.

Materials and methods

Neuropathic pain

All procedures involving mice were carried out in compliance with the Canadian Council on Animal Care guidelines and were pre-approved by the McGill University Animal Care Committee. C57BL/6J female mice, at 8 weeks of age, underwent the bilateral SNI surgical procedure as described previously (Decosterd and Woolf, 2000; Shields et al., 2003) to induce neuropathic pain. Briefly, under 2% isoflurane anesthesia, the lateral surface skin of the thigh was shaved and incised. The biceps femoris muscle was incised to expose the sciatic nerve just below its branching point. The tibial and common peroneal branches were tightly ligated using 7-0 silicone coated silk (Covidien, S-1768K) and a 3–4 mm portion of each of the ligated branches was sectioned and removed distal to the ligation point. Finally, the muscle and the skin incisions were closed using 6-0 Vicryl suture (Ethicon, J489G). During the entire process, great care was taken to leave the sural branch unharmed. The mouse was returned to its home-cage for recovery. Sham animals were used as controls, where the surgical procedure was carried out identically but all three branches of the sciatic nerve were left untouched and unharmed. The animals were sacrificed 30 days post-surgery, and DRG and dorsal horn of the spinal cord samples were extracted. Tissues from 10 animals were pooled per sample and 2 independent replicates were made for each of the four conditions.

Harvesting of DRG and dorsal horn of spinal cord

To collect DRG and dorsal horn of the spinal cord, animals were sacrificed by brief isoflurane anesthesia followed by decapitation. The animal was secured on a bed of dry ice and the spinal cord was exposed and doused with RNA*later* stabilization solution (Ambion, AM7020). Lumbar DRG (level L3–L5) were excised for all animals. Next, the lumbar region of the spinal cord at which the L3–L5 DRG branch into was excised and placed on a bed of dry ice/metal plate and allowed to freeze after which it was cut along the frontal plane to separate the dorsal horn section. The DRG and dorsal horn were collected in non-stick, RNase free microcentrifuge tubes (Ambion, AM12450), immediately snap-frozen in liquid nitrogen, and stored at −80 °C until further processing.

Ribosomal profiling

Tissue homogenization and cell lysis

Flash frozen DRG and dorsal horn tissue was lysed in ice-cold cell lysis buffer (1% Polysome buffer (20 mM TrisCl (pH 7.4), 150 mM NaCl,

5 mM $MgCl_2$, 1 mM DTT and 100 μg/ml cyclohexamide, 8% glycerol), 1% Triton X-100 and 25 U/ml Turbo DNase I) in a glass homogenizer system. The total lysate was divided into two fractions. A fraction containing at least 150 μg of total RNA was reserved for ribosome footprinting (RFP fraction) and the remaining (at least 100 μg) was processed for mRNA-Seq.

Obtaining ribosome footprints (RFPs)

Ribosome footprinting was carried out as previously described (Ingolia et al., 2012) with minor modifications. Briefly the RFP fraction was subjected to RNase I treatment (Ambion, AM2295) at a concentration of 2 U/μg of RNA, at 4 °C for 45 min with end over end mixing and quenched for 5 min by adding 4U SUPERaseIn (Ambion, AM2696) for every 5 U of RNase I. Monosomes were pelleted by ultracentrifugation (Beckman Coulter, Optima MAX-UP)) through a 34% sucrose cushion (in polysome buffer) at 70,000 RPM for 3 h at 4 °C. The resulting RNA pellet was resuspended in 600 μl Tris Cl (pH 7) and RNA was extracted by double acid Phenol and one Chloroform extraction, precipitated by 1 vol Isopropanol and 1/9 vol 3 M NaOAc (pH%.5) and 2 μl Glycoblue (15 mg/mg stock, Invitrogen, AM9515) at −80 °C overnight followed by centrifugation at 20,000*g* at 4 °C for 30 min. Purified RNA was resolved on a 15% polyacrylamide urea gel (Invitrogen, EC6885BOX) and bands corresponding to 28–32 nucleotides, containing the desired ribosome footprints (RFPs), was excised and extracted for RNA using Costar Spin-X column (Sigma, CLS8160).

Random RNA fragmentation of cytoplasmic RNA

Poly (A)+ mRNAs were purified from 100 μg of cytoplasmic RNA, using magnetic oligo-dT DynaBeads. The purified RNA was then subjected to alkaline fragmentation by treating it with an equal volume of 2× alkaline fragmentation solution (2 mM EDTA, 10 mM Na_2CO_3, 90 mM $NaHCO_3$, pH 9.2) for 20 min at 95 °C. The reaction was stopped by addition of the precipitation solution (300 mM NaOAc pH 5.5 and 2 μl GlycoBlue), followed by Isopropanol. Fragmented mRNAs were size-selected on a denaturing 10% polyacrylamide-urea gel and the bands corresponding to 30–50 nucleotides were excised, eluted, and precipitated with Isopropanol.

Library preparation for sequencing

Fragmented mRNA and RFPs were subjected to PNK dephosphorylation and 10 pmol of the dephosphorylated RNA fragments were used for ligation to a pre-adenylated and 3′-blocked linker, followed by separation on a 10% polyacrylamide urea gel. Linker ligated bands were excised and extracted for RNA, which was reverse transcribed using oNTI223 adapter (Illumina) and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instruction manual. The resulting cDNA was purified by size selection on a 10% polyacrylamide Tris/Borate/EDTA-urea (TBE-urea) gel. The cDNA was then circularized using CircLigase (Epicentre, CL4111K). Products arising from ribosomal sequences were depleted using biotinylated rDNA complementary oligos (Ingolia et al., 2012) and MyOne Streptavidin C1 dynabeads. The remaining products were amplified by PCR (11 cycles) using indexed primers, size-selected on a 8% polyacrylamide gel and purified. At these intermediate steps, bands in the gels that were very close to the fragment size + adapter were excised and purified. The resulting cDNA library samples were analyzed on an Agilent Bioanalyzer High Sensitivity DNA chip to confirm the size and concentration and then sequenced using the non Strand–Specific, single-read 50 (SR50) on the Illumina HiSeq 2500 Sequencing platform according to the manufacturer's instructions, with sequencing primer oNTI202 (5CGACAGGTTCAGAGTTCTACAGTCCGACGATC).

Bioinformatics analysis of ribosomal footprinting data

Raw sequencing data were de-multiplexed by the sequencing facility (Genome Quebec). Sequences were analyzed using a custom developed bioinformatics pipeline adapted from Ingolia et al. (2012) as described in Silva Amorim et al. (2018). In brief, reads were adapter-trimmed, contaminant sequences (rRNA, tRNA) were removed using bowtie with optimised parameters for ribosome profiling as per Ingolia et al. (2009) and reads were aligned to a reference mouse genome (GRCm38.p5). Since the RNA-seq and ribosome footprint assays were paired for each sample of the four conditions (DRG_SNI; spinal cord_SNI; DRG_Sham and spinal cord_Sham), the RNA-seq data were used to normalize the footprint numbers to derive the Translation efficiency (TE).

Reads Per Kilobase of transcript per Million mapped reads (RPKM) was calculated using an in-house R-script described in Ingolia et al. (2009) for each transcript. TE for each transcript was calculated by dividing RPKM values of the RFP libraries by RPKM values of the total mRNA libraries for each of the two sample condition replicates and then averaged. Z-score, P-values and FDR were calculated for all transcripts as in Silva Amorim et al. (2018). Genes with < 128 reads were discarded. A Supplementary Table 4 includes RPKM abundances for all genes for all experiments. Raw RNA-seq data is available upon request.

IPA

Pathway Analysis was performed using the Ingenuity Pathway Analysis Software (IPA; Qiagen; version 42012434). Datasets previously filtered to include only differentially expressed and differentially translated genes were submitted to IPA. Location and Type information were obtained from the IPA annotated datasets to determine the % of genes from each dataset belonging to individual subcellular localization and molecular type/function categories. Data was plotted as% of genes in each category, with category "other" not shown. IPA annotated datasets were submitted to Core Analysis with analysis parameters set to include "Direct and indirect interactions" and "Experimentally observed data only". Network data was obtained for all datasets and a Molecular Activity Predictor (MAP) analysis was applied based on the differentially regulated genes belonging to each individual network.

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Conflict of interest

There is no Conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ynpai.2018.04.001.

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