UNIVERSITY OF TURIN

DEPARTMENT OF CLINICAL AND BIOLOGICAL SCIENCES

DOCTORAL SCHOOL IN LIFE AND HEALTH SCIENCES

Ph.D. PROGRAMME IN EXPERIMENTAL MEDICINE AND THERAPY

XXXIII CYCLE

EFFECTS OF THE MECHANICAL STIMULATION ON THE PERIPHERAL NERVOUS SYSTEM

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ACADEMIC YEARS OF ENROLMENT: **2017-2021**

CODE OF SCIENTIFIC DISCIPLINE: **BIO16 and MED48**

GLOSSARY

ITALIAN	**ENGLISH**
Dipartimento di Scienze Cliniche e Biologiche	Department of Biological and Clinical Sciences
Dottorato di ricerca in Medicina e Terapia Sperimentale	PhD Programme in Experimental Medicine and Therapy
Ciclo XXXIII	Cycle XXXIII
Effetti dello stimolo mecchanico sul sistema nervoso periferico	Effects of mechanical stimuli on the peripheral nervous system
Tesi presentata da Giacomo Carta	Thesis' author Giacomo Carta
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Coordinatore del Dottorato Prof. Pasquale Pagliaro	PhD Programme Co-ordinator Prof. Pasquale Pagliaro
Anni Accademici 2018-2021	Academic years of enrolment 2018-2021
Settore Scientifico Disciplinare di Afferenza: BIO16 e MED48	Code of scientific discipline BIO16 e MED48

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ABSTRACT

Nerve injuries and diseases are a rising problem leading to disability due to sensory and motor impairments often associated with neuropathic pain and in particular mechanical allodynia and hyperalgesia. The neurodynamic treatment (NDT) consisting of selective uniaxial nerve repeated tension protocols has been described to effectively reduce mechanical allodynia and hyperalgesia in neuropathic pain patients. Nevertheless, even if some studies on *in vivo* models reported the ability of NDT to promote pain modulation most of the biological effects involved are still unknown. Moreover, no standardized protocol of treatment is available.

Neurodynamic tests are also reliable for the assessment of neuropathies in somatic nerves but no test is available for the autonomic nervous system, in particular the vagus nerve (VN). My thesis is focused on defining if NDT could promote biological changes related to nerve regeneration and mechanical pain modulation in somatic nerves. As reported in the first article in the thesis we have found that many clinical studies use flossing nerve techniques, but only a few clinical and most preclinical studies assess the effects of tensions techniques, which can induce changes in the peripheral and central nervous system related to neural plasticity and pain modulation. This led us to explore the dose-dependent pro-regenerative and antiapoptotic effects of repeated tensions on sensory and motor neurons as described in the second article. In the third article presented, we explored the *in vivo* and *ex vivo* effects of NDT with a dosage defined by the *in vitro* to be more effective in neurite outgrowth cell survival and

differentiation. These experiments revealed that the NDT effects on sensory recovery and pain modulation were selectively induced by neuronal responses to mechanical stimulations and not to be dependent on other non-neural tissue. Moreover, the most relevant results obtained are that NDT increases the length of the neurites in sensory neurons, and after a nerve injury regulates the TACAN gene expression in the DRG, a mechanosensitive receptor, shared between humans and rats, responsible for mechanical allodynia and hyperalgesia. Finally, in the last article presented, through a cross-sectional double-blind sex-balanced study, we developed and explored the effects on symptoms and heart rate of a VN tensioning test in humans providing a validated clinical test for parasympathetic dysfunctions and diseases that was lacking.

In conclusion, the results of my Ph.D. thesis show that repeated nerve tensioning, from a therapeutic point of view, promotes nerve regeneration processes, sustained by neurons, can speed up the sensory-motor recovery after a nerve injury, and induces modulation of mechanical allodynia and hyperalgesia by affecting the gene expression of the mechanosensitive channel TACAN in the DRG. Also, from a diagnostic point of view, the neurodynamic test of the VN is a validated and reliable test to assess clinical conditions in which autonomic neuropathies and dysfunctions are suspected.

CHAPTER 1

INTRODUCTION

1.1 Nerve anatomy

The peripheral nervous system (PNS) is located in every part of the human body and connects the innervated tissues (target tissues) to the central nervous system (CNS) (Garrett et al., 2021). The PNS' main functions are sensory, motor and autonomic and are related to the types of neurons by which it is composed. Sensory functions correspond to the codification of stimuli in the peripheral tissues, the transduction of them in electrical signals, and the modulation of those signals through the synapsis with neurons located in the CNS. Motor functions consist of the conduction of electrical signals produced in the CNS that reach the muscle and the release of acetylcholine that induces muscle contraction. Autonomic functions control our organs and glands, are fundamental for the survival and homeostasis of the body, and are regulated by short and long loops of autonomic neurons that can be divided into parasympathetic and sympathetic depending on the type of neurotransmitters they release, the location of the cell body and the length of the axons (Jiman et al., 2020).

The PNS is mainly composed of axons (Figure 1A) of neurons with their cell bodies located in ganglia or inside the CNS, Schwann cells that compose the peripheral glia, blood vessels that give the proper blood supply, and connective tissue that organizes and protects those delicate structures from the mechanical stresses (Kerns et al., 2019) induced by a physiological condition like joint

movements and muscle contraction or by interacting with the surrounding environment like landing from a jump.

To properly deal with mechanical strain and compressions, the PNS has an intraneural wavy (Figure 1B) structure able to maintain the integrity of the axons when nerves are stretched or compressed (Liu et al., 2012). Contrary to soft tissues like muscles, ligaments, and joint capsules, nerves move independently from surrounding tissues as shown by ultrasound and cadaveric studies. The PNS slides on its longitudinal axis similarly to tendons but with a very low degree of motion which for example is around 4.5 mm at the posterior midthigh for the sciatic nerve when in sitting position the knee extension is performed (Ellis et al., 2018), and 12.5 mm when the Straight leg raise test is performed (Ridehalgh et al., 2015). Also, very small degrees of lateral displacement of the sciatic nerve at the deep gluteal space during hip rotations had been described to be around 0.8-3 mm (Balius et al., 2021).

Thanks to the wavy pattern of the nerve fibers the PNS can deal with mild compression and stretch-induced physiological movements (Figure 1B), but prolonged or acute high degree compressions or stretches are deleterious for its anatomical integrity and function (Bueno and Shah, 2008).

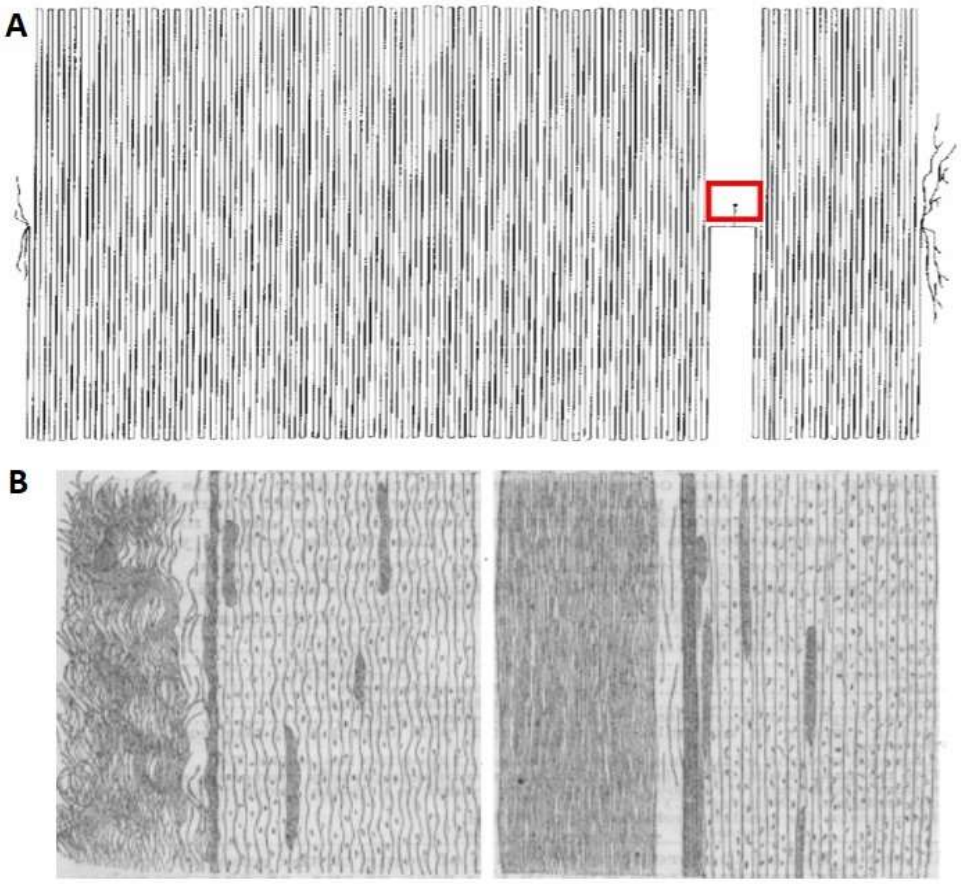

Figure 1.(A) The figure reported in the 1999 publication by Marshall Devor decribes the length of the peripheral axon reaching the target tissue (left) and the central axon reaching the spinal cord (or brain stem) of a human DRG neuron with a 50 µm diameter soma, in the red square. (B) the figure reported in the 1883 publication by Jhon Mashall describes the macroscopic effects of mechanical tension on a longitudinal section of a somatic nerve on the left the unstretched nerve and on the right the stretched nerve(Marshall, 1883).

1.2 Nerve injuries and regeneration

The structures that protect and isolate the axons and also nerve fibers in the PNS can be injured, and nerve traumas occur in approximately 3% of patients arriving at an emergency room after a physical trauma (Nadi et al., 2019) and are two and a half times more frequent than spinal cord injuries (Kumar et al., 2018; Tapp et al., 2019; Padovano et al., 2020). Contrary to the central neurons

the peripheral nerves have the unique ability to regenerate (Fregnan et al., 2020; Jara et al., 2020). Indeed, the injured axons distal to the site of the trauma undergo the process called Wallerian degeneration, which is a complex biological phenomenon that involves Schwann cells activation and neuronal reorganization in the way that distal nerve debris is removed and proximal axon regeneration is promoted till the target tissues is reached (Geuna et al., 2009; Fregnan et al., 2012; Cintron-Colon et al., 2022). The nerve regeneration requires time since peripheral nerve regeneration speed is around 1 mm/day and several biological and environmental factors can affect this process (Yamada et al., 2009; Geuna et al., 2016). Indeed, peripheral nerve regeneration is affected by a variety of factors and the most relevant are the type of lesion (partial or total)(Temiz et al., 2021), site of lesion (proximal or distal)(Sulaiman and Gordon, 2013), loss of nerve substance (>3mm)(Ducic et al., 2017), intraneural scar formation (Tos et al., 2015), age of the patient and tobacco use (Rodriguez-Fontan et al., 2020). Since nerve regeneration is fundamental to restore the loss of sensory, motor, and autonomic functions, due to the nerve injury, and a complete recovery is generally very rare, it is basilar to consider the anatomical integrity of the nerve as a fundamental goal to restore its function (Navarro et al., 2007; Niedermair et al., 2020; Taylor et al., 2021).

1.3 Neuropathic pain

Neuropathic pain has been defined by the international association for the study of Pain (IASP) as "pain arising from a lesion like a nerve injury or disease like radiculopathy affecting the somatosensory system", and it affects one person on ten worldwide (Van Hecke et al., 2014; Machado-Duque et al., 2020). In particular, persistent neuropathic pain develops in more than half of the patients undergone nerve surgery (Miclescu et al., 2019), and from 48% to 74% of the patients suffering from back pain develop neuropathic pain (Harrisson et al., 2020). Notably, back pain, which is often associated with peripheral nerves inflammation, is still for three decades the leading cause of disability worldwide, possibly impacting society more than psychiatric, oncologic, and cardiovascular diseases combined (Hoy et al., 2014; Vos et al., 2016; Abajobir et al., 2017). Neuropathic back pain has indeed a very high cost in Italy, considering that the direct health-related cost is €2,046.67/per patient per year and the total cost (direct and indirect costs) per patient of €9,305/year (Liedgens et al., 2016). Chronic neuropathic back pain alarmingly affects people independently from their biological variables like sex, smoke, and body mass index (Juniper et al., 2009; Edit et al., 2013; Bekar et al., 2014; Nuttall, 2015; Bendayan et al., 2017; Kreddig and Hasenbring, 2017; Wong et al., 2017; Jonsdottir et al., 2019; Hogans et al., 2021; Varallo et al., 2021), and social aspects like salary or educational level (Lallukka et al., 2014) leading to disability and loss of social participation (Keene et al., 2021). Chronic back pain has a tremendous impact on individual disability, social interactions, generating opioid addiction, and

macroeconomic issues (Dagenais et al., 2008; Beth Darnall, 2018; Mutubuki et al., 2020; Tölle et al., 2021). For example, chronic back pain pushes people to retire from work prematurely and stay at home inactively more than neoplasms, respiratory, cardiovascular diseases, and asthma combined (Schofield et al., 2008).

Chronic and acute neuropathic pain is a rising condition, with an increased incidence and prevalence in post-covid-19 patients (Attal et al., 2021; Meyer-Frießem et al., 2021; Odozor et al., 2021), and nowadays the large improvement in medical interventions and increased opioid prescription did not reduce the impact of neuropathic pain on the world population (Loeser and Cahana, 2013a; Van Hecke et al., 2014; Beth Darnall, 2018; Blyth, 2018; Machado-Duque et al., 2020; Yeh et al., 2020; Hogans et al., 2021). Indeed, is still difficult for clinicians to make a diagnosis, define and maintain an adequate therapy when neuropathic pain is involved (Loeser and Cahana, 2013b; Beth Darnall, 2018; Blyth, 2018; Machado-Duque et al., 2020). It is also difficult to treat neuropathic pain with the available drugs, mainly antidepressants and antiepileptic, which are indicated for short-term treatments (one to three months) and are effective for one on two or sixteen patients (Finnerup et al., 2015, 2018; Terkelsen et al., 2017). Therefore, it is of paramount importance to provide new effective drugs for the known mechanisms involved in mechanical allodynia and to understand which mechanisms of effective non-pharmacological interventions are modulated if unknown.

1.4 Neuropathic pain and mechanical allodynia

Allodynia's current definition by the IASP is "pain due to a stimulus that does not normally provoke pain". In particular, it is a stimulus-dependent process and the most common and disabling condition in neuropathic pain patients is allodynia induced by mechanical stimuli, defined as mechanical allodynia (Jensen and Finnerup, 2014; Lolignier et al., 2014). Indeed, mechanical allodynia can be described as perceiving light touch as painful or pain evoked by performing a physiological movement or a gentle muscle contraction. Patients suffering from neuropathic back pain complain that bending to reach an object on the ground or moving an object from one part to another of the house produces pain even if those tasks did not provoke pain in the week before the nerve inflammation onset.

The exact etiology that produces mechanical allodynia is still undefined. However, it is clear that after a nerve injury unmyelinated and myelinated primary afferent neurons increase their activity sustaining a predominantly peripheral neuropathic pain process with stimuli dependent abnormal responses like mechanical allodynia and hyperalgesia (Woolf and Salter, 2000; Campbell and Meyer, 2006; Jensen and Finnerup, 2014; Truini, 2017). In particular, these processes are linked to changes in neurochemical, anatomical, electrophysiological properties or protein and gene expression in the DRG neurons (Shi et al., 2011; Kim et al., 2012; Xu et al., 2016; Prato et al., 2017; Truini, 2017; Beaulieu-Laroche et al., 2020; Bogen et al., 2020; Yeh et al., 2020).

A considerable number of studies have revealed that changes in the DRG neurons gene expression modulate neuropathic pain and in particular, allodynia and hyperalgesia, which are disabling stimuli-dependent pain phenomena (Jensen and Finnerup, 2014). Neuropathic mechanical allodynia and hyperalgesia had been described to be related to specific genes expression in the DRG responsible for regulating the mechanosensitive transmembrane channels for low threshold stimuli like PIEZO1, PIEZO2 (Kim et al., 2012; Qi et al., 2015; Zhao et al., 2016; Song et al., 2019; Wang et al., 2019a; Roh et al., 2020), or for high threshold stimuli like TACAN (Tmem120A) (Cavanaugh et al., 2009; Beaulieu-Laroche et al., 2020; Bogen et al., 2020), for Schwann cells activation and proliferation after a nerve injury like c-Jun (Zhuang et al., 2006; Sanna et al., 2020), and finally for the expression of Toll-like receptor-2 (TLR2), a receptor modulating the macrophages response (Yang et al.; Jurga et al., 2016; Cobos et al., 2018). Indeed, it has been shown in healthy mice that the PIEZO1 and PIEZO2 increased activity induced by subcutaneous administration of a PIEZO1-specific agonist (Yoda1) or PIEZO1 and PIEZO2 overexpression in the DRGs in transgenic mice can induce mechanical allodynia (Qi et al., 2015; Zhang et al., 2019). On the opposite, the injection of methyl-β-cyclodextrin or depletion of PIEZO1 and PIEZO2 suppresses mechanical allodynia in a mice neuropathic pain model of nerve chronic constraint injury (CCI) (Borbiro and Rohacs, 2017; Wang et al., 2019a). Furthermore, it has been shown that suppression of TACAN expression with siRNA in the DRG of CCI rats induces mechanical allodynia suppression (Bogen et al., 2020). Also, the injection of Lipopolysaccharide from Rhodobacter sphaeroides, a TLR2 antagonist,

suppresses mechanical allodynia in murine models of CCI (Jurga et al., 2016; Cobos et al., 2018). The c-Jun expression has been also recently linked to mechanical pain by knocking out selectively JNK1, 2, and 3 in the DRG in mice (Sanna et al., 2020). Notably, the processes assessed in murine models especially the expression of genes and proteins related to the phenomenon of allodynia, cell survival, or death are expressed in both murine and human DRG neurons (Kremer et al., 2018; Zaltsman et al., 2019; Beaulieu-Laroche et al., 2020; Roh et al., 2020; Wang et al., 2020b), and as described by Stephens and colleagues these changes are independent of the subject sex (Stephens et al., 2019).

1.5 Neurodynamic tests development

A restriction of nerve movement, its compression, or neuropathy (Lundborg and Dahlin, 1996; Gao et al., 2013) can itself generate inflammation and pathology. It has also been shown that a subset of peptidergic C-fiber nociceptors can be sensitized by inflammatory mediators in mammalians, inducing an alteration of the mechanical force transduction in electrical signals, causing neuropathic pain with mechanical hyperalgesia and mechanical allodynia (Jensen and Finnerup, 2014; Prato et al., 2017). Sensory neurons in somatic nerves can transduce mechanical stimuli due to the presence of mechanosensitive ion channels exposed on their cell membranes (Coste et al., 2010; Song et al., 2019; Beaulieu-Laroche et al., 2020). The first manual test to stretch a somatic nerve was developed and described by Charles Lasegue in "Considerations on Sciatica"

to help clinicians in the diagnosis of Sciatica (Lasegue, 1864) by rising the patients' leg with the knee extended. Only more than one century later tests to stretch the nerves in the upper limbs and the femoral nerve was developed (Vanti et al., 2011; Lai et al., 2012; Nee et al., 2012a; Bueno-Gracia et al., 2016). Cadaveric and in vivo studies with different imaging techniques had shown the nerve tension tests are biomechanically plausible (Manvell et al., 2015; Shacklock et al., 2016; Tawa et al., 2017)

The pioneering works of Dennis Bray and colleagues in the late 70s of the last century had shown that mechanical stimuli can affect the growth and orientation of axons in sensory neurons (Bray, 1979, 1984). Nowadays many studies had been published showing that neurons are mechanosensitive and mechanoresponsive and that mechanical stimuli are crucial for neurodevelopment and nerve repair (Pfister et al., 2006a, 2008; Loverde et al., 2011; Magou et al., 2011; Higgins et al., 2013). Unfortunately, those data had never been adopted to develop modern neurodynamic concepts. Only in the last two decades some in vivo experiments conducted on small mammals, nerve injury models have detected that NDT can affect healing processes in the peripheral tissues and processes linked to neural plasticity in the nerves and the CNS as described below. The manual nerve stretch was adopted as a therapeutic tool, by exposing the spinal root and stretching for 5-6 minutes, and it was described to be an effective treatment to reduce pain in drug-resistant sciatica patients since the end of the 18th century (Macfarlane, 1878; Marshall, 1883). Historically it was believed that the nervous system may lack the ability to stretch and move, and NDT was advocated as a therapeutic option to restore

mechanical homeostasis and perfusion (Brown et al., 2011; Gilbert et al., 2015b, 2015a; Boudier-Revéret et al., 2017). Neurodynamic tests had been developed to increase the load on somatic nerves and resistance perceived by the assessor during the manuevers was defined as a hallmark to discriminate the neural or non-neural origin of symptoms by the administration of sensitization manuevers distal or proximal to the site where symptoms were referred (Vanti et al., 2011; Lai et al., 2012; Manvell et al., 2015; Leoni et al., 2016).

1.6 Neurodynamic from the clinic to the bench

The selective repeated administration of uniaxial tensions to somatic nerves also known as neurodynamic treatment (NDT) has shown to be effective in short and long-term pain modulation and nerve conduction improvements detected by electrophysiology in long term neuropathic pain patients suffering from carpal tunnel syndrome (Wolny et al., 2017; Wolny and Linek, 2018, 2019; Talebi et al., 2020; Núñez de Arenas-Arroyo et al., 2021) or radiculopathies of somatic nerves in the upper or lower limbs (Basson et al., 2015; Mahmoud ELDesoky, 2016; Yamin et al., 2016; Rajalaxmi M.V., Lavanya R., Kirupa K., Divya Mary S.M., 2020; Yun et al., 2020). The NDT effects are obtained by two types of techniques the sliding and the tensioning techniques (Coppieters and Butler, 2008). Sliding techniques utilize combinations of joint and limb movements designed to promote nerve movement towards one end of the nerve tract, whilst tensioning techniques move the ends of a nerve tract in opposite directions thereby imposing (Coppieters et al., 2009; Ellis et al., 2012; Neto et al., 2017).

Most preclinical studies and a few clinical trials adopted the tensioning technique to describe the effects of NDT described on pain, nerve morphology, and protein expression in the nervous system in Figure 2 (Villafañe et al., 2012; Arumugam et al., 2014; Santos et al., 2014; Da Silva et al., 2015; Giardini et al., 2017; Lima et al., 2017; Zhu et al., 2018). For this reason, to provide clinically relevant and clinically translatable data on the NDT effects also in our studies, only the tensioning technique was adopted.

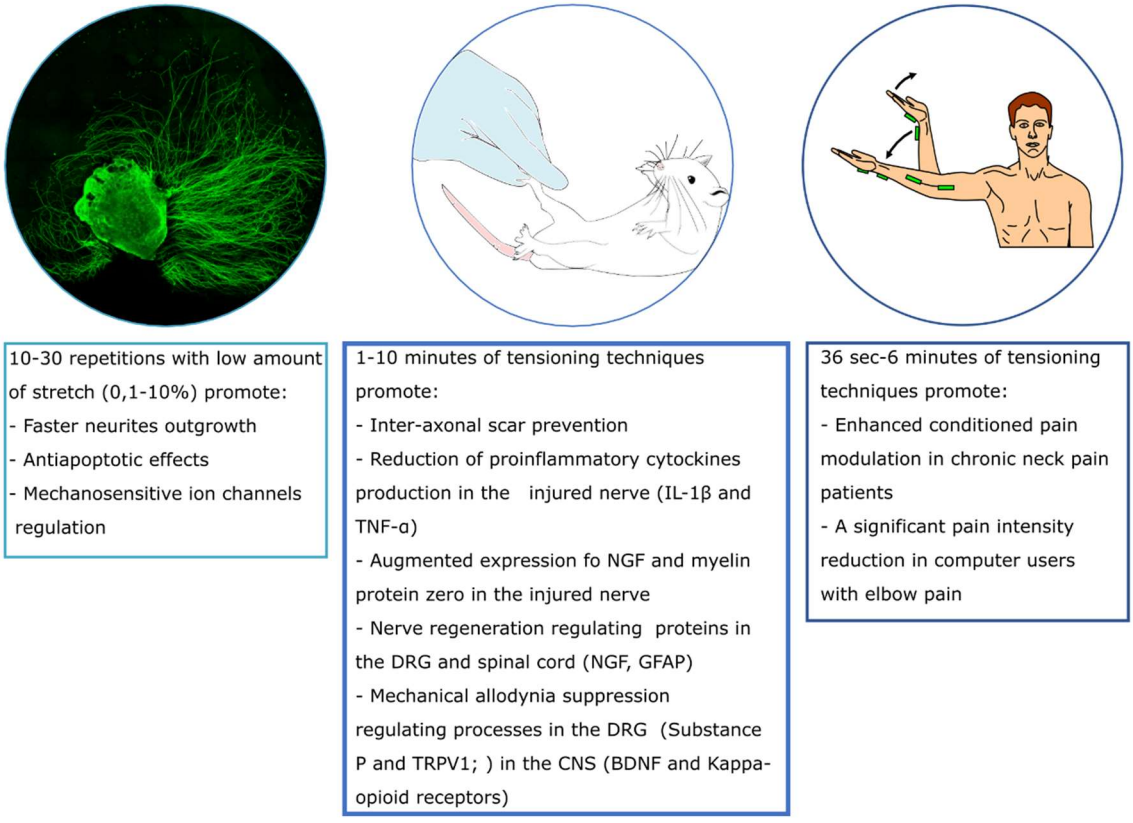

Figure 2. Effects of repeated mechanical tension from preclinical and clinical researches. From the left are reported in each column the protocol durations and the effects of *in vitro* (left), *in vivo* (middle), and clinical studies (right) adopting the nerve tensioning technique.

NDT significantly reduces pain and disability with long-term effects in neuropathic pain patients (Basson et al., 2015; Mahmoud ELDesoky, 2016;

Yamin et al., 2016; Rajalaxmi M.V., Lavanya R., Kirupa K., Divya Mary S.M., 2020; Yun et al., 2020). Also, hypoalgesic and antiallodynic short-term effects have been reported respectively in asymptomatic (Beltran-Alacreu et al., 2015; Gamelas et al., 2019; Martins et al., 2019) and neuropathic pain patients (Villafañe et al., 2012; Arumugam et al., 2014), suggesting a possible pain gating effect induced by repeated nerve mechanical tailored stretch. Since Sellheim pioneered the paravertebral block in 1905, the peripheral nerves and the DRG had become a therapeutic target for pain suppression, but no active role in pain gating of this structure had been hypothesized since the discovery of genes regulations described above (Adriani, 1980; Boezaart et al., 2009; Hussain et al., 2018). To define if one or more of these genes were regulated by the NDT was one of the aims of the research reported in this thesis.

A restriction of nerve movement, its compression, or neuropathy (Lundborg and Dahlin, 1996) can itself generate nerve inflammation and pathology in the target tissues (Gao et al., 2013). In particular, peptidergic nerve fibers can be sensitized by inflammatory mediators, causing neuropathic pain detectable through the presence of mechanical hyperalgesia and mechanical allodynia (Jensen and Finnerup, 2014; Beaulieu-Laroche et al., 2020; Bogen et al., 2020). Peripheral nerves selective tension tests or neurodynamic tests are bed-side examinations reliable clinical tests validated for neuropathies detection of somatic nerves (Taenzer et al., 2000; Wasan et al., 2011; Verwoerd et al., 2016; Bueno-Gracia et al., 2017; Ekedahl et al., 2018; Koulidis et al., 2019). These tests are also adopted as treatment and assess the nerve response to mechanical stimuli which are transduced by stretch-sensitive ion channels in

peripheral nerves also present in parasympathetic axons and cell body membranes (Beaulieu-Laroche et al., 2020; Bogen et al., 2020). Several clinical trials have shown that NDT is an effective treatment to increase nerve functions and reduce pain in patients with neuropathies of the somatic nerves (Nee et al., 2012b; Torres et al., 2015; Wolny et al., 2016; Basson et al., 2020; Plaza-Manzano et al., 2020). Also, several preclinical studies on murine sciatic neuropathy models have shown that NDT resolves mechanical and thermal allodynia, which are very common and disabling symptoms among neuropathic pain patients (Ramos et al., 2020). NDT affects the protein expression of the Nerve Growth Factor (NGF) and Myelin Protein Zero (MPZ) in the nerve distal to the ligature (Da Silva et al., 2015), and in the homologous DRG and the spinal cord metameres involved by the somatic nerve lesion with pain modulation effects (Bertolini et al., 2009; Santos et al., 2012; Zhu et al., 2018). A recent literature review (Lutke Schipholt et al., 2021) reported that neural tensioning technique positively influenced various neuro-immune responses at the peripheral nerve injury site, but also at the corresponding DRG, spinal cord, and higher brain centers (Figure 2). Interestingly, the tensioning techniques used in these animal studies have shown remarkable results. For example, in rats with induced painful diabetic neuropathy, tensioning techniques alleviated mechanical hypersensitivity on the paw of the treated side compared to the untreated side or compared to animals who did not receive NDT (Zhu et al., 2018). This improvement correlated with lower concentrations of pro-

inflammatory cytokines (IL-1β and TNF-α) in the sciatic nerve and its branches only on the treated side.

At the level of the DRG, tensioning techniques resulted in a decrease of Substance-P and transient receptor potential vanilloid 1 (TRPV1) expression, and an increase in m-opioid receptor expression in rats with a sciatic nerve injury (Santos et al., 2012). These are relevant changes, as TRPV1 upregulation contributes to mechanical allodynia and thermal hyperalgesia; Substance-P plays an important role in the development of chronic pain (Jang et al., 2004) and is responsible for the development of hyperalgesia in murine models (Hoot et al., 2011). Another study revealed that tensioning techniques were associated with a decrease in nerve growth factor (NGF) and glial fibrillary acidic protein (GFAP) in the DRG (Santos et al., 2012). Suppression of NGF overproduction in the DRG has been shown to attenuate neuropathic pain following a chronic constriction injury to the sciatic nerve (Dai et al., 2020). GFAP is a molecular marker for glial cell activity (astrocytes) and activated glial cells facilitate pain neurotransmission (Dubový et al., 2018b). A decrease in GFAP expression following tensioning techniques was also observed in the lumbar spinal cord. These changes were associated with a reduction in mechanical and thermal hyperalgesia and allodynia in the paw of the treated side (Santos et al., 2012).

Glial cells and brain-derived neurotrophic factor (GDNF and BDNF) expression are increased in the midbrain following a sciatic nerve injury causing neuropathic pain in rats. Glial cells are implicated in the development of persistent pain and BDNF released from activated microglia contributes to the nociceptive transmission. Remarkably, tensioning techniques resulted in a reduction of

GDNF and BDNF expression in the midbrain equal to uninjured rats (Giardini et al., 2017). Also, tensioning techniques resulted in an increased expression of endogenous opioid receptors (Kappa-opioid receptors) in the periaqueductal gray (Santos et al., 2014). Furthermore, the rats who received tensioning techniques had increased tibialis anterior muscle strength and improved their locomotion compared to nontreated injured rats.

Studies applying tensioning techniques following a sciatic nerve crush injury or a chronic constriction injury in rats have shown a successful preventive effect against intraneural scar formation (Lima et al., 2017), anti-allodynic and neurotrophic effects able to speed up the nerve regeneration processes (Santos et al., 2012; Da Silva et al., 2015). In particular, distal to the injury site, it was observed an increased axon number with myelin sheaths of normal thickness and less inter-axonal fibrosis after NDT has been shown in rats compared to controls with the same sciatic nerve injury who did not receive the intervention (Da Silva et al., 2015). Although there is a growing understanding of how mechanical forces influence tissues (Mueller and Maluf, 2002; Topp and Boyd, 2006), there is quite poor evidence on biological processes involved when they are applied to nerves. In addition, an extreme heterogeneity of NDT protocols available in the literature is not supportive of an appropriate, effective, and safe use of NDT in clinical practice. Indeed, the available NDT protocols are different for the amount of nerve elongation (from 0.8% to 15% of the total nerve rest length)(Driscoll et al., 2002; Coppieters et al., 2006; Sharma et al., 2016), for the elongation speed (from one to five seconds) (Wang et al., 2015; Sharma et al., 2016), for the number of stimuli (from three to three series of 60 repetitions)

(Torres et al., 2015; Wolny et al., 2016), and the stretch duration (from one to 30 seconds) (Torres et al., 2015; Sharma et al., 2016). For these reasons, a standardized NDT protocol is suitable for neuropathic pain management and the detection of the effects related to allodynia and hyperalgesia in the nervous system is of paramount importance.

1.7 Neurodynamic and the autonomic nervous system

The vagus nerve is the longest cranial nerve in our body (Cunningham and Martínez, 2021). It connects the brain to our organs in the thoracic and abdominal cavities and it exclusively rules the parasympathetic responses in the target tissues and organs in the neck, thorax, and upper abdomen (Zandstra et al., 2021). The vagus nerve composes the so-called brain-gut axis and its integrity is fundamental for a person's wellbeing and survival (Fung et al., 2017). Autonomic peripheral neuropathies are cryptic conditions impacting the brain-gut communications increasing the risk of neurodegenerative diseases and other acute life-threatening conditions like heart attack, atrial fibrillation, and sudden cardiac death. Autonomic neuropathies are conditions difficult to be detected that increase hemodynamic instability (Ang, L. et al., 2020), post-surgery complications (Lankhorst et al., 2015; Suarez-Roca et al., 2019), and sudden death in obese and diabetic patients (Freeman, 2005; Santos Breder and Sposito, 2019; Williams et al., 2019; Malaty et al., 2021). Patients with COVID-19 have an increased prevalence of cardiac arrhythmias (Ho et al., 2020) with an estimated incidence of 15% in post-COVID-19 patients (Malaty et al., 2021).

Indeed, autonomic neuropathies are a raising health problem and it is fundamental to have a reliable clinical tool that investigates selectively VN neuropathies.

Since NDTs are effective in stimulating positive effects in peripheral nerves it would be useful to validate a VN neurodynamic test and to investigate its effects on these pathophysiological conditions and other conditions like diabetes-related gastrointestinal alterations, cardiac neuropathies, and arrhythmias secondary to coronavirus infection (Garamendi-Ruiz and Gómez-Esteban, 2019; Santos Breder and Sposito, 2019; Malaty et al., 2021).

No clinical data are available on mechanical stimulation of the autonomic nervous system but, it has been established that the invasive mechanical stimulation on the surgically exposed cervical tract of the vagus nerve (VN) for 5 minutes significantly ameliorates survival rates in sepsis models by reducing the serum level of tumor necrosis factor α (TNFα) (Huston et al., 2007). These data on the mechanical responsiveness of the VN are promising for the development of a neurodynamic test of this cranial nerve with relevant and multiple clinical applications. Also, it has been established that the invasive and non-invasive stimulation on the cervical tract of the vagus nerve (VN) ameliorates survival rates in sepsis models (Huston et al., 2007), promotes heart and lungs regeneration in preclinical models (Brandt et al., 2019; Chen et al., 2020), HR variability in cardiological patients (Kobayashi et al., 2013), and symptoms improved in people with pharmacoresistant problems such as acute and chronic pain, dementia, psychiatric illness, consciousness disorder and epilepsy (Kirchner et al., 2000; Schachter, 2006; Corazzol et al., 2017; Breit et

al., 2018; Dong and Feng, 2018; Johnson and Wilson, 2018). NDT induces augmented nerve activation through the production of antidromic action potentials produced by stretch-sensitive ion channels in peripheral nerves also present in the VN that are exposed on the axons and cell body membranes (Zeng et al., 2018; Huo et al., 2021), for these reasons the validation of a neurodynamic test of the VN could be very impacting on several health issues difficult to be assessed and treated by modern medicine.

CHAPTER 2

AIM OF THE STUDY

The aim of this Ph.D. thesis is twofold: first, to explore and deepen the biological mechanisms induced by NDT able to promote nerve regeneration and pain modulation to define a standardized NDT protocol; second, to validate a vagus nerve neurodynamic test, to assess autonomic neuropathies and reporting the normative data on symptoms and changes in heart rate induced by the test in males and females.

To reach the first aim we used *in vitro* (sensory and motor cell lines), *ex-vivo* (DRG explants), and *in vivo* models (median and ulnar crush nerve injury in rats). In particular, for *in vitro* and *ex-vivo* experiments, NDT was performed using a bioreactor, built *ad hoc*, that allowed to administer uniaxial repeated tension protocols on sensory and motor neurons cell lines and DRG explants. Starting from the parameters of NDT described in the literature (Driscoll et al., 2002; Coppieters et al., 2006; Torres et al., 2015; Wang et al., 2015; Sharma et al., 2016; Wolny et al., 2016) we aimed to define a protocol of repeated mechanical stretch (NDT) to define the most effective dosage, translatable in clinical practice, by assessing a possible dose-dependent effect. Morphological changes, protein expression related to cell survival, and the expression of genes related to mechanical allodynia and hyperalgesia in the DRG were assessed. NDT for *in vivo* experiments, adopting a median and ulnar nerve crush injury model in rats, was administered by hand performing a combination of neck, arm, and

hand movements able to increase the tension on the injured neural tissue. In all experiments, tension was administered until initial resistance was felt by the clinician, since it is a hallmark used in clinical practice for neurodynamic protocols, and to monitor the animal behaviors the treatment was administered to awake animals. Behavioral tests were adopted to assess the NDT effects on mechanical pressure, mechanical pain, and motor tasks involving gross and fine movements of the upper limbs. We have assessed through morphological analysis the nerve portion distal to the injury site, and the gene expression of TACAN and TLR2 in the DRG, which are receptors involved in the condition of mechanical allodynia and hyperalgesia.

As regards the second aim of this thesis, considering that VN neuropathies are life-threatening conditions often very difficult to be screened and none of the tests available in the clinic are selective to study the pathophysiology of the VN, we decided to validate nerve selective tension tests or neurodynamic tests of the VN since these are bedside examinations and reliable clinical tests able to detect neuropathies of the somatic nerves (Wasan et al., 2011; Bueno-Gracia et al., 2016; Verwoerd et al., 2016; Ekedahl et al., 2018; Koulidis et al., 2019). Taking advantage of the VN anatomy and innervation, we intended to collect normative data for heart rate (HR) variations induced by the test in males and females and to describe the relationship between symptoms induced during the test and any autonomic dysfunction-related symptom.

CHAPTER 3

RESULTS

3.1 Paper n°1

Neurodynamics: is tension contentious?

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Journal of Manual & Manipulative Therapy, 2021

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Journal of Manual & Manipulative Therapy

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/yjmt20

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To cite this article: Richard Ellis, Giacomo Carta, Ricardo J. Andrade & Michel W. Coppieters (2021): Neurodynamics: is tension contentious?, Journal of Manual & Manipulative Therapy, DOI: 10.1080/10669817.2021.2001736

To link to this article: https://doi.org/10.1080/10669817.2021.2001736

Published online: 16 Nov 2021.

JOURNAL OF MANUAL & MANIPULATIVE THERAPY
https://doi.org/10.1080/10669817.2021.2001736

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Neurodynamics: is tension contentious?

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ABSTRACT

Tensioning techniqueswere the first neurodynamic techniques used therapeutically in the management of people with neuropathies. This article aims to provide a balanced evidence-informed view on the effects of optimal tensile loading on peripheral nerves and the use of tensioning techniques. Whilst the early use of neurodynamics was centered within a mechanical paradigm, research into the working mechanisms of tensioning techniques revealed neuroimmune, neurophysiological, and neurochemical effects. *In-vitro* and *ex-vivo* research confirms that tensile loading is required for mechanical adaptation of healthy and healing neurons and nerves. Moreover, elimination of tensile load can have detrimental effects on the nervous system. Beneficial effects of tensile loading and tensioning techniques, contributing to restored homeostasis at the entrapment site, dorsal root ganglia and spinal cord, include neuronal cell differentiation, neurite outgrowth and orientation, increased endogenous opioid receptors, reduced fibrosis and intraneural scar formation, improved nerve regeneration and remyelination, increased muscle power and locomotion, less mechanical and thermal hyperalgesia and allodynia, and improved conditioned pain modulation. However, animal and cellular models also show that 'excessive' tensile forces have negative effects on the nervous system. Although robust and designed to withstand mechanical load, the nervous system is equally a delicate system. Mechanical loads that can be easily handled by a healthy nervous system, may be sufficient to aggravate clinical symptoms in patients. This paper aims to contribute to a more balanced view regarding the use of neurodynamics and more specifically tensioning techniques.

KEYWORDS

Neurodynamics; tensioning techniques; neuropathic pain; mononeuropathy; polyneuropathy; radiculopathy; carpal tunnel syndrome

Introduction

Neurodynamic techniques are commonly utilized with either an assessment focus (i.e. to assess the mechanosensitivity of the nervous system) or treatment focus. Within a treatment paradigm, neurodynamic techniques refer to therapeutic methods (manual techniques or exercises) which (1) facilitate movement between the nervous system and its interfacing tissues (e.g. by mobilizing the nervous system itself, including the internal neural connective tissue layers, or the structures that surround the nervous system) or (2) reduce the mechanical loading on the nervous system (e.g. adopting a posture or joint position that unloads the nervous system). The aim of these therapeutic techniques is to restore the altered homeostasis in and around the nervous system [1].

When considering techniques that are aimed at mobilizing the nervous system itself, a biomechanical distinction can be made between 'tensioning techniques' and 'sliding techniques' [2]. Both aim to mobilize the nervous system [3,4], but tensioning techniques are associated with a considerable increase in nerve strain, whereas with sliding techniques the nervous system can be mobilized without substantial increases in strain [2,5,6].

Historically, tensioning techniques were the first described neurodynamic treatment techniques. They were derived from the neurodynamic tests (previously called neural *tension* tests). Because tensioning techniques use elements of neurodynamic tests that are aimed to reproduce or provoke the patient's symptoms [7], they may be contraindicated. In order to mobilize the nervous system, but without provoking or exacerbating symptoms, sliding techniques were developed [5]. A sliding technique consists of two or more joint movements whereby movements which load the nervous system are simultaneously counterbalanced by movements that unload the nervous system. For example, throwing a dart can be considered a sliding technique for the median nerve (and a tensioning technique for the ulnar nerve).

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A backhand frisbee throw can be considered a tensioning technique for the median nerve (and a sliding technique for the ulnar nerve) (Figure 1).

Because sliding techniques are associated with greater nerve excursion with less strain [2–4,8], a common misconception is that sliding techniques are clinically superior to tensioning techniques. They are indeed biomechanically different, but there is no clinical evidence that one type of technique is more effective than another. Each type of technique most likely has its place depending on the stages of tissue healing and aims of the intervention.

Traditionally, neural mobilization has been based within a mechanical paradigm. This is not surprising, as 'entrapment' neuropathy suggests a mechanical cause, and longitudinal and transverse nerve movement is reduced in conditions, such as carpal tunnel syndrome [9] and cervical radiculopathy [10]. However, there is now a greater understanding that the effects of neural mobilization are also – and perhaps predominantly – neurophysiological, neurochemical and neuroimmune.

Interestingly, the beneficial effects of neurodynamic techniques in animal models of nerve injury have all been demonstrated following tensioning techniques [11–16]. The aim of this article is therefore to provide a re-appraisal of neurodynamic tensioning techniques. We will focus on the effects of tensile loading on neurons and nerves *in-vitro* and *ex-vivo*, from animal models and human trials, to highlight beneficial effects of moderate nerve tension. We already want to emphasize that by no means are we advocating a re-introduction of nerve stretching or vigorous neurodynamic techniques. We always have and will continue to advocate to err on the side of caution in the management of people with neuropathies and to base treatment and technique selection on sound clinical reasoning. We hope this paper will provide important context for this clinical reasoning process for clinicians who use (or do not use) neurodynamic techniques in their clinical practice.

A structure designed to handle tension

During functional activities, the peripheral nervous system needs to be able to accommodate substantial amounts of nerve tension and elongation. Composite limb movements typically expose peripheral nerves to approximately 5 to 10% strain [17], although some authors report much higher increases up to 20% strain

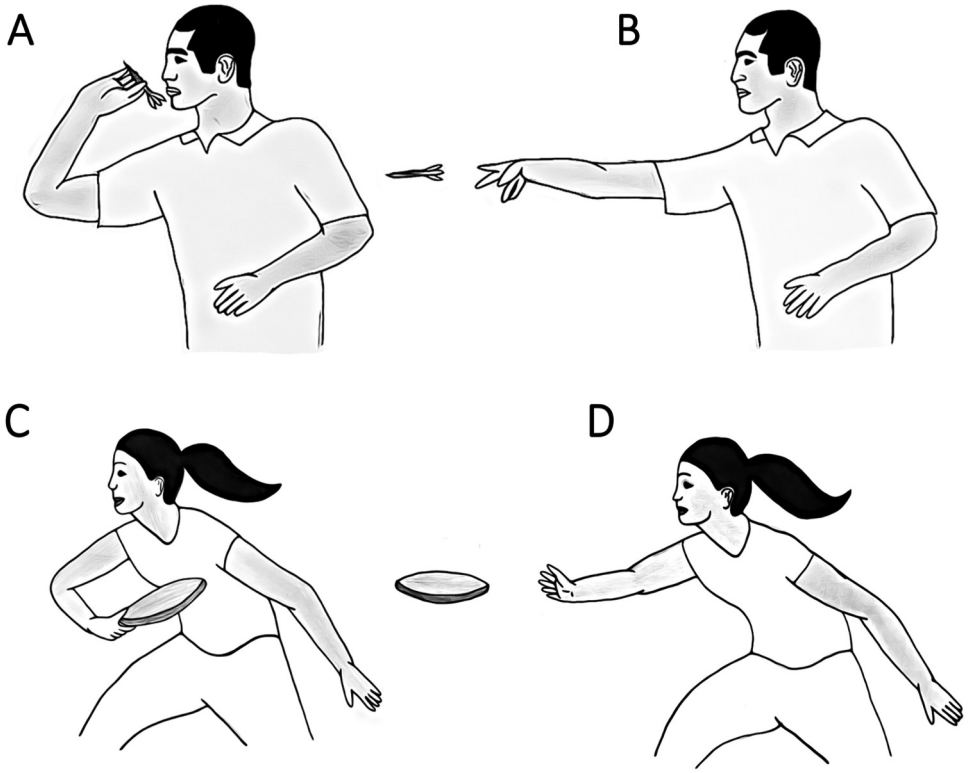

Figure 1. Examples of functional sliding and tensioning techniques. Throwing a dart is a sliding technique for the median nerve (A: wrist extension loads the median nerve; elbow flexion simultaneously unloads the median nerve; B: Elbow extension loads the median nerve; wrist flexion simultaneously unloads the median nerve), but a tensioning technique for the ulnar nerve (A: wrist extension and elbow flexion both load the ulnar nerve; B: elbow extension and wrist flexion both unload the ulnar nerve). Conversely, a frisbee backhand throw is a sliding technique for the ulnar nerve (C: elbow flexion loads the ulnar nerve and wrist flexion simultaneously unloads the ulnar nerve; D: wrist extension loads the ulnar nerve; elbow extension simultaneously unloads the ulnar nerve), but a tensioning technique for the median nerve (C: wrist and elbow flexion both unload the median nerve; D: wrist and elbow extension both load the median nerve).

[18–20]. Because form and function follow each other, the nervous system is structurally designed to handle nerve strain well.

Peripheral nerves are heterogeneous structures. They exhibit non-linear viscoelastic responses to tensile loading [21,22]. Furthermore, although the well-organized, multilayered structure of the extracellular matrix bears considerable mechanical loads, the response of peripheral nerves to mechanical load is also characterized by their ability to glide, bend and twist [22]. Neuroprotective architectural features, such as their unique undulating pattern, may also offer additional protective strength [23]. In addition, mechanical properties of peripheral nerves are not equal along their length [24], revealing a complex tissue ultrastructure [25]. A recent model of nerve layer connections has been proposed to explain the complex (whole) nerve response to stretch [26]. This model suggests that the mesoneurium, epineurium, and perineurium are coupled via viscoelastic physical connections and interact with a loosely coupled perineurium and endoneurium, allowing axons to glide and unravel throughout the length of the nerve. Collectively, these mechanical features allow nerves to straighten without bearing significant stresses while maintaining functional and structural integrity of the delicate axons within [26].

Beneficial effects of tensile loading

The following sections present findings from *in-vitro, ex-vivo* (including cadaveric), and animal and human *in-vivo* research which has assessed the effects of tensile loading on nerves (Figure 2.).

1. *Evidence from the Petri dish*

Mechanical stimuli are fundamental for tissue healing since they affect cell differentiation and tissue renewal in physiological and pathological conditions, from embryo development to wound healing [27–29]. Mechanical load applied directly to nerves can activate intracellular processes responsible for nerve myelination and nerve homeostasis. *In-vitro* experiments performed on human and rodent neurons have shown that stretch promotes cell differentiation (a process in which immature neurons acquire an adult phenotype with neurites connecting to other surrounding neurons, via neurites outgrowth). This process is fundamental in nerve repair following injury. For example, 7 days of intermittent mechanical stretch (10% strain at 0.25 Hz, for 120 minutes/day), applied to sensory neurons maintained in culture for an extended period of time (human neuroblastoma cell line; SH-SY5Y)), promoted processes linked to nerve regeneration (i.e.

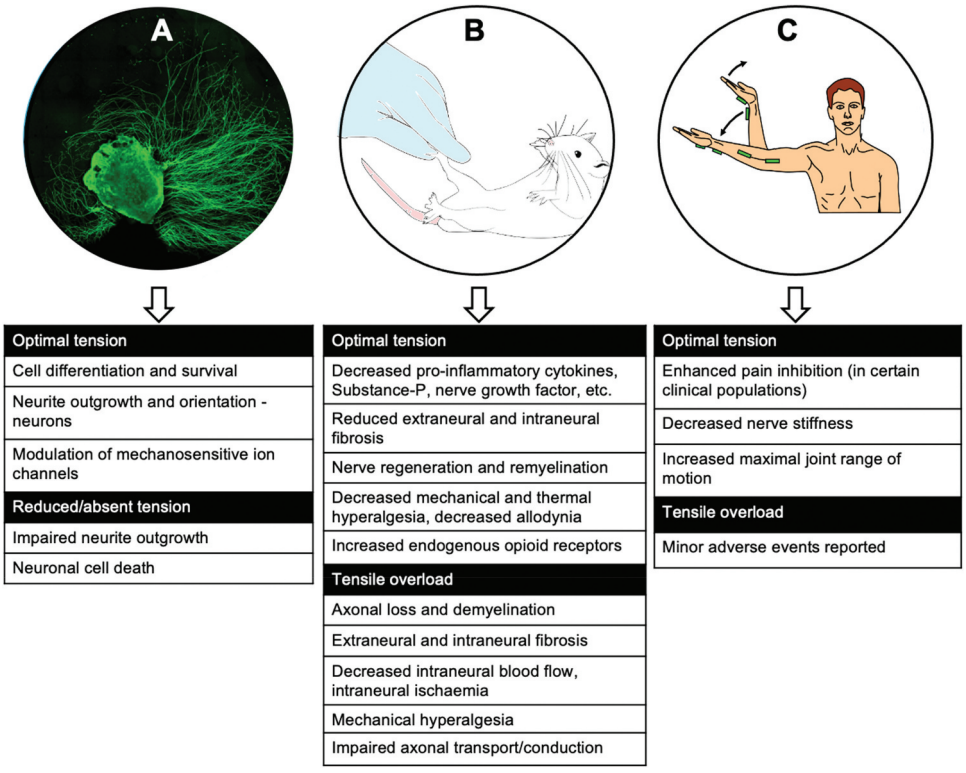

Figure 2. The effects of tensile loading of the nervous system from (A) evidence from the Petri dish, (B) evidence from animal models, and (C) evidence from human trials.

neurite outgrowth and cell differentiation) [30]. A study examining sensory and motor neurons demonstrated that repeated application of tensile load (between 0.1–1% strain) resulted in dose-dependent neurite outgrowth, cell differentiation, and modulation of mechanosensitive ion channel expression with a plausible anti-nociceptive effect [31,32].

Progressive uniaxial tension for 14 days applied to *ex-vivo* DRG explants from rats significantly enhanced the outgrowth and orientation of neurites [33–35]. A tailored mechanical stretch (with a strain of 0.1–1% for cultured neurons and up to 10% for DRG explants) had positive effects on neurite outgrowth and pain modulation [27,30,36,37]. Higher levels of stretch had negative effects on neurons, such as impaired neurite regeneration and increased neuronal death [27,33,34,36–38].

2. Evidence from animal models

Following a peripheral nerve injury, a cascade of neuro-immune responses occurs resulting in neuroinflammation and neuromodulation [39]. In severe nerve injuries, this occurs not only at the injury site, but also more proximal along the neuraxis: at the corresponding DRG [40], the dorsal horn of the spinal cord [41] and higher brain centers, such as in the midbrain [42], thalamus [43], nucleus accumbens, and prefrontal cortex [44]. Neuro-immune responses are defined as substances or processes (such as neuropeptides, cytokines, hormones and altered gene expression) involved in interactions between the immune system and the nervous system [45]. These neuro-immune responses play an important role in the generation and maintenance of neuropathic pain following a nerve injury. Most of these insights originate from animal models of peripheral nerve injuries associated with severe axonal damage. More recently however, local and remote neuro-immune responses (e.g. in the DRG and spinal cord) have been demonstrated in people with lumbar radiculopathy [46].

A recent literature review [47] revealed that neural mobilization positively influenced various neuro-immune responses at the peripheral nerve injury site, but also at the corresponding DRG, spinal cord and higher brain centers. Interestingly, the type of treatment techniques used in these animal studies all mimicked tensioning techniques, with remarkable results. For example, in rats with induced painful diabetic neuropathy, tensioning techniques alleviated mechanical hypersensitivity on the paw of the treated side compared to the untreated side or compared to animals who did not receive neural mobilization [13]. This improvement correlated with lower concentrations of pro-inflammatory cytokines (IL-1β and TNF-α) in the sciatic nerve and its branches only on the treated side.

At the level of the DRG, tensioning techniques resulted in a decrease of Substance-P and transient receptor potential vanilloid 1 (TRPV1) expression, and an increase in m-opioid receptor expression in rats with a sciatic nerve injury [12]. These are relevant changes, as TRPV1 upregulation contributes to mechanical allodynia and thermal hyperalgesia; Substance-P plays an important role in the development of chronic pain [48] and is responsible for the development of hyperalgesia in rats [49]. Another study revealed that tensioning techniques were associated with a decrease in nerve growth factor (NGF) and glial fibrillary acidic protein (GFAP) in the DRG [12]. Suppression of NGF overproduction in the DRG has shown to be related to neuropathic pain attenuation following a chronic constriction injury to the sciatic nerve [50]. GFAP is a molecular marker for glial cell activity (astrocytes) and glial cell activation is a phenomenon linked to neuropathic pain [51]. A decrease in GFAP expression following tensioning techniques was also observed in the lumbar spinal cord, associated with a reduction in mechanical and thermal hyperalgesia and allodynia [12].

Glial cells and brain-derived neurotrophic factor (BDNF) expression are increased in the midbrain following a sciatic nerve injury causing neuropathic pain in rats [14]. Glial cells are implicated in the development of persistent pain and BDNF released from activated microglia contributes to the nociceptive transmission. Remarkably, tensioning techniques resulted in a normalization of glial cells and BDNF expression [14]. Also in the midbrain, tensioning techniques resulted in an increase in endogenous opioid receptors (Kappa-opioid receptors) in the periaqueductal gray [16]. Furthermore, the rats who received tensioning techniques had increased tibialis anterior muscle strength and better locomotion compared to injured rats who did not receive neural mobilization [16].

Tensioning techniques following a sciatic nerve crush injury or a chronic constriction injury in rats resulted in reduced intraneural scar formation [15], anti-allodynic and neurotrophic effects able to speed up the nerve regeneration processes [11,12]. A severe sciatic nerve constriction injury can cause significant axonal loss (i.e. Wallerian degeneration) distal to the injury site. Increased numbers of axons with myelin sheaths of normal thickness and less inter-axonal fibrosis after treatment (tensioning techniques) have been shown in rats compared to controls with the same sciatic nerve injury who did not receive the intervention [11]. In contrast, when injured nerves are not exposed to mechanical stimuli, nerve regeneration

diminishes due to increased intraneural scar tissue formation, leading to mechanical allodynia and hyperalgesia [11,12,15].

3. Evidence from human trials

In larger human clinical trials, neural mobilization is typically part of a multimodal intervention [52–55]. If neural mobilization is evaluated as a unimodal intervention, different neurodynamic techniques are often combined [52,53], making it impossible to isolate the effects of tensioning techniques. A few smaller studies that evaluate the immediate effects of tensioning techniques are, however, available. Tensioning techniques enhanced conditioned pain modulation in people with chronic neck pain [56]. Tensioning techniques had no effect on pain intensity in people with chronic neck pain [56], and decreased pain intensity in computer users with elbow pain [57].

Ultrasound shear wave elastography investigations in healthy young adults revealed that nerve stiffness adapts to both short and long-term tensile mechanical stimuli [58,59] (Figure 3). For example, sciatic nerve stiffness at the onset of stretch pain decreased following maintaining a nerve tension position (long-sitting, maintained twice for 3 minutes) [58]. Similarly, a randomized controlled trial revealed a decrease in sciatic and tibial nerves' stiffness following a neural tensioning style regime (total loading stimuli of 7.5 hours over 12 weeks), compared to a muscle stretching style regime [59].

There is a growing body of evidence of *in-vivo* studies supporting that mechanical properties of human peripheral nerves are altered in various peripheral neuropathies, such as those associated with nerve compression [60], metabolic syndrome [61], and radiculopathies [62–64]. Interestingly, a preliminary study reported that a 3-min slump (static) stretch resulted in an immediate decrease in sciatic nerve stiffness in people with unilateral sciatica [65].

Too much of a good thing?

As described above, mechanical loading, including tensile forces, is fundamental to maintain the homeostasis in the nervous system. However, the response is influenced by the characteristics of tensile loading, such as the magnitude, duration, rate of loading and frequency [66]. For example, 8% nerve strain exposed to an animal nerve [67,68] caused immediate reduction of intraneural blood flow, with total occlusion (leading to ischemia) at 15% strain (if sustained for up to one hour) [68–70]. Nerve conduction becomes impaired from 8% strain of an animal nerve [67] with

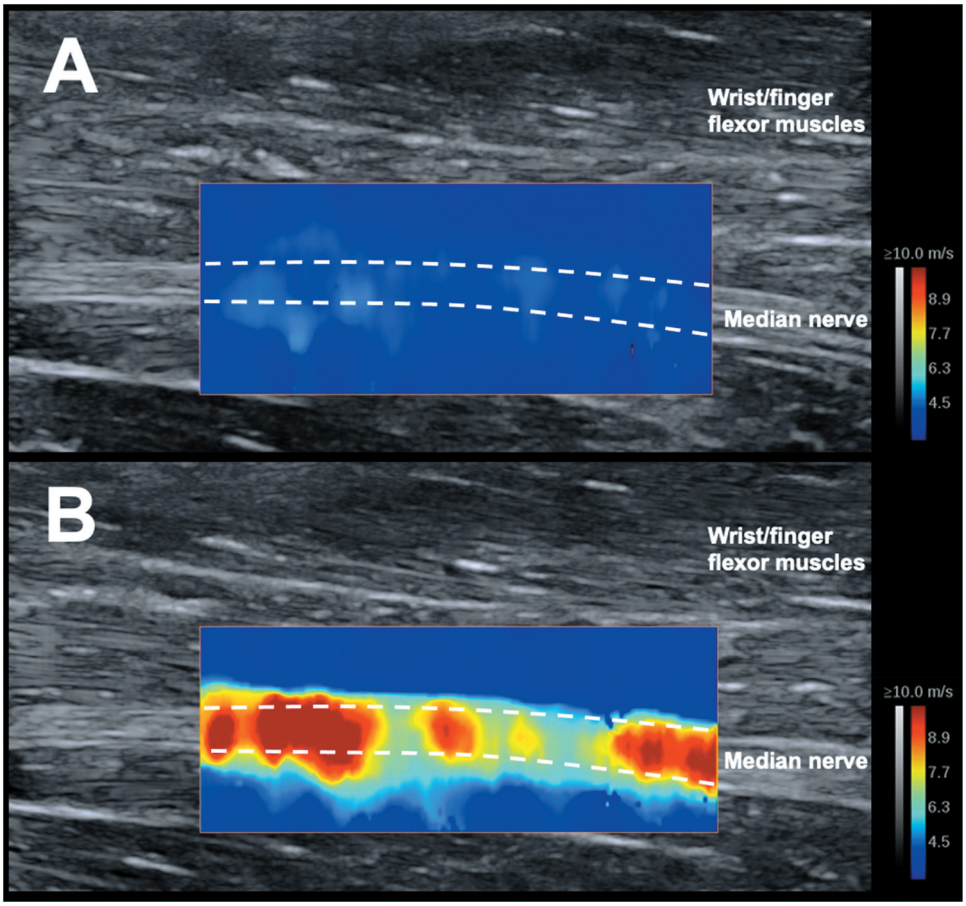

Figure 3. The median nerve, imaged using shear wave elastography, at the level of the mid-forearm when (A) relaxed and (B) when on stretch. Note: colored elastogram presents nerve shear wave velocity (metres/sec) (an index of nerve stiffness).

conduction block from 10% to 12% strain, sustained (sustained for up to one hour) [70]. Furthermore, as little as 3–5% strain of experimentally inflamed animal nerve resulted in increased neural mechanosensitivity [71]. Nerve elongation has also been shown to decrease the cross-sectional area of a peripheral nerve [72], a phenomenon believed to result in increased intraneural pressure with associated adverse effects [22,68,73]. To the authors' knowledge, there are no studies that have investigated the effect of 'excessive' tensile forces on neuroimmune responses both locally and at remote sites along the neuraxis, but it is likely that symptom flare following too much tensile loading, which often has a delayed onset, is also related to an increase in neuroinflammation.

Clinical reflections

From the research described above, what do these values of nerve tension and strain mean for clinical practice? During forward-bending in standing, sciatic nerve strain has been reported to reach 10.5% strain [74], whilst composite movements of the upper limb expose the median [20], radial [19] and ulnar [18] nerves to 18%, 12% and 10% strain respectively. During neurodynamic testing, it has been reported that the straight-leg raise test increased tibial nerve strain to 12.5%, whilst the median nerve neurodynamic test exposed the distal median nerve (proximal to the wrist) to 4% strain [17]. However, some care is required when interpreting these values. Comparison of nerve strain between and within different methods (i.e. cadaver versus animal) is difficult as strain calculations require an accurate measure of nerve length at rest, and this is different between studies, which may explain the sometimes vastly different strain values reported in the literature.

Systematic reviews with and without meta-analyses reveal the clinical efficacy of neurodynamic techniques (which typically combine sliding and tensioning exercises, and techniques to mobilize the structures that surround the nervous system) for various conditions [75–79]. Basic science studies revealed the potential working mechanisms of neurodynamic techniques (see above). However, the translation of these findings to clinical recommendations remains challenging, if not impossible. For example, all studies that used animal models to document the effects of tensioning techniques used nerve injury models that are much more severe than the neuropathies patients who seek physiotherapy care present with. The nerve injuries are also uniformly the same for all animals, with a selective lesion to the nerve without damage to joints or intervertebral discs, and animals are typically young without comorbidities.

It has also been difficult to directly determine nerve strain from human research, which presents a significant limitation (to date) when interpreting the effects of tensile forces upon the nervous system. *In-vivo*, human studies have examined shear-strain at the nerve-muscle interface [80], ultrasound shear wave elastography to measure nerve shear wave velocity (an index of nerve stiffness) [59,81] (Figure 3), and indirect measures of nerve strain calculated from excursion values [74]. Future technologies that allow direct quantification of nerve strain through human research will be of great interest to this field.

The rate of tension application is important to consider also. It is believed that sudden stretching trauma has the potential to be more damaging compared to more gradual tension applied within appropriate limits [67]. For example, fast eccentric muscle contractions have been shown to induce functional and structural damage in interfacing animal [82,83] and human [84] nerves. Clinically, neurodynamic techniques are typically promoted to be performed as dynamic techniques rather than sustained or static holds [85,86]. Furthermore, tensioning techniques are promoted within limits of perceived passive resistance [53,87], a feature that has been adopted also in animal models with beneficial results [27,34]. Making evidence-based recommendations regarding the precise frequency and number of repetitions of neurodynamic techniques, including tensioning techniques, is problematic given the large variability reported in the literature [85,88]. For example, a systematic review of lower-limb neural mobilization techniques for healthy people and people with low back pain revealed technique application duration between 60 and 300 seconds with repetitions ranging between 1 and 45 [88].

As mentioned earlier, tensioning techniques are considered more biomechanically challenging than sliding techniques, and as such careful clinical reasoning is essential when using and prescribing them, particularly in regard to mitigating symptom flare and/or clinical deterioration [79]. Although there are many benefits of tensile loading to the nervous system, there is also a risk that movement-based techniques, in particular tensioning techniques, may be temporarily aggravating peripheral neuropathic pain [89].

Conclusion

The studies summarized within this article illustrate the beneficial effects of optimal mechanical load to healthy and pathological nerves. The research shows that repeated mechanical tension on animal peripheral nerves applied by tensioning techniques

has positive effects on nerve biomechanics, nerve repair and nerve regeneration processes, promoting multi-level changes in the peripheral and central nervous systems. Notably, these changes are induced in the cells of the peripheral and central nervous system and are linked to pain modulation and normal nerve function restoration. However, the optimal tension dosage to apply in clinical conditions remains the challenge. Not enough tension may result in little effects, whereas too much tension will undoubtedly exacerbate symptoms. It should be noted, that the optimal dosage for applying nerve tension in people with neuropathies has not been established and is unlikely to be established any time soon. A dosage that works well in one patient, may be too much or too little for another person. Therefore, judicious use of neurodynamic techniques, including tensioning techniques, should always consider multiple aspects and how they interact, such as the clinical presentation, nature of the symptoms, levels of irritability, pathophysiology, stages of healing, beliefs of the patient. Frequent re-assessments continue to play an important role.

Acknowledgments

Our thanks to Lilah Ellis for the provision of illustrations for Figure 1.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The author(s) reported there is no funding associated with the work featured in this article.

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Ricardo is a Griffith University Postdoctoral Research Fellow at Menzies Health Institute Queensland and a member of Griffith Centre of Biomedical and Rehabilitation Engineering (GCORE) in Australia. Ricardo's personal research interests are in understanding the integrated contributions of structure and biomechanics of peripheral nerves to neural function, dysfunction, and regeneration. His research expertise spans neuromusculoskeletal biomechanics, advanced neuroimaging methods, and clinical neurophysiology. Ricardo's current research focuses on understanding the mechanisms underlying nerve damage in various peripheral neuropathies, with a particular focus on chemotherapy-induced peripheral neuropathy, and seeks to enhance the early diagnosis and management of peripheral neuropathies and chronic neuropathic pain.

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Michel is Menzies Foundation Professor of Health Research at Griffith University in Brisbane (Australia), where he directs the Musculoskeletal Health and Persistent Pain Research Group (https://mskhealthlab.com.au). The Group's state-of-the-art research facility has quickly become one of the best pain labs in Australia and beyond. Although the focus has traditionally been on common musculoskeletal conditions, such as back pain and neck pain, we also research important conditions and side-effects that affect the musculoskeletal system, such as diabetic neuropathy, spinal cord injury and chemotherapy-induced peripheral neuropathy. We conduct lab-based research, as well as clinical research in the community, primary care, and hospital settings. Our research aims to (1) better understand the pathobiology of musculoskeletal and neurological conditions, (2) improve diagnostic procedures, (3) develop and evaluate biologically plausible interventions and (4) assess whether success of these interventions can be explained by reversal of the original pathobiological processes. Michel has successfully supervised many national and international PhD students. He has an excellent track record in mentoring early and mid-career researchers and clinicians, and is well-respected nationally and internationally.

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References

[1] Coppieters M, and Nee RJ. Neurodynamic management of the peripheral nervous system. In:Jull G, Moore A, and Falla D, et al. editors. Grieve's Modern Musculoskeletal Physiotherapy. 4th ed Elsevier:2015; 287–297 .
[2] Coppieters MW, Butler DS. Do 'sliders' slide and 'tensioners' tension? An analysis of neurodynamic techniques and considerations regarding their application. Manual Ther. 2008;13(3):213–221.
[3] Coppieters MW, Andersen LS, Johansen R, et al. Excursion of the sciatic nerve during nerve mobilization exercises: an in vivo cross-sectional study using dynamic ultrasound imaging. J Orthop Sports Phys Ther. 2015;45(10):731–737.
[4] Ellis RF, Hing WA, McNair PJ. Comparison of longitudinal sciatic nerve movement with different mobilization exercises: an in vivo study utilizing ultrasound imaging. J Orthop Sports Phys Ther. 2012;42(8):667–675.
[5] Coppieters MW, Alshami AM. Longitudinal excursion and strain in the median nerve during novel nerve gliding exercises for carpal tunnel syndrome. J Orthop Res. 2007;25(7):972–980.
[6] Coppieters MW, Alshami AM, Babri AS, et al. Strain and excursion of the sciatic, tibial, and plantar nerves during a modified straight leg raising test. J Orthop Res. 2006;24(9):1883–1889.
[7] Nee RJ, Jull GA, Vicenzino B, et al. The validity of upper-limb neurodynamic tests for detecting peripheral neuropathic pain. J Orthop Sports Phys Ther. 2012;42(5):413–424.
[8] Coppieters MW, Hough AD, Dilley A. Different nerve-gliding exercises induce different magnitudes of median nerve longitudinal excursion: an in vivo study using dynamic ultrasound imaging. J Orthop Sports Phys Ther. 2009;39(3):164–171.
[9] Ellis R, Blyth R, Arnold N, et al. Is there a relationship between impaired median nerve excursion and carpal tunnel syndrome? A systematic review. J Hand Ther. 2017;30(1):3–12.
[10] Thoomes E, Ellis R, Dilley A, et al. Excursion of the median nerve during a contra-lateral cervical lateral glide movement in people with and without cervical radiculopathy. Musculoskelet Sci Pract. 2021;52. DOI:10.1016/j.msksp.2021.102349.
[11] Da Silva JT, Dos Santos FM, Giardini AC, et al. Neural mobilization promotes nerve regeneration by nerve growth factor and myelin protein zero increased after sciatic nerve injury. Growth Factors. 2015;33(1):8–13.
[12] Santos FM, Silva JT, Giardini AC, et al. Neural mobilization reverses behavioral and cellular changes that characterize neuropathic pain in rats. Mol Pain. 2012;8. DOI:10.1186/1744-8069-8-57.
[13] Zhu GC, Tsai KL, Chen YW, et al. Neural mobilization attenuates mechanical allodynia and decreases proinflammatory cytokine concentrations in rats with painful diabetic neuropathy. Phys Ther. 2018;98(4):214–222.
[14] Giardini AC, Santos FMD, Da Silva JT, et al. Neural mobilization treatment decreases glial cells and brain-derived neurotrophic factor expression in the central nervous system in rats with neuropathic pain induced by CCI in rats. Pain Res Manag. 2017;2017. DOI:10.1155/2017/7429761.
[15] Ê L, Santana H, Medrado A, et al. Neurodynamic mobilization reduces intraneural fibrosis after sciatic crush lesion in rats. Braz J Med Human Health. 2017;5(2):54–62.
[16] Santos FM, Grecco LH, Pereira MG, et al. The neural mobilization technique modulates the expression of endogenous opioids in the periaqueductal gray and improves muscle strength and mobility in rats with neuropathic pain. Behav Brain Funct. 2014;10(1):1.
[17] Nee RJ, Yang CH, Liang CC, et al. Impact of order of movement on nerve strain and longitudinal excursion: abiomechanical study with implications for neurodynamic test sequencing. Manual Ther. 2010;15 (4):376–381.
[18] Wright TW, Glowczewskie F, Cowin D, et al. Ulnar nerve excursion and strain at the elbow and wrist associated with upper extremity motion. J Hand Surg Am. 2001;26A(4):655–662.
[19] Wright TW, Glowczewskie F, Cowin D, et al. Radial nerve excursion and strain at the elbow and wrist associated with upper-extremity motion. J Hand Surg Am. 2005;30(5):990–996.
[20] Wright TW, Glowczewskie F, Wheeler D, et al. Excursion and strain of the median nerve. J Bone Joint Surg Am. 1996;78(12):1897–1903.
[21] Ma Z, Hu S, Tan JS, et al. In vitro and in vivo mechanical properties of human ulnar and median nerves. J Biomed Mater Res A. 2013;101(9):2718–2725.
[22] Topp KS, Boyd BS. Structure and biomechanics of peripheral nerves: nerve responses to physical stresses and implications for physical therapist practice. Phys Ther. 2006;86(1):92–109.
[23] Shah SB. Tissue biomechanics: whales have some nerve. Curr Biol. 2017 Mar 06;27(5):R177–R179.
[24] Phillips JB, Smit X, De Zoysa N, et al. Peripheral nerves in the rat exhibit localized heterogeneity of tensile properties during limb movement. J Physiol. 2004;557(3):879–887.
[25] Mason S, Phillips JB. An ultrastructural and biochemical analysis of collagen in rat peripheral nerves: the relationship between fibril diameter and mechanical properties. J Peripher Nerv Syst. 2011;16(3):261–269.
[26] Sung J, Sikora-Klak J, Adachi SY, et al. Decoupled epineurial and axonal deformation in mouse median and ulnar nerves. Muscle Nerve. 2019;59(5):619–628.
[27] Carta G, Gambarotta G, Fornasari BE, et al. The neurodynamic treatment induces biological changes in sensory and motor neurons in vitro. Sci Rep. 2021;11. DOI:10.1038/s41598-021-92682-2.
[28] Panciera T, Azzolin L, Cordenonsi M, et al. Mechanobiology of YAP and TAZ in physiology and disease. Nat Rev Mol Cell Biol. 2017;18(12):758–770.
[29] Passini FS, Jaeger PK, Saab AS, et al. Shear-stress sensing by PIEZO1 regulates tendon stiffness in rodents and influences jumping performance in humans. Nat Biomed Eng. 2021. DOI:10.1038/s41551-021-00716-x.
[30] Higgins S, Lee JS, Ha L, et al. Inducing neurite outgrowth by mechanical cell stretch. Biores Open Access. 2013;2(3):212–216.
[31] van Wilgen CP, Testa M. Giving insight in pain research of tomorrow! Pain Rep. 2019;4(3):e753.

[32] Zhang M, Wang Y, Geng J, et al. Mechanically activated piezo channels mediate touch and suppress acute mechanical pain response in mice. Cell Rep. 2019;26 (6):1419–1431.e4.
[33] Loverde JR, Ozoka VC, Aquino R, et al. Live imaging of axon stretch growth in embryonic and adult neurons. J Neurotrauma. 2011;28(11):2389–2403.
[34] Loverde JR, Pfister BJ. Developmental axon stretch stimulates neuron growth while maintaining normal electrical activity, intracellular calcium flux, and somatic morphology. Front Cell Neurosci. 2015;9. DOI:10.3389/fncel.2015.00308
[35] Pfister BJ, Iwata A, Taylor AG, et al. Development of transplantable nervous tissue constructs comprised of stretch-grown axons. J Neurosci Meth. 2006;153 (1):95–103.
[36] Gladman SJ, Ward RE, Michael-Titus AT, et al. The effect of mechanical strain or hypoxia on cell death in subpopulations of rat dorsal root ganglion neurons in vitro. Neuroscience. 2010;171(2):577–587.
[37] Zhou M, Hu M, He S, et al. Effects of RSC96 schwann cell-derived exosomes on proliferation, senescence, and apoptosis of dorsal root ganglion cells in Vitro. Med Sci Monit. 2018;24:7841–7849.
[38] Kampanis V, Tolou-Dabbaghian B, Zhou L, et al. Cyclic stretch of either PNS or CNS located nerves can stimulate neurite outgrowth. Cells. 2020;10(1):1.
[39] Calvo M, Bennett DLH. The mechanisms of microgliosis and pain following peripheral nerve injury. Exp Neurol. 2012;234(2):271–282.
[40] Hu P, McLachlan EM. Macrophage and lymphocyte invasion of dorsal root ganglia after peripheral nerve lesions in the rat. Neuroscience. 2002;112(1):23–38.
[41] Watkins LR, Maier SF. Beyond neurons: evidence that immune and glial cells contribute to pathological pain states. Physiol Rev. 2002;82(4):981–1011.
[42] Mor D, Bembrick AL, Austin PJ, et al. Anatomically specific patterns of glial activation in the periaqueductal gray of the sub-population of rats showing pain and disability following chronic constriction injury of the sciatic nerve. Neuroscience. 2010;166 (4):1167–1184.
[43] Schmid AB, Nee RJ, Coppieters MW. Reappraising entrapment neuropathies-Mechanisms, diagnosis and management. Manual Ther. 2013;18(6):449–457.
[44] Fiore NT, Austin PJ. Are the emergence of affective disturbances in neuropathic pain states contingent on supraspinal neuroinflammation? Brain Behav Immun. 2016;56:397–411.
[45] Chapman CR, Tuckett RP, Song CW. Pain and stress in a systems perspective: reciprocal neural, endocrine, and immune interactions. J Pain. 2008;9(2):122–145.
[46] Albrecht DS, Ahmed SU, Kettner NW, et al. Neuroinflammation of the spinal cord and nerve roots in chronic radicular pain patients. Pain. 2018;159(5):968–977.
[47] Lutke Schipholt I, Coppieters M, Meijer O, et al. Effects of joint and nerve mobilisation on neuroimmune responses in animals and humans with neuromusculoskeletal conditions: a systematic review and meta-analysis. Pain Rep. 2021;6(2):e927.
[48] Gao YJ, Zhang YQ, Zhao ZQ. Involvement of spinal neurokinin-1 receptors in the maintenance but not induction of carrageenan-induced thermal hyperalgesia in the rat. Brain Res Bull. 2003;61(6):587–593.
[49] Hoot MR, Sim-Selley LJ, Selley DE, et al. Chronic neuropathic pain in mice reduces μ-opioid receptor-mediated G-protein activity in the thalamus. Brain Res. 2011;1406:1–7.
[50] Dai WL, Yan B, Bao YN, et al. Suppression of peripheral NGF attenuates neuropathic pain induced by chronic constriction injury through the TAK1-MAPK/NF-κB signaling pathways. Cell Commun Signal. 2020;18(1):1.
[51] Dubový P, Klusáková I, Hradilová-Svíženská I, et al. Activation of astrocytes and microglial cells and CCL2/CCR2 upregulation in the dorsolateral and ventrolateral nuclei of periaqueductal gray and rostral ventromedial medulla following different types of sciatic nerve injury. Front Cell Neurosci. 2018;12. DOI:10.3389/fncel.2018.00040.
[52] Ferreira G, Stieven F, Araujo F, et al. Neurodynamic treatment did not improve pain and disability at two weeks in patients with chronic nerve-related leg pain: a randomised trial. J Physiother. 2016;62(4):197–202.
[53] Nee RJ, Vicenzino B, Jull GA, et al. Neural tissue management provides immediate clinically relevant benefits without harmful effects for patients with nerve-related neck and arm pain: a randomised trial. J Physiother. 2012;58(1):23–31.
[54] Wolny T, Linek P. Long-term patient observation after conservative treatment of carpal tunnel syndrome: a summary of two randomised controlled trials. PeerJ. 2019;2019:11.
[55] Ayub A, Osama M, Shakil UR, et al. Effects of active versus passive upper extremity neural mobilization combined with mechanical traction and joint mobilization in females with cervical radiculopathy: a randomized controlled trial. J Back Musculoskelet Rehabil. 2019;32(5):725–730.
[56] Fernandez-Carnero J, Sierra-Silvestre E, Beltran-Alacreu H, et al. Neural tension technique improves immediate conditioned pain modulation in patients with chronic neck pain: a randomized clinical trial. Pain Med. 2019;20(6):1227–1235.
[57] Arumugam V, Selvam S, MacDermid JC. Radial nerve mobilization reduces lateral elbow pain and provides short-term relief in computer users. Open Orthop J. 2014;17(8):368–371.
[58] Andrade RJ, Freitas SR, Hug F, et al. The potential role of sciatic nerve stiffness in the limitation of maximal ankle range of motion. Sci Rep. 2018;8(1):14532.
[59] Andrade RJ, Freitas SR, Hug F, et al. Chronic effects of muscle and nerve-directed stretching on tissue mechanics. J Appl Physiol. 2020;129(5):1011–1023.
[60] Kantarci F, Ustabasioglu FE, Delil S, et al. Median nerve stiffness measurement by shear wave elastography: a potential sonographic method in the diagnosis of carpal tunnel syndrome. Eur Radiol. 2014;24(2):434–440.
[61] Dikici AS, Ustabasioglu FE, Delil S, et al. Evaluation of the tibial nerve with shear-wave elastography: a potential sonographic method for the diagnosis of diabetic peripheral neuropathy. Radiology. 2017;282(2):494–501.
[62] Celebi UO, Burulday V, Ozveren MF, et al. Sonoelastographic evaluation of the sciatic nerve in patients with unilateral lumbar disc herniation. Skeletal Radiol. 2019;48(1):129–136.
[63] Neto T, Freitas SR, Andrade RJ, et al. Noninvasive measurement of sciatic nerve stiffness in patients with chronic low back related leg pain using shear wave elastography. J Ultrasound Med. 2019;38(1):157–164.

[64] Wang Q, Zhang H, Zhang J, et al. The relationship of the shear wave elastography findings of patients with unilateral lumbar disc herniation and clinical characteristics. BMC Musculoskelet Disord. 2019;20(1):438.
[65] Neto T, Freitas SR, Andrade RJ, et al. Shear wave elastographic investigation of the immediate effects of slump neurodynamics in people with sciatica. J Ultrasound Med. 2020;39(4):675–681.
[66] Bueno FR, Shah SB. Implications of tensile loading for the tissue engineering of nerves. Tissue Eng Part B Rev. 2008;14(3):219–233.
[67] Driscoll PJ, Glasby MA, Lawson GM. An in vivo study of peripheral nerves in continuity: biomechanical and physiological responses to elongation. J Orthop Res. 2002;20(2):370–375.
[68] Lundborg G, Rydevik B. Effects of stretching the tibial nerve of the rabbit. A preliminary study of the intraneural circulation and the barrier function of the perineurium. J Bone Joint Surg Br. 1973;55(2):390–401.
[69] Ogata K, Naito M. Blood flow of peripheral nerve effects of dissection, stretching and compression. J Hand Surg. 1986;11B(1):10–14.
[70] Wall EJ, Massie JB, Kwan MK, et al. Experimental stretch neuropathy. Changes in nerve conduction under tension. J Bone Joint Surg Br. 1992;74-B (1):126–129.
[71] Dilley A, Lynn B, Pang SJ. Pressure and stretch mechanosensitivity of peripheral nerve fibres following local inflammation of the nerve trunk. Pain. 2005;117 (3):462–472.
[72] Pagnez MAM, Corrêa LA, Almeida RS, et al. The variation of cross-sectional area of the sciatic nerve in flexion-distraction technique: a cross-sectional study. J Manipulative Physiol Ther. 2019;42(2):108–116.
[73] Millesi H, Zoch G, and Reihsner R. Mechanical properties of peripheral nerves. Clin Orthop Relat Res. 1995; 5 (314):76–83.
[74] Ellis R, Richards N, Archer L, et al. Assessing sciatic nerve excursion and strain with ultrasound imaging during forward bending. Ultrasound Med Biol. 2021;47 (9):2560–2569.
[75] Basson A, Olivier B, Ellis R, et al. The effectiveness of neural mobilization for neuromusculoskeletal conditions: a systematic review and meta-Analysis. J Orthop Sports Phys Ther. 2017;47(9):593–615.
[76] Ellis RF, Hing WA. Neural mobilization: a systematic review of randomized controlled trials with an analysis of therapeutic efficacy. J Man Manip Ther. 2008;16 (1):8–22.
[77] Lim YH, Chee DY, Girdler S, et al. Median nerve mobilization techniques in the treatment of carpal tunnel syndrome: a systematic review. J Hand Ther. 2017;30 (4):397–406.
[78] Neto T, Freitas SR, Andrade RJ, et al. Sciatic nerve stiffness is not changed immediately after a slump neurodynamics technique. Muscles Ligaments Tendons J. 2017;7(3):583–589.
[79] Su Y, Lim ECW. Does evidence support the use of neural tissue management to reduce pain and disability in nerve-related chronic musculoskeletal pain? A systematic review with meta-analysis. Clin J Pain. 2016;32(11):991–1104.
[80] Ellis R, Rohan M, Hitt JR, et al. Ultrasound elastography measurement of sciatic nerve displacement and shear strain during active and passive knee extension. J Ultrasound Med. 2018;37(8):2091–2103.
[81] Andrade RJ, Nordez A, Hug F, et al. Non-invasive assessment of sciatic nerve stiffness during human ankle motion using ultrasound shear wave elastography. J Biomech. 2016;49(3):326–331.
[82] Kouzaki K, Kobayashi M, Nakamura KI, et al. Repeated bouts of fast eccentric contraction produce sciatic nerve damage in rats. Muscle Nerve. 2016;54 (5):936–942.
[83] Lee K, Kouzaki K, Ochi E, et al. Eccentric contractions of gastrocnemius muscle-induced nerve damage in rats. Muscle Nerve. 2014;50(1):87–94.
[84] Ochi E, Ueda H, Tsuchiya Y, et al. Eccentric contraction–induced muscle damage in human flexor pollicis brevis is accompanied by impairment of motor nerve. Scand J Med Sci Sports. 2020;30(3):462–471.
[85] Ridehalgh C, Barnard K. Principles of nerve treatment. In: Petty NJ, Barnard K, editors. Principles of musculoskeletal treatment and management: a handbook for therapists. 3rd ed ed. Edinburgh: Elsevier; 2018. p. 180–194.
[86] Shacklock MO. Clinical neurodynamics: a new system of neuromusculoskeletal treatment. Oxford: Butterworth Heinemann; 2005.
[87] Rodríguez-Sanz D, López-López D, Unda-Solano F, et al. Effects of median nerve neural mobilization in treating cervicobrachial pain: a randomized waiting list–controlled clinical trial. Pain Pract. 2018;18(4):431–442.
[88] Neto T, Freitas SR, Marques M, et al. Effects of lower body quadrant neural mobilization in healthy and low back pain populations: a systematic review and meta-analysis. Musculoskelet Sci Pract. 2017;27:14–22.
[89] Zusman M. Mechanisms of peripheral neuropathic pain: implications for musculoskeletal physiotherapy. Phys Ther Rev. 2008;13(5):313–323.

3.2 Paper n°2

The neurodynamic treatment induces biological changes in sensory and motor neurons in vitro

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Scientific Reports, 2021

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The neurodynamic treatment induces biological changes in sensory and motor neurons in vitro

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Nerves are subjected to tensile forces in various paradigms such as injury and regeneration, joint movement, and rehabilitation treatments, as in the case of neurodynamic treatment (NDT). The NDT induces selective uniaxial repeated tension on the nerve and was described to be an effective treatment to reduce pain in patients. Nevertheless, the biological mechanisms activated by the NDT promoting the healing processes of the nerve are yet still unknown. Moreover, a dose–response analysis to define a standard protocol of treatment is unavailable. In this study, we aimed to define in vitro whether NDT protocols could induce selective biological effects on sensory and motor neurons, also investigating the possible involved molecular mechanisms taking a role behind this change. The obtained results demonstrate that NDT induced significant dose-dependent changes promoting cell differentiation, neurite outgrowth, and neuron survival, especially in nociceptive neurons. Notably, NDT significantly upregulated PIEZO1 gene expression. A gene that is coding for an ion channel that is expressed both in murine and human sensory neurons and is related to mechanical stimuli transduction and pain suppression. Other genes involved in mechanical allodynia related to neuroinflammation were not modified by NDT. The results of the present study contribute to increase the knowledge behind the biological mechanisms activated in response to NDT and to understand its efficacy in improving nerve regenerational physiological processes and pain reduction.

Every day, nerves are subjected to tensile forces as a consequence of joint movements or rehabilitation treatments after a nerve injury[1]. In many cases, the positive effects of applying tensile forces on nerves are exploited to facilitate the healing process[2–8]. For example, the neurodynamic treatment (NDT), which is a non-pharmacological intervention consisting of a combination of physiological movements that induce selective repeated uniaxial tension on the nerve[2,9,10], was described to be an effective treatment to reduce pain in drug-resistant sciatica patients[11,12] since the end of the eighteenth century. Several clinical trials have shown that NDT is an effective treatment to increase nerve functions and reduce pain in patients with neuropathies of the somatic nerves[13–17]. Also, several preclinical studies on murine sciatic neuropathy models have shown that NDT resolves mechanical and thermal allodynia, which are very common and disabling symptoms among neuropathic pain patients[18]. NDT affects the protein expression of Nerve Growth Factor (NGF) in the homologous dorsal root ganglia (DRG) and the spinal cord metameres involved by the nerve lesion[3,19,20]. Although there is a growing understanding of how mechanical forces influence tissues[21,22], there is quite poor evidence on biological processes involved during this phenomenon on nerves. The recent discoveries on nerve-related neuropathic models have identified the specific gene expression in the DRG and the spinal cord related to neuropathic pain and mechanical allodynia. TLR2 (Toll-like receptor-2) and YAP (Yes-associated protein) are significantly upregulated in the DRG neurons in several neuropathic pain models, and their suppression is linked to the suppression of mechanical allodynia[23–25]. In particular, TLR2, is a receptor mediating the macrophage recognition of ligands from microbes that promote inflammation in the DRG and spinal cord neurons. Several studies have shown that TRL2 depletion induces mechanical allodynia suppression through the signalling pathway of myeloid differentiation factor-88 adaptor

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protein (MyD88)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in DRG cells[26]. YAP is a transcriptional regulator, its overexpression and nuclear accumulation in the DRG and spinal cord neurons, like motor neurons, is associated to neuroinflammation after a nerve injury and neuropathic pain leading to mechanical hypersensitivity in naive animals[24,27]. Also, the nerve injury-dependent mechanical allodynia is suppressed in YAP knock-down rats and using siRNA against YAP in the spinal cord. The expression of c-JUN (a gene involved in nerve degeneration) in the DRG and the spinal cord has been linked to neuropathic pain conditions and neuroinflammation[28,29]. Also, c-Jun in these neurons has been identified as one of the main hub genes in several neuropathic pain conditions and its suppression is linked to pain relief in several neuropathic pain animal models[30–33]. The gene expression in DRG neurons encoding for mechanosensitive ion channels has been shown to affect the perception of mechanical painful stimuli. In particular, TACAN suppression, an ion channel that is co-localized with TRPV1 (transient receptor potential cation channel subfamily V member 1) in non-peptidergic nociceptors in the human and murine DRGs[34–36], is significantly linked to the suppression of mechanical hyperalgesia induced by local and systemic inflammation[36]. Furthermore, the upregulation in the DRG of PIEZO1, an ion channel that is responsible for the sensation of non-noxious stretch and compression forces, is linked to the mechanical pain suppression in humans and animal models of neuropathic pain[37–39].

NDT protocols available in literature are extremely heterogeneous concerning the number of repeated stimuli (from three to three series of 60 repetitions)[14,16], the amount of nerve elongation (from 0.8 to 15% of the total nerve rest length)[40–42], the elongation speed (from one to five seconds)[6,42] and the time of stretch duration (from 1 to 30 s)[16,42]. Despite the positive results obtained from clinical and preclinical studies that link NDT-induced pain reduction and allodynia improvement to changes in the peripheral and central nervous system, fundamental aspects to obtain standardized treatment protocols have not yet been defined. To overcome this lack of knowledge, the aim of the present research is threefold: (1) to define whether a single set of repeated mechanical stimuli could induce biological and morphological changes in neurons; (2) to define whether NTD could lead to negative effects on neurons and (3) to investigate whether those changes have a dose-dependent behavior.

In the field of biomedical research in vitro experiments are mandatory to avoid animal use as much as possible[9]. Nevertheless, to obtain in vitro data suitable to be translated in humans, it is fundamental to adopt a biological model with the same features of human cells. Taking into account this aspect, to assess the effects of NDT, we selected a mouse motor neuron-like cell line (NSC-34)[43–46] and a rat nociceptive sensory neuron-like cell line (50B11)[47,48] as biological models. Indeed, although not all the biological processes are shared among different species, in our research we focused on genes linked to neuropathic pain and stress mechanisms that are expressed in mice, rats, and humans[35,37,49–51].

Results

Effects of neurodynamic treatment protocols on cell morphology. Morphological analyses were performed to assess specifically the possible beneficial or toxic effects of NDT protocols on cell differentiation, a process that is related to nerve healing and sensitive to environmental stimuli[2,45,47,52–55]. Also, effects on neurite growth, a process that is fundamental for neural repair and target tissue re-innervation[7,45,47], and neurites orientation[2,56–61].

Representative images of NSC-34 (motor) and 50B11 (sensory) neurons for each type of experimental protocol are reported in Fig. 1A. A significant and dose-dependent response of NDT protocols was detectable for both cell lines (Fig. 1B). The differentiation ratio was significantly higher in NSC-34 cells treated with both LR and HR protocols (Fig. 1B, Table 1 Supplementary for statistical analysis) than in cells belonging to CTR IN or CTR OUT groups. In particular, the median value of the differentiation ratio was 12.00 points (95% CI: 5.25–25) in the LR protocol, 8.6 points (95% CI: 5.5–30) in the HR protocol, and 2.11 points in the CTR OUT protocol (95% CI: 1.33–4). The CTR IN protocol differentiation ratio of 2.00 points (95% CI: 1.47–2) was not statistically different from the CTR OUT. Similar results were detected in 50B11 cells with a significantly higher differentiation rate in LR and HR protocols compared to CTR IN and CTR OUT groups (Fig. 1B, Table 1 Supplementary for statistical analysis). Notably, the differentiation ratio of the LR group was significantly higher than the CTR OUT protocols of 11.33 points and of 7.29 points in the HR protocol. Also, the CTR IN protocol has shown a differentiation ratio significantly higher than the CTR OUT protocol of about 5.19 points.

No significant differences were detected on neurite length for all experimental groups compared to CTR OUT in motor neurons (Fig. 1B, Table 1 Supplementary for statistical analysis). However, a significantly higher neurite length of about 60 µm was detected for the LR and HR protocols compared to the CTR IN group. Also, the sensory neurons treated with LR and HR protocols had significantly longer neurites when compared to those observed in the CTR OUT protocol. The neurites were significantly longer than those of the CTR OUT protocol by about 63.03 µm in the LR protocol and by about 31.28 µm in the HR protocol. Interestingly, the neurites length of the CTR IN protocol was significantly greater than that of the CTR OUT protocol of about 35.62 µm. No significant differences were detected between all protocols on neurites orientation for motor and sensory neurons (Table 1 Supplementary for statistical analysis). In an experimental design where the neuronal models received two different NDT protocols where the CTR OUT represents the sham group, the results showed that both NDT treatments induce a significant increase in cell differentiation and neurites outgrowth, while neurite orientation is not affected.

Effects of neurodynamic treatment protocols on cell apoptosis. Proteins were analyzed to assess specifically the possible beneficial or deleterious effects of NDT protocols on neuron survival and death. In particular, the expression of Bax (a pro-apoptotic protein) and Bcl2 (an anti-apoptotic protein) was quantified. A not-pro-apoptotic behavior was induced on sensory and motor neurons by NDT protocols showing the absence of negative effects on cell survival (Fig. 2). Surprisingly, the Bax and Bcl-2 ratio revealed relevant changes in the

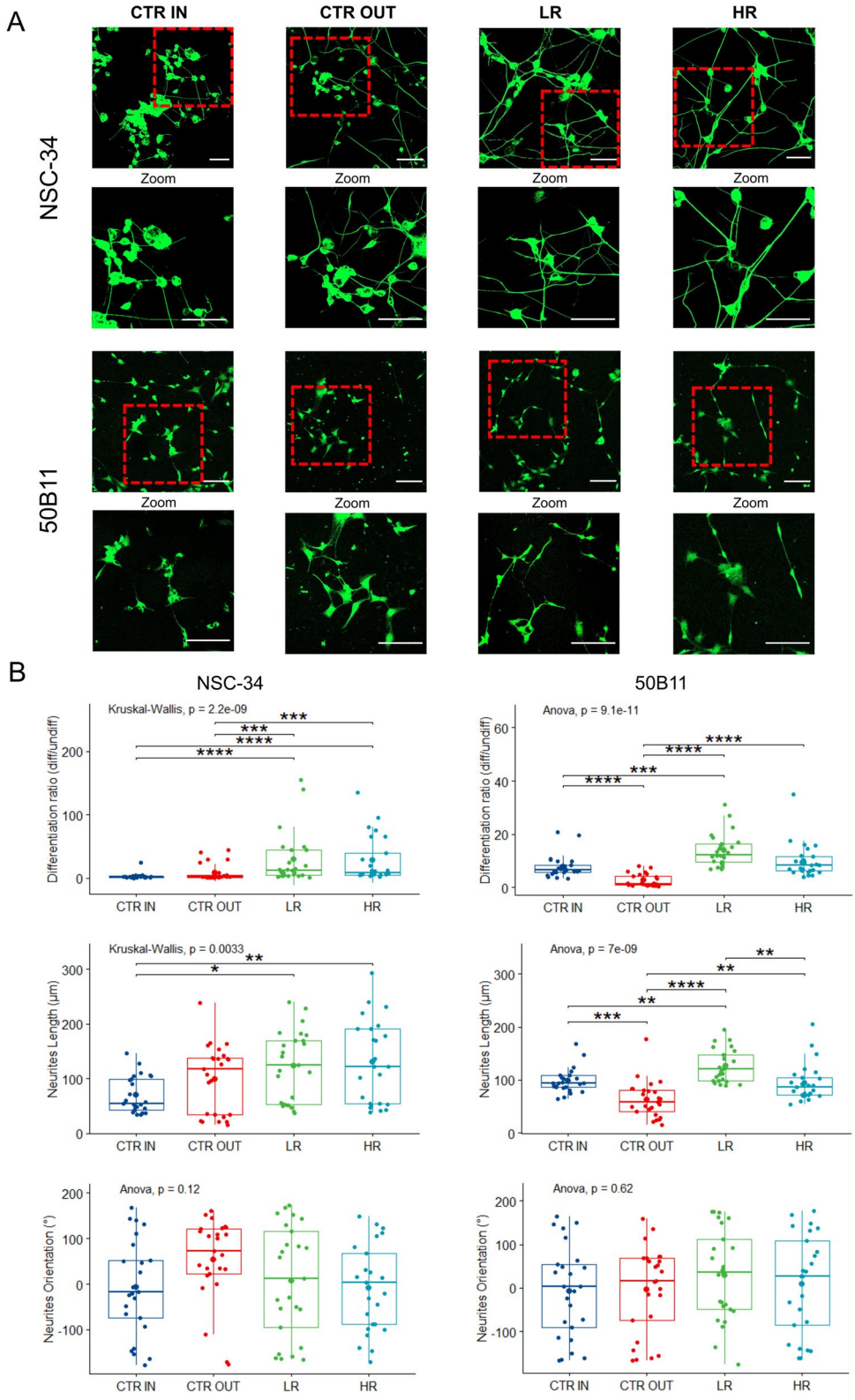

Figure 1. Effects of different neurodynamic treatment protocols on cell morphology. (**A**) Representative images of NSC-34 and 50B11 neurons stained with βIII-tubulin are reported for each type of experimental protocol. Scale bar: 100 µm. Red boxed regions correspond to zoom panels (down), which highlight the cells features following the treatment. (**B**) Quantitative analysis of differentiation ratio, neurites length, and neurites orientation. Values in the graphics are expressed as mean ± SD. For normally distributed data with comparable variances, One-way ANOVA was carried out, while Kruskal–Wallis-test was used for nonparametric data; asterisks show statistically significant differences; $p \leq 0.01$, $^{***}p \leq 0.001$, and $^{****}p \leq 0.000$.

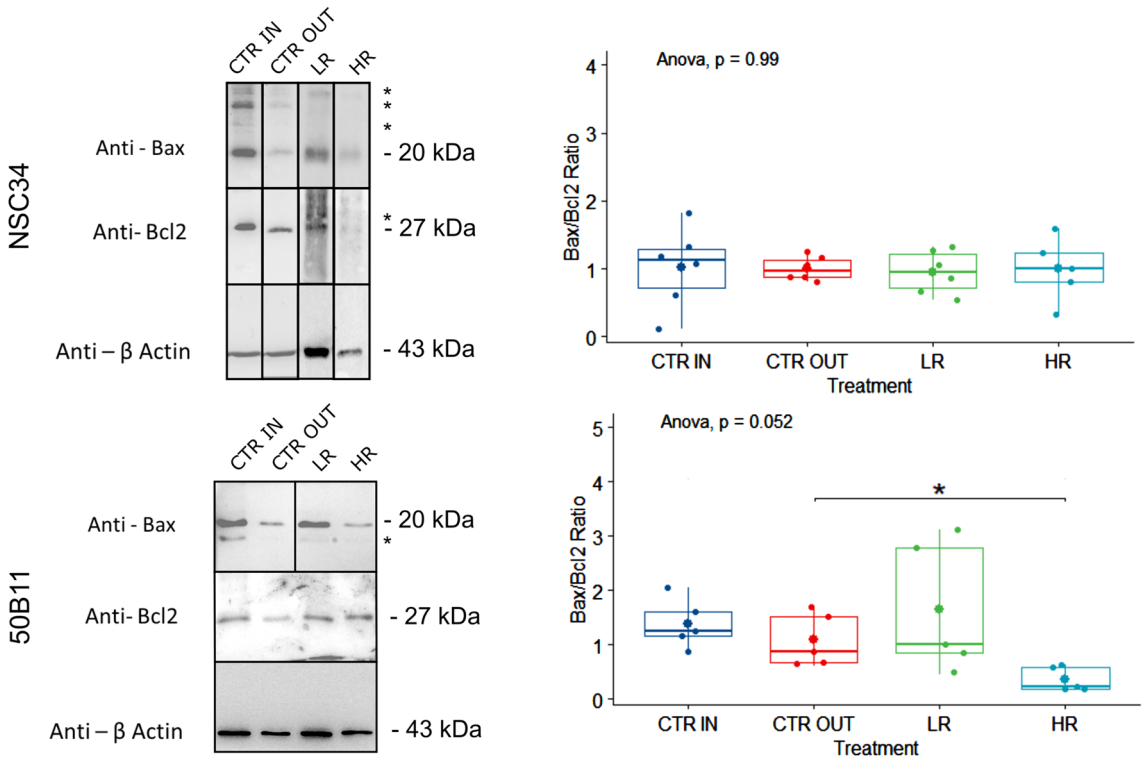

Figure 2. Effects of different neurodynamic treatment protocols on protein expression. Protein expression analysis for Bax and Bcl2 markers on untreated neuronal cells (CTR IN), cells positioned in the bioreactor but untreated (CTR OUT), and cells treated with low (LR) and high (HR) repetitions of neurodynamic treatment. Experiments were carried out in biological quintuplicate (n = 5). On the left, representative western blots are shown; actin was used as a loading control. Upper panels correspond to NSC-34 cells; lower panels correspond to 50B11 cells. Asterisks (*) identify unspecific bands. The samples derive from the same experiment and gels/blots were processed in parallel. Full-length blots and gels are presented in Supplementary Figs. 1–2. In the right panels, the quantitative analysis of all samples is shown. Values in the graphics are expressed as mean ± SD. One-way ANOVA was carried out (data are normally distributed with comparable variances); asterisk shows the statistically significant difference (*$p \leq 0.05$).

survival cell profile induced by NDT protocols: a dose-dependent response and anti-apoptotic behavior was found for 50B11 cells with a significant effect for HR protocol compared to CTR OUT ($F[3, 20] = 3.18$; $p < 0.05$; 95% CI: − 1.21 to 0.54; $\eta_p^2 = 0.374$, large effect). No significant difference was detectable between the CTR IN and the CTR OUT protocols ($F[3, 20] = 0.68$; $p = 0.902$; 95% CI: − 0.96 to 1.56; $\eta_p^2 = 0.374$, large effect). No significant anti-apoptotic effects were detected for NDT and CTR IN protocols on NSC-34 cells ($F[3, 20] = 0.03$; $p = 0.992$; $\eta_p^2 = 0.005$, small effect).

Effects of neurodynamic treatment protocols on gene expression. Gene expression linked to immune response mediated mechanical allodynia (TLR2) and neuropathic pain (YAP, c-JUN) was evaluated in sensory and motor neurons after two different NDT protocols (HR and LR; Fig. 3A). Differently, gene expression linked to mechanical hyperalgesia (TACAN) and mechanical stimuli detection (PIEZO1) was evaluated only in sensory neurons (50B11) since those ion channels play a key role in the sensory stimuli transduction from the periphery to the central nervous system (Fig. 3B).

No significant difference between all experimental groups was detectable for TLR2 expression in sensory neurons (50B11) ($F[3, 20] = 3.61$; $p < 0.03$; $\eta_p^2 = 0.351$, large effect) and motor neurons (NSC-34) ($F[3, 20] = 1.90$; $p = 0.161$; $\eta_p^2 = 0.214$, large effect). Moreover, significant differences of YAP expression were not detectable between the different protocols in both sensory ($F[3, 20] = 0.37$; $p = 0.77$; $\eta_p^2 = 0.053$, small effect) and motor neurons ($F[3, 20] = 1.92$; $p = 0.158$; $\eta_p^2 = 0.214$, large effect).

The c-JUN expression revealed no significant differences between all experimental groups in both 50B11 and NSC-34 (respectively $F[3, 20] = 0.77$; $p = 0.524$; $\eta_p^2 = 0.103$, small effect; and $F[3, 20] = 0.77$; $p = 0.12$; $\eta_p^2 = 0.237$, large effect). Since TACAN and PIEZO1 ion channel functions are related to sensory stimuli transduction, only 50B11 cell line was assessed for the expression of those genes.

The expression of ion channels linked to mechanical hyperalgesia (TACAN) revealed no significant differences, among all protocols ($F[3, 20] = 2.42$; $p = 0.098$; $\eta_p^2 = 0.267$, large effect).

Interestingly, a significant dose- dependent behavior was detected between the HR and CTR IN protocols ($F[3, 20] = 4.18$; $p = 0.013$; $\eta_p^2 = 0.382$, large effect) and between HR and CTR OUT protocols for PIEZO1 expression ($F[3, 20] = 4.11$; $p = 0.019$; $\eta_p^2 = 0.381$, large effect).

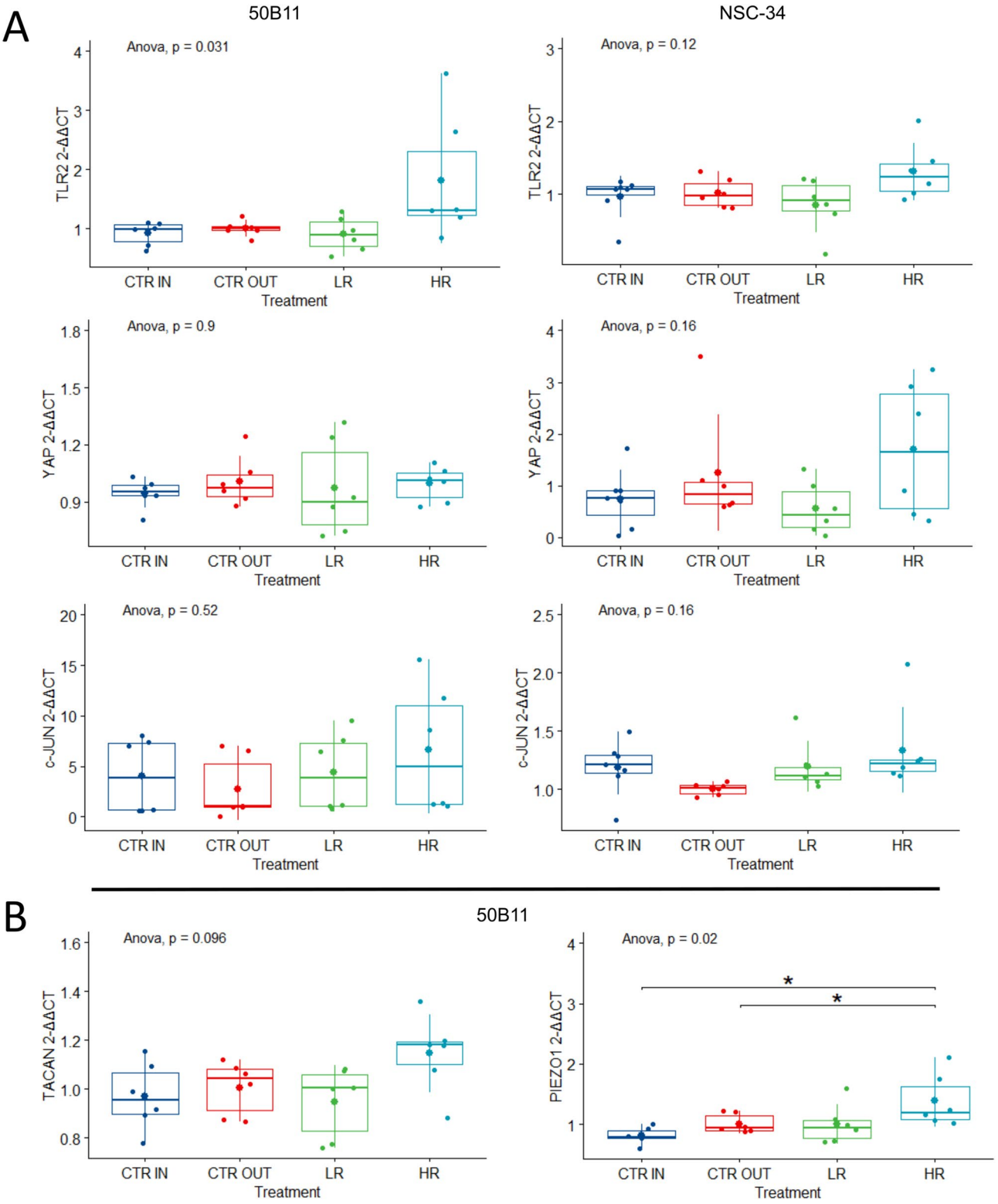

Figure 3. Quantitative gene expression analysis of mechanical allodynia and neuropathic pain markers. Relative quantification ($2^{-\Delta\Delta Ct}$) of genes was evaluated by qRT-PCR to assess benefits or side effects induced by neurodynamic protocols on sensory and motor neurons. TATA-binding protein (TBP) was used as a housekeeping gene to normalize data. All data were calibrated to CTR OUT samples. Values in the graphics are expressed as mean ± SD. One-way ANOVA was carried out (data are normally distributed with comparable variances). (**A**) Expression of TLR2, YAP, and c-JUN is reported to assess the effects of NDT on mechanical allodynia and neuropathic pain. No statistically significant differences with CTR OUT were observed. (**B**) Expression of TACAN and PIEZO1 is reported to assess the effects of NDT on mechanical hyperalgesia and mechanical anti-nociception in sensory neurons 50B11; asterisks show statistically significant differences ($^*p \leq 0.05$).

To summarize, the gene expression results showed no significant changes induced by NDT on genes involved in neuroinflammation and neuropathic pain (TLR2, YAP, c-JUN) in both motor and sensory nociceptive neurons revealing no effects in promoting allodynia or mechanical pain. A significant positive effect was detected for the HR protocol in the sensory nociceptive neurons with an upregulation of PIEZO1, a low threshold mechanosensitive ion channel, responsible for mechanical pain suppression and non-noxious mechanical stimuli transduction.

Discussion

In this study, two different dosages of NDT were tested on sensory and motor neurons to deepen the knowledge of the biological mechanisms activated in response to repeated tensile forces administration.

To our knowledge, this is the first in vitro model of NDT using motor and sensory neuron cell lines and it shows that a treatment, commonly used in rehabilitation programs to relieve pain and reduce disability in patients, promotes neuroplasticity and regenerative processes selectively on neurons without any detectable negative effect.

Higgins and colleagues have previously demonstrated using a human neuroblastoma cell line (SH-SY5Y), that seven days of continuous progressive mechanical stimuli promote differentiation and neurite outgrowth similar to NGF administration[2]. Unlike other experiments, in this study, we have shown that a single session of mechanical stimuli (NDT) is sufficient to affect the cell differentiation in both sensory and motor neurons. We have also determined that neurite outgrowth is strongly promoted more in sensory than in motor neurons by both NDT tested protocols. As regards the orientation of regenerating axons, we hypothesized that the induced uniaxial tension with our bioreactor could influence the orientation of axonal growth leading to fiber alignment. Indeed, several in vitro studies have shown that continuous linear mechanical stretch can guide the neurites to follow the direction of the administered force[2,58,59]. In our study, both in sensory and motor neurons, the neurites orientation was not affected by the uniaxial tension created by the NDT. This was probably due to the fact that our protocols were administered once, and not for days or weeks as was performed in other studies, and that the stretch was intermittent and not progressive. To better understand the biological effects of mechanical stimuli on neurons, we analyzed proteins linked to cell survival and genes linked to mechanical allodynia and hyperalgesia that have been described in neuropathic pain models.

The cell survival analysis performed, evaluating the ratio between Bax (a pro-apoptotic protein) and Bcl2 (an anti-apoptotic protein) expression, demonstrated that 30 repetitions of mechanical stimuli (HR protocol) had a significant anti-apoptotic effect in sensory nociceptive neurons, promoting cell wellbeing.

Based on the in vivo experimental results on neuropathic pain models treated with NDT[3,6,7,18,20], a pain modulation effect was described in particular on mechanical pain and mechanical allodynia. Since allodynia is linked to an upregulation of YAP, TLR2, or c-JUN[23–25,28,29,62] (genes linked to neuroinflammation and immune response) in the DRG and spinal cord, we assessed in motor and sensory neurons their expression profiles.

The lack of significant differences between the experimental protocols has shown that the NDT protocols were not able to promote pro-allodynic responses mediated by the activation of TLR2, YAP, and c-Jun. Otherwise, the analysis performed on sensory nociceptive neurons, derived from rat DRG (50B11), revealed a beneficial effect of NDT in terms of upregulation of PIEZO1 due to HR protocol administration, in accordance with data reported in previous studies that have shown that the PIEZO1 mechanism can suppress mechanical pain[37–39]. Moreover, our data also suggest that NDT does not promote mechanical allodynia as it does not modulate TACAN expression, which is mainly involved in inflammation-mediated mechanical pain[35,36]. Its worth mentioning that all these molecules and their mechanisms are common in rodents and humans[37–39,49–51] giving our results a high translatability to clinical conditions.

In summary, we have for the first time assessed the effects of NDT protocol dosages on neuronal cells showing positive dose-dependent response linked to morphology, protein, and gene expression levels. Considering that our experiments were not performed on neuropathic pain models, our observations related to the upregulation of the PIEZO1 gene, can only lead us to hypothesize a specific pathway activated by mechanical stimuli for pain modulation. Indeed, further future studies on ex vivo and in vivo neuropathic pain models will be essential to confirm if this is the key-pathway that NDT activates to induce a pain modulation effect.

Methods

Bioreactor description. As suggested by Sherman and colleagues, a nerve stretch model requires a tool to obtain a controllable, repeatable, mechanical insult[63]. Therefore, to have a reasonable translational power, since the NDT is performed by the assessor's hands, we had to consider the manual administration of the mechanical stimuli as a fundamental feature of our bioreactor. Since to our knowledge, no bioreactors available on the market can produce uniaxial repeated stretch by hand, we built a bioreactor ad hoc (Fig. 4A, B). A detailed description of the bioreactor can be found in Table 1. Bioreactor sterilization was performed under a cell culture hood by an ethanol 99% wash and with a UV lamp for 30 min.

Cell culture. Neuroblastoma × spinal cord cells (NSC-34) were grown in Dulbecco's Modified Eagle's Medium (DMEM, containing 4.5 g/l glucose) with the addition of 10% heat-inactivated fetal bovine serum (FBS; INVITROGEN), 1% (4 mM) L-glutamine, 1% (0.1 mg/ml) streptomycin, and 100 U/ml penicillin[64]. The differentiation medium consisted of DMEM-F12 supplemented with 4 mM L-glutamine, 0.1 mg/ml streptomycin, 100 U/ml penicillin, 1% FBS, and 1 µM retinoic acid.

Immortalized nociceptive sensory neuronal cell line obtained from dorsal root ganglia (50B11) were grown in neurobasal medium (LIFE TECHNOLOGIS, GIBCO) supplemented with 10% FBS (INVITROGEN), 2% B27 (Life Technologies), 0.22% glucose and 0.2 mM glutamine[65]. The differentiation medium consisted of Neurobasal medium added with 50 µM forskolin. Where not specified, reagents were provided by Sigma-Aldrich.

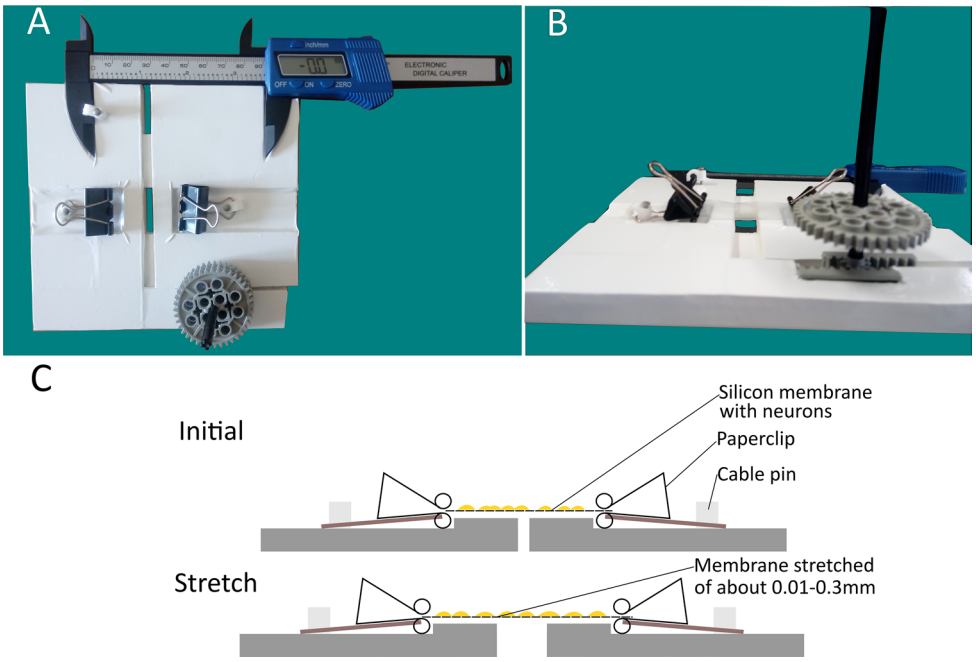

Figure 4. The Bioreactor for in vitro Neurodynamic treatment model. (**A**) The device consists of three 3 mm Forex layers covered with UV-resistant D-c-fix® and divided into two parts puzzled possibly to be driven horizontally by the user to induce neurodynamic treatment protocols. (**B**) Type I collagen pre-coated silicon membrane cut in rectangular shape is clipped into the device and the stretch is administered by hand when turning the wheel to the right. (**C**) The device and the membrane are shown in cross-section. Initially, the membrane is positioned in the device and clipped. To induce the stretch, the wheel is turned to the right, stretching uniaxially the membranes with constant gravity force. Stretch is repeated by turning the wheel to the left until the initial position is reached and turned again to the right with different repetitions depending on the treatment protocols.

Mechanical and physical features of the bioreactor
Speed and strain of the uniaxial stretch are regulated by hand with negligible shear stresses on other planes
Gravity is kept constant
Suitable to be used under a class II cell culture hoods
Materials are compatible with 99% ethanol washes and UV sterilization process
Small dimensions are useful to handle the device (9.7 mm high, 140 mm large and 134.5 mm long), to reduce the possible risk of contamination, and to avoid the waste of materials
Possibility of visually monitoring the amount of membrane elongation (electronic caliber)
Materials commercially available to build the bioreactor
A triple layer of 3 mm *FOREX* (expanded PVC) for the core structure
UV resistant plastic sheet 0.09 mm thick *D-C-FIX* for the cover
LEGO wheels (one 8 teeth; three 24 teeth) and bars (five 100 mm bars) to produce the bioreactor movements
Two paperclips to keep the silicon membranes attached to the bioreactor
Three plastic cable pins with stainless steel nails covered in nickel to keep the clips and the caliber jointly liable with the bioreactor core
A carbon fiber composite digital thickness gauge with an accuracy of ± 0.1 mm/ ± 0.004 was added to the bioreactor for real-time visual feedback of the elongation induced by the user

Table 1. Detailed features of the bioreactor used for mechanical stimulation.

NSC-34 and 50B11 cells were seeded on type I collagen pre-coated silicon membranes (FLEXCELL)[2] at a density of 15×10^3 cells per cm^2. The medium was changed into a differentiation medium two days after seeding the culture. The differentiation medium was changed every two days.

NSC-34 cells differentiate after 2 days and maintain their differentiation state for 8 days[45] while 50B11 cells differentiate after 2 days and maintain their differentiation state for 4 days[47,48].

Cell stretch model parameters. To assess the effects of NDT, the parameters of mechanical stimuli administration were standardized. The silicon membrane elongation was defined as "strain" and the amount of membrane elongation reported as a percentage value was defined as "strain rate". The strain rate was standardized and refined starting from parameters described in literature. In particular, Clark and colleagues have described from in vivo experiments that the optimal nerve elongation promoting nerve regeneration must be less than the 12% of the nerve length, between 6 and 9%[66]. Nevertheless, silicon membranes compared to nerves have no elastic reserve, meaning that mechanical stimuli applied to the membranes are directly transmitted to the membranes of the neurons seeded on it. Indeed, the stretch force reached when a resistance was perceived by the bioreactor user was quantified using a mean value of the strain induced and was determined to be between 28 and 283 Pa. Those values are lower than the mean strain resistance of the cell membrane of motor neurons (500 Pa) and of sensory neurons (3000 Pa)[67–69]. As a matter of fact, during the NDT treatment, the tissue resistance perceived by the clinician, while the test maneuvers are performed, is used as a standardized hallmark to start the treatment[16,70–72]. For this reason, the amount of the strain rate was set to a range of 0.1–1% of the distance between the two clips of the bioreactor in which the membrane was inserted (31.7 mm; see Fig. 4C). This amount of strain corresponded to the measured onset of the resistance to a strain of the membranes, clipped in the bioreactor, perceivable by the bioreactor user. Notably, the strain rate adopted was between 0.02 and 0.5% of the membrane length, which as described by Rivera & Shah, is the normal mechanical resistance range of peripheral nerves[1] Moreover, a higher amount of speed, elongation, and duration of the stretch in preliminary trials induced toxic effects leading to massive cell detachment in 10–30 min after the administration of the protocol. The dosage and speed for the Low Repetitions (LR) protocol were set as described by Wang and colleagues since the parameters were fitting with the criteria reported above and they were also effective as a neurodynamic protocol in preventing muscle atrophy after sciatic nerve injury in rabbits[6]. To study a possible dose–response behaviour on neurons a High Repetitions (HR) protocol was performed, fitting with the same parameters described above, but threefold more intense. Both these protocols could be also suitable for future ex vivo and in vivo studies on neuropathic pain mechanisms. An estimated sample of 7 membranes from each group of treatment (effect size f = 0.90; Power [1 − β err prob] = 0.95; α = 0.05; Actual power = 0.97) was evaluated.

Treatment. NSC-34 and 50B11 cells seeded on silicon membranes pre-coated with type I collagen (FLEXCELL)[2], were incubated 48 h in differentiation medium; then, they were moved into the bioreactor and treated with the protocols reported below and then returned into the differentiation medium till the end of the experiment (two and five days after the procedure, for respectively 50B11 and NSC-34 cells).

A Low Repetitions (LR) protocol of NDT was administered to the membranes as described by Wang and colleagues[6], with a cycle of 1 s of stretch (strain rate of about 0.1 and 1% of the membrane rest length), with 5 s to return at the starting position for 10 times. A High Repetitions (HR) protocol followed the same stretch parameters of the LR, but 30 repetitions were administered.

To assess the environmental effects of nourishment privation[57], induced by the treatment protocols reported above, a sham control, in which membranes were taken off from the medium and positioned in the bioreactor for 90 s (calculated as the mean amount of time between LR and HR protocols in which the membranes were left out of the medium), without the application of mechanical stimuli, was included in the experiment. This experimental group was named "control out" (CTR OUT).

Finally, it was defined the "control in" (CTR IN) protocol, in which membranes were left in the differentiation medium for all the duration of the experiment; the comparison with CTR OUT protocol allowed to assess the effect of taking the membranes out off the medium. Positive effects were defined as those changes linked to nerve regeneration or pain suppression like cell survival, neurite outgrowth, and modulation of genes linked to neuropathic pain and mechanical allodynia. On the opposite, negative effects were defined as those changes induced by the NDT and linked to apoptosis and regulation of the genes linked to pain promotion.

Immunofluorescent imaging. After 5 and 2 days of culture for NSC-34 and 50B11 respectively, cells were fixed in 4% PFA for 15 min, washed in 0.1 M phosphate buffer (pH 7.2), and processed for immunofluorescence analysis. Samples were permeabilized and blocked in 0.1% Triton X-100, 10% normal goat serum (NGS, VECTOR LABORATORIES INC.), 0.02% NaN3 in Dulbecco's phosphate-buffered saline (PBS; without Ca + + and Mg + +) for 1 h and incubated overnight with anti-βIII-tubulin (mouse, monoclonal, 1:1000, SIGMA-ALDRICH) primary antibody in PBS; then, after 3 washes at room temperature (RT) in 0.1 M phosphate buffer (pH 7.2), the secondary antibody goat anti-mouse Alexa fluor 488 (1:200, MOLECULAR PROBEC) in PBS was incubated for 1 h at RT. Cells were mounted with a Dako fluorescence mounting medium, the long edges of the membranes were mounted parallel to the long edge of the coverslips. Images were acquired using a Zeiss LSM800 confocal laser microscopy system (ZEISS, Jena, Germany) in a 40 × magnification.

An estimated sample of 25 images, in which all cells were assessed, from each group of treatment (effect size f = 0.40; Power [1 − β err prob] = 0.95; α = 0.05; Actual power = 0.95) was analyzed. The images were randomly acquired from the central portion of the silicon membranes, from a blinded assessor. From confocal cell images, measurements were performed by ImageJ software.

Differentiation ratio, neurite length, and orientation. The undifferentiated and differentiated cells were manually counted following the criteria described by Maier and colleagues[45], considering that differentiated cells are characterized by having more than two neurites with at least one of them demonstrating a length that is at least two folds longer than the smallest cell diameter; values were calculated by the ratio between numbers of differentiated and undifferentiated cells for each image. Neurite lengths and orientations were manually measured in all cells as described by Song and colleagues[56], starting from the soma to the end of each neurite.

Gene	Sequence	Amplicon length (bp)	Accession number
TLR2	Forward: 5′-CAAACTGGAGACTCTGGAAGC AGG-3′ Reverse: 5′-CACACAGGTAGCTGTCTGCC-3′	125	NM_198769.2 NM_011905.3
YAP	Forward: 5′-CTTCCTGATGGATGGGAG CAAGC-3′ Reverse: 5′-CTGGTTCATGGCAAAACGAGG GTC-3′	120	NM_001034002.2 NM_001171147.1
c-JUN	Forward: 5′-ACGACCTTCTACGACGAT GCCC-3′ Reverse: 5′- GGGTCGGCCAGGTTCAAGG-3′	116	NM_010591.2 NM_021835.3
PIEZO1	Forward: 5′-ACTCCTGGCCGGCCTCCC-3′ Reverse: 5′-AGGCGACCTGTGTGACCTGG-3′	122	NM_001077200
TACAN	Forward: 5′-TGCAGCAGGACTTCCAAGGTA TCC-3′ Reverse: 5′-CGCTTCTTCTGGCGTGTGATA GAG-3′	115	NM_001010945.1
Antibodies for Western blot analysis			
	Code	**Dilution**	**Host**	**Source**
Primary antibodies				
actin	A5316	1:4000	Mouse	Sigma
Bax	SC-23959	1:600	Rabbit	Santa Cruz Biotechnology Inc
BcL2	SC-492	1:200	Rabbit	Santa Cruz Biotechnology Inc
Secondary antibodies				
HRP conjugated-anti-rabbit	7074	1:15,000	Goat	Cell Signaling
HRP conjugated-anti-mouse	7076	1:15,000	Goat	Cell Signaling

Table 2. Sequences of primers used for quantitative real-time PCR and antibodies used for western blot analysis.

Only the longest neurite of each differentiated neuron was acquired and the orientation of a neurite was defined as the angle relative to the stretch force direction and the neurite outgrowth[56].

RNA isolation, cDNA preparation, and quantitative real-time PCR. RNA extraction, retro-transcription, and quantitative real-time PCR (qRT-PCR) were performed as previously described[73], using 0.75 µg RNA/sample for retro-transcription. The average value of CTR OUT ΔCt was used as a Ct calibrator for relative quantification. Data normalization was performed adopting TBP (TATA box binding protein) as a housekeeping gene as described in our previous research[74].

The assessed genes are reported in Table 2; in sensory neurons 50B11 only PIEZO1 and TACAN expression was assessed to detect any effect of NDT on mechanical pain suppression or promotion.

Western blot analysis. Following RNA extraction, total proteins were extracted with TRIzol Reagent according to the manufacturer's instructions (INVITROGEN, THERMOFISHER). The Laemli buffer (2.5% sodium dodecyl sulfate, 0.125 M Tris-HCl, pH 6.8) at 100 °C was used to dissolve the protein pellet. To determine the protein concentration, the Bicinchoninic Acid assay kit (SIGMA) was used where equal protein quantities (50 µg) were loaded into each lane after being resolved by 12% SDS-PAGE. Western blot analysis was carried out as previously described[75]. Table 2 reports the list of primary and secondary antibodies.

Statistical analysis. Statistical analyses were performed using R Statistical Software 2020 version (Foundation for Statistical Computing, Vienna, Austria)[76]. The analyses were performed using the car[77] and rstatix[78] packages, and plots reported in the manuscript figures were performed using the ggpbur package[79].

If the normality of the variables assessed was observed (Levene test), One Way Analysis Of Variance (ANOVA) was adopted to assess differences between normally distributed continuous variables with Tukey post-hoc correction. Not normally distributed variables were assessed using the Dunn Kruskal–Wallis multiple comparisons with Bonferroni post-hoc method. The effect size was estimated adopting the partial eta squared (η_p^2; 0.010 = small effect; 0.059 = medium effect and 0.138 = large effect)[80] and for non-normally distributed variables, otherwise epsilon squared (ε_p^2) was used (effects: small < 0.08, medium 0.08–0.26, large > 0.26). Confidence Intervals (CI) at 95% were calculated and a statistical significance level of 0.05 was implemented throughout. Values were expressed as mean ± SD (standard deviation). The level of significance was set at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), and $p \leq 0.0000$ (****).

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Received: 12 March 2021; Accepted: 14 June 2021
Published online: 24 June 2021

References

1. Bueno, F. R. & Shah, S. B. Implications of tensile loading for the tissue engineering of nerves. *Tissue Eng. Part B Rev.* https://doi.org/10.1089/ten.teb.2008.0020 (2008).
2. Higgins, S., Lee, J. S., Ha, L. & Lim, J. Y. Inducing neurite outgrowth by mechanical cell stretch. *Biores. Open Access* https://doi.org/10.1089/biores.2013.0008 (2013).
3. Santos, F. M. *et al.* Neural mobilization reverses behavioral and cellular changes that characterize neuropathic pain in rats. *Mol. Pain* https://doi.org/10.1186/1744-8069-8-57 (2012).
4. Loverde, J. R. & Pfister, B. J. Developmental axon stretch stimulates neuron growth while maintaining normal electrical activity, intracellular calcium flux, and somatic morphology. *Front. Cell. Neurosci.* https://doi.org/10.3389/fncel.2015.00308 (2015).
5. Lima, Ê. M., Santana, H. H. S., Medrado, A. P., Martinez, A. M. B. & Baptista, A. F. Neurodynamic mobilization reduces intraneural fibrosis after sciatic crush lesion in rats. *Braz. J. Med. Hum. Heal.* https://doi.org/10.17267/2317-3386bjmhh.v5i2.1303 (2017).
6. Wang, Y. *et al.* The effects of different tensile parameters for the neurodynamic mobilization technique on tricipital muscle wet weight and MuRf-1 expression in rabbits with sciatic nerve injury. *J. Neuroeng. Rehabil.* https://doi.org/10.1186/s12984-015-0034-4 (2015).
7. Da Silva, J. T. *et al.* Neural mobilization promotes nerve regeneration by nerve growth factor and myelin protein zero increased after sciatic nerve injury. *Growth Factors* https://doi.org/10.3109/08977194.2014.953630 (2015).
8. Santos, F. M., Silva, J. T., Rocha, I. R. C., Martins, D. O. & Chacur, M. Non-pharmacological treatment affects neuropeptide expression in neuropathic pain model. *Brain Res.* https://doi.org/10.1016/j.brainres.2018.02.034 (2018).
9. Geuna, S., Raimondo, S., Fregnan, F., Haastert-Talini, K. & Grothe, C. *In vitro* models for peripheral nerve regeneration. *Eur. J. Neurosci.* **43**, 287–296 (2016).
10. Lolignier, S., Eijkelkamp, N. & Wood, J. N. Mechanical allodynia. *Pflug. Arch. Eur. J. Physiol.* **467**, 133–139 (2014).
11. Macfarlane, A. W. Case of sciatica, treated by nerve-stretching. *Lancet* https://doi.org/10.1016/S0140-6736(02)40843-4 (1878).
12. Marshall, J. Bradshaw lecture on nerve-stretching for the relief or cure of pain. *Br. Med. J.* https://doi.org/10.1136/bmj.2.1198.1173 (1883).
13. Basson, C. A., Stewart, A., Mudzi, W. & Musenge, E. Effect of neural mobilization on nerve-related neck and arm pain: A randomized controlled trial. *Physiother. Can.* https://doi.org/10.3138/ptc-2018-0056 (2020).
14. Wolny, T., Saulicz, E., Linek, P., Myśliwiec, A. & Saulicz, M. Effect of manual therapy and neurodynamic techniques vs ultrasound and laser on 2PD in patients with CTS: A randomized controlled trial. *J. Hand Ther.* https://doi.org/10.1016/j.jht.2016.03.006 (2016).
15. Plaza-Manzano, G. *et al.* Effects of adding a neurodynamic mobilization to motor control training in patients with lumbar radiculopathy due to disc herniation: A randomized clinical trial. *Am. J. Phys. Med. Rehabil.* https://doi.org/10.1097/PHM.0000000000001295 (2020).
16. Torres, J. R. *et al.* Results of an active neurodynamic mobilization program in patients with fibromyalgia syndrome: A randomized controlled trial. *Arch. Phys. Med. Rehabil.* https://doi.org/10.1016/j.apmr.2015.06.008 (2015).
17. Nee, R. J., Vicenzino, B., Jull, G. A., Cleland, J. A. & Coppieters, M. W. Neural tissue management provides immediate clinically relevant benefits without harmful effects for patients with nerve-related neck and arm pain: A randomised trial. *J. Physiother.* https://doi.org/10.1016/S1836-9553(12)70069-3 (2012).
18. Ramos, M. *et al.* Effects of neural mobilization on individuals with chronic low back pain. *Braz. J. Pain* **3**, 1–8 (2020).
19. Bertolini, G. R. F., Silva, T. S., Trindade, D. L., Ciena, A. P. & Carvalho, A. R. Neural mobilization and static stretching in an experimental sciatica model: An experimental study. *Braz. J. Phys. Ther.* https://doi.org/10.1590/s1413-35552009005000062 (2009).
20. Zhu, G. C., Tsai, K. L., Chen, Y. W. & Hung, C. H. Neural mobilization attenuates mechanical allodynia and decreases proinflammatory cytokine concentrations in rats with painful diabetic neuropathy. *Phys. Ther.* https://doi.org/10.1093/ptj/pzx124 (2018).
21. Mueller, M. J. & Maluf, K. S. Tissue adaptation to physical stress: A proposed 'Physical Stress Theory' to guide physical therapist practice, education, and research. *Phys. Ther.* https://doi.org/10.1093/ptj/82.4.383 (2002).
22. Topp, K. S. & Boyd, B. S. Structure and biomechanics of peripheral nerves: Nerve responses to physical stresses and implications for physical therapist practice. *Phys. Ther.* https://doi.org/10.1093/ptj/86.1.92 (2006).
23. Cobos, E. J. *et al.* Mechanistic differences in neuropathic pain modalities revealed by correlating behavior with global expression profiling. *Cell Rep.* https://doi.org/10.1016/j.celrep.2018.01.006 (2018).
24. Xu, N. *et al.* Inhibition of YAP/TAZ activity in spinal cord suppresses neuropathic pain. *J. Neurosci.* https://doi.org/10.1523/JNEUROSCI.0800-16.2016 (2016).
25. Jurga, A. M. *et al.* Blockade of toll-like receptors (TLR2, TLR4) attenuates pain and potentiates buprenorphine analgesia in a rat neuropathic pain model. *Neural Plast.* https://doi.org/10.1155/2016/5238730 (2016).
26. Yang, H. *et al.* Anti-inflammatory protein TSG-6 secreted by bone marrow mesenchymal stem cells attenuates neuropathic pain by inhibiting the TLR2/MyD88/NF-κB signaling pathway in spinal microglia. *J. Neuroinflamm.* https://doi.org/10.1186/s12974-020-1731-x (2020).
27. Li, N. *et al.* Spinal expression of Hippo signaling components YAP and TAZ following peripheral nerve injury in rats. *Brain Res.* **1535**, 137–147 (2013).
28. Sanna, M. D., Manassero, G., Vercelli, A., Herdegen, T. & Galeotti, N. The isoform-specific functions of the c-Jun N-terminal kinase (JNK) in a mouse model of antiretroviral-induced painful peripheral neuropathy. *Eur. J. Pharmacol.* https://doi.org/10.1016/j.ejphar.2020.173161 (2020).
29. Zhuang, Z. Y. *et al.* A peptide c-Jun N-terminal kinase (JNK) inhibitor blocks mechanical allodynia after spinal nerve ligation: Respective roles of JNK activation in primary sensory neurons and spinal astrocytes for neuropathic pain development and maintenance. *J. Neurosci.* https://doi.org/10.1523/JNEUROSCI.5290-05.2006 (2006).
30. Gu, Y. *et al.* Identification of potential mechanism and hub genes for neuropathic pain by expression-based genome-wide association study. *J. Cell. Biochem.* https://doi.org/10.1002/jcb.27766 (2019).
31. Di Cesare Mannelli, L. *et al.* The neuropathy-protective agent acetyl-l-carnitine activates protein kinase C-γ and MAPKs in a rat model of neuropathic pain. *Neuroscience.* https://doi.org/10.1016/j.neuroscience.2009.11.021 (2010).
32. Oh, S. H. *et al.* Nefopam downregulates autophagy and c-Jun N-terminal kinase activity in the regulation of neuropathic pain development following spinal nerve ligation. *BMC Anesthesiol.* https://doi.org/10.1186/s12871-018-0559-8 (2018).
33. Sanna, M. D., Manassero, G., Vercelli, A., Herdegen, T. & Galeotti, N. The isoform-specific functions of the c-Jun N-terminal kinase (JNK) in a mouse model of antiretroviral-induced painful peripheral neuropathy. *Eur. J. Pharmacol.* **880**, 173161 (2020).
34. Cavanaugh, D. J. *et al.* Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. *Proc. Natl. Acad. Sci. U. S. A.* https://doi.org/10.1073/pnas.0901507106 (2009).
35. Beaulieu-Laroche, L. *et al.* TACAN is an ion channel involved in sensing mechanical pain. *Cell* https://doi.org/10.1016/j.cell.2020.01.033 (2020).
36. Bonet, I. J. M., Araldi, D., Bogen, O. & Levine, J. D. Involvement of TACAN, a mechanotransducing ion channel, in inflammatory but not neuropathic hyperalgesia in the rat. *J. Pain* https://doi.org/10.1016/j.jpain.2020.11.004 (2020).

37. Roh, J. *et al.* Functional expression of piezo1 in dorsal root ganglion (DRG) neurons. *Int. J. Mol. Sci.* https://doi.org/10.3390/ijms21113834 (2020).
38. Song, Y. *et al.* The mechanosensitive ion channel piezo inhibits axon regeneration. *Neuron* https://doi.org/10.1016/j.neuron.2019.01.050 (2019).
39. Kim, S. E., Coste, B., Chadha, A., Cook, B. & Patapoutian, A. The role of Drosophila Piezo in mechanical nociception. *Nature* https://doi.org/10.1038/nature10801 (2012).
40. Coppieters, M. W. *et al.* Strain and excursion of the sciatic, tibial and plantar nerves during a modified straight leg raising test. *J. Orthop. Res.* https://doi.org/10.1002/jor.20210 (2006).
41. Driscoll, P. J., Glasby, M. A. & Lawson, G. M. An in vivo study of peripheral nerves in continuity: Biomechanical and physiological responses to elongation. *J. Orthop. Res.* https://doi.org/10.1016/S0736-0266(01)00104-8 (2002).
42. Sharma, S., Balthillaya, G., Rao, R. & Mani, R. Short term effectiveness of neural sliders and neural tensioners as an adjunct to static stretching of hamstrings on knee extension angle in healthy individuals: A randomized controlled trial. *Phys. Ther. Sport* https://doi.org/10.1016/j.ptsp.2015.03.003 (2016).
43. Miyagishi, H. *et al.* Prostaglandin E2-induced cell death is mediated by activation of EP2 receptors in motor neuron-like NSC-34 cells. *J. Pharmacol. Sci.* https://doi.org/10.1254/jphs.12274SC (2013).
44. Eggett, C. J. *et al.* Development and characterisation of a glutamate-sensitive motor neurone cell line. *J. Neurochem.* https://doi.org/10.1046/j.1471-4159.2000.0741895.x (2000).
45. Maier, O. *et al.* Differentiated NSC-34 motoneuron-like cells as experimental model for cholinergic neurodegeneration. *Neurochem. Int.* https://doi.org/10.1016/j.neuint.2013.03.008 (2013).
46. Sabitha, K. R., Sanjay, D., Savita, B., Raju, T. R. & Laxmi, T. R. Electrophysiological characterization of Nsc-34 cell line using microelectrode array. *J. Neurol. Sci.* https://doi.org/10.1016/j.jns.2016.09.038 (2016).
47. Chen, W., Mi, R., Haughey, N., Oz, M. & Höke, A. Immortalization and characterization of a nociceptive dorsal root ganglion sensory neuronal line. *J. Peripher. Nerv. Syst.* https://doi.org/10.1111/j.1529-8027.2007.00131.x (2007).
48. Gnavi, S. *et. al.* In vitro evaluation of gelatin and chitosan electrospun fibres as an artificial guide in peripheral nerve repair: a comparative study. *J. Tissue. Eng. Regen. Med.* https://doi.org/10.1002/term.2351 (2018).
49. Kremer, M. *et al.* A dual noradrenergic mechanism for the relief of neuropathic allodynia by the antidepressant drugs duloxetine and amitriptyline. *J. Neurosci.* https://doi.org/10.1523/JNEUROSCI.1004-18.2018 (2018).
50. Zaltsman, Y., Masuko, S., Bensen, J. J. & Kiessling, L. L. Angiomotin regulates YAP localization during neural differentiation of human pluripotent stem cells. *Stem Cell Rep.* https://doi.org/10.1016/j.stemcr.2019.03.009 (2019).
51. Wang, Q. S. *et al.* MiR-139 protects against oxygen-glucose deprivation/reoxygenation (OGD/R)-induced nerve injury through targeting c-Jun to inhibit NLRP3 inflammasome activation. *J. Stroke Cerebrovasc. Dis.* https://doi.org/10.1016/j.jstrokecerebrovasdis.2020.105037 (2020).
52. Muratori, L. *et al.* Generation of new neurons in dorsal root ganglia in adult rats after peripheral nerve crush injury. *Neural Plast.* https://doi.org/10.1155/2015/860546 (2015).
53. Hu, F. *et al.* Neuronally differentiated adipose-derived stem cells and aligned PHBV nanofiber nerve scaffolds promote sciatic nerve regeneration. *Biochem. Biophys. Res. Commun.* https://doi.org/10.1016/j.bbrc.2017.05.119 (2017).
54. Sun, Y. *et al.* Enhanced nerve cell proliferation and differentiation on electrically conductive scaffolds embedded with graphene and carbon nanotubes. *J. Biomed. Mater. Res. Part A* https://doi.org/10.1002/jbm.a.37016 (2020).
55. Sigerson, C. D., Dipollina, C. J. & Fornaro, M. Effects of chemical and physical cues in enhancing neuritogenesis and peripheral nerve regeneration. *Neural Regen. Res.* https://doi.org/10.4103/1673-5374.177717 (2016).
56. Song, M. & Uhrich, K. E. Optimal micropattern dimensions enhance neurite outgrowth rates, lengths, and orientations. *Ann. Biomed. Eng.* **35**, 1812–1820 (2007).
57. Lin, M. Y. *et al.* Biophysical stimulation induces demyelination via an integrin-dependent mechanism. *Ann. Neurol.* https://doi.org/10.1002/ana.23592 (2012).
58. Feng, Z. Q. *et al.* Mechanical tension applied to substrate films specifies location of neuritogenesis and promotes major neurite growth at the expense of minor neurite development. *J. Biomed. Mater. Res. Part A* https://doi.org/10.1002/jbm.a.35627 (2016).
59. Loverde, J. R., Ozoka, V. C., Aquino, R., Lin, L. & Pfister, B. J. Live imaging of axon stretch growth in embryonic and adult neurons. *J. Neurotrauma* https://doi.org/10.1089/neu.2010.1598 (2011).
60. Pfister, L. A., Alther, E., Papaloïzos, M., Merkle, H. P. & Gander, B. Controlled nerve growth factor release from multi-ply alginate/chitosan-based nerve conduits. *Eur. J. Pharm. Biopharm.* **69**, 563–572 (2008).
61. Pfister, B. J. *et al.* Development of transplantable nervous tissue constructs comprised of stretch-grown axons. *J. Neurosci. Methods* https://doi.org/10.1016/j.jneumeth.2005.10.012 (2006).
62. Di Cesare Mannelli, L., Ghelardini, C., Toscano, A., Pacini, A. & Bartolini, A. The neuropathy-protective agent acetyl-l-carnitine activates protein kinase C-γ and MAPKs in a rat model of neuropathic pain. *Neuroscience* https://doi.org/10.1016/j.neuroscience.2009.11.021 (2010).
63. Sherman, S. A. *et al.* Stretch injury of human induced pluripotent stem cell derived neurons in a 96 well format. *Sci. Rep.* https://doi.org/10.1038/srep34097 (2016).
64. Johann, S., Dahm, M., Kipp, M., Zahn, U. & Beyer, C. Regulation of choline acetyltransferase expression by 17β-oestradiol in NSC-34 cells and in the spinal cord. *J. Neuroendocrinol.* https://doi.org/10.1111/j.1365-2826.2011.02192.x (2011).
65. Gnavi, S. *et al.* The effect of electrospun gelatin fibers alignment on schwann cell and axon behavior and organization in the perspective of artificial nerve design. *Int. J. Mol. Sci.* https://doi.org/10.3390/ijms160612925 (2015).
66. Clark, W. L., Trumble, T. E., Swiontkowski, M. F. & Tencer, A. F. Nerve tension and blood flow in a rat model of immediate and delayed repairs. *J. Hand Surg. Am.* https://doi.org/10.1016/0363-5023(92)90316-H (1992).
67. Koch, D., Rosoff, W. J., Jiang, J., Geller, H. M. & Urbach, J. S. Strength in the periphery: Growth cone biomechanics and substrate rigidity response in peripheral and central nervous system neurons. *Biophys. J.* https://doi.org/10.1016/j.bpj.2011.12.025 (2012).
68. Spedden, E., White, J. D., Naumova, E. N., Kaplan, D. L. & Staii, C. Elasticity maps of living neurons measured by combined fluorescence and atomic force microscopy. *Biophys. J.* https://doi.org/10.1016/j.bpj.2012.08.005 (2012).
69. Moore, P. & Staii, C. Cytoskeletal dynamics of neurons measured by combined fluorescence and atomic force microscopy. *MRS Adv.* https://doi.org/10.1557/adv.2018.101 (2018).
70. Boyd, B. S., Wanek, L., Gray, A. T. & Topp, K. S. Mechanosensitivity of the lower extremity nervous system during straight-leg raise neurodynamic testing in healthy individuals. *J. Orthop. Sports Phys. Ther.* **39**, 780–790 (2009).
71. Basson, A. *et al.* The effectiveness of neural mobilization for neuromusculoskeletal conditions: A systematic review and meta-Analysis. *J. Orthop. Sports Phys. Ther.* https://doi.org/10.2519/jospt.2017.7117 (2017).
72. Ayub, A., Osama, M. & Shakeel, A. Effects of active versus passive upper extremity neural mobilization combined with mechanical traction and joint mobilization in females with cervical radiculopathy: A randomized controlled trial. *J. Back Musculoskelet. Rehabil.* https://doi.org/10.3233/BMR-170887 (2019).
73. Ronchi, G. *et al.* The Neuregulin1/ErbB system is selectively regulated during peripheral nerve degeneration and regeneration. *Eur. J. Neurosci.* https://doi.org/10.1111/ejn.12974 (2016).
74. Morano, M. *et al.* Modulation of the Neuregulin 1/ErbB system after skeletal muscle denervation and reinnervation. *Sci. Rep.* https://doi.org/10.1038/s41598-018-23454-8 (2018).

75. Gambarotta, G. *et al.* ErbB4 expression in neural progenitor cells (ST14A) is necessary to mediate neuregulin-1β1-induced migration. *J. Biol. Chem.* https://doi.org/10.1074/jbc.M408374200 (2004).
76. R Development Core Team. *R: A Language and Environment for Statistical Computing* (R Foundation for Statistical Computing, 2017).
77. Fox, J. & Weisberg, S. *Package 'Car'. Companion to Applied Regression* 2nd edn. (Sage, 2011).
78. Kassambara, A. *et al.* Global miRNA expression analysis identifies novel key regulators of plasma cell differentiation and malignant plasma cell. *Nucleic Acids Res.* https://doi.org/10.1093/nar/gkx327 (2017).
79. Kassambara, A. *Package 'ggpubr': 'ggplot2' Based Publication Ready Plots*. R Packag. version 0.4.0 (2020).
80. Richardson, J. T. E. Eta squared and partial eta squared as measures of effect size in educational research. *Educ. Res. Rev.* **6**, 135–147 (2011).

Acknowledgements

This work was partially funded by Ricerca Finalizzata 2018 (RF-2018-12366887). We thank Dr. Filippo Carta for the precious suggestions on materials chosen to build the device. We also thank Dr. Riccardo Carta for the help in the projecting and production of the device. We also thank Dr. Sebastian Rademacher and Prof. Dr. Peter Claus, to have kindly provided the Neuroblastoma spinal cord cells (NSC-34), and Prof. Ahmet Hoke to have kindly provided the immortalized nociceptive sensory neuronal cell line obtained from dorsal root ganglia (50B11).

Author contributions

G.C. and F.F.: study design; S.G., S.R., G.G.: experiment supervision; G.C., F.F., L.M.: immunohistochemistry analysis; G.C., G.G., B.E.F., M.E.S.: RNA and protein extraction and analysis; G.C., G.G., S.R.: data analysis and interpretation, statistical analysis; G.C. and F.F.: figure preparation and manuscript drafting; S.R., G.G., S.G., B.E.F., L.M.: manuscript revision and editing. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-92682-2.

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3.3 Paper n°3

The neurodynamic treatment promotes mechanical pain modulation in sensory neurons and nerve regeneration in rats

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Submitted, 2022

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Competing interests: The author(s) declare no competing interests.

Abstract

Somatic nerve injuries are a rising problem leading to disability associated with neuropathic pain commonly reported as mechanical allodynia (MA) and hyperalgesia. Growing evidence has shown that these symptoms are strongly dependent on specific processes in the dorsal root ganglia (DRG). The neurodynamic treatment (NDT), consisting of selective uniaxial nerve repeated tension protocols, effectively reduces pain and disability in neuropathic pain patients[31]. Nevertheless, the biological mechanisms induced by the NDT in nerve regeneration promotion and pain modulation remain poorly characterized. We aimed to define both *in vivo* and *ex vivo,* how NDT could promote nerve regeneration and modulate the processes in the DRG linked to MA and hyperalgesia.

For *in vivo* experiments, we adopted the rat unilateral median and ulnar nerve crush model. We compared to control rats (animals subjected only to nerve injury), animals treated with NDT for 3 weeks after injury, and animals treated with NDT both 10 sessions before and 3 weeks after nerve injury. Behavioural tests, morphological and morphometrical analysis, gene and protein expression analysis revealed that NDT promotes nerve regeneration processes, speeds up sensory-motor recovery, and modulates mechanical pain by affecting, in the DRG, the expression of TACAN, a mechanosensitive receptor, shared between humans and rats, responsible for MA and hyperalgesia. The DRG explant *ex vivo* model has shown that NDT increases neurite regrowth and confirmed the modulation of TACAN. In conclusion, the NDT can be safely adopted in clinical practice after nerve injuries to promote functional recovery and pain modulation.

Keywords: Neuropathic pain, neurodynamic, nociceptor, allodynia, regeneration, motor recovery, non-pharmacological treatment, dorsal root ganglia, gene expression.

Introduction

Neuropathic pain and disability are common conditions in patients suffering from peripheral nervous system (PNS) injuries or pathologies [40,77]. In particular, when PNS injuries occur, they are often associated with sensory and motor impairment or loss of function distal to the lesion site. Unfortunately, the interventional procedure improvement and opioid prescriptions did not reduce the burden of neuropathic pain which is still a clinical challenge in terms of making a diagnosis, defining and maintaining an adequate therapy [11,55,56].

After a nerve injury, unmyelinated and myelinated primary afferent neurons increase their activity sustaining a predominantly peripheral neuropathic pain process with stimuli that are dependent on abnormal responses like mechanical allodynia (MA) and hyperalgesia [46,87,96]. These conditions are related respectively to a pain perception for non-painful pressure or stretch and exaggerated pain for painful stimuli. These processes are linked to changes in neurochemical, anatomical, electrophysiological properties or protein and gene expression in the dorsal root ganglia (DRG) neurons [9,13,63,73,87,102]. More in detail, MA and hyperalgesia are related to the expression of specific genes in the DRG, like the high threshold mechanosensitive channel TACAN (Tmem120A) [9,13,23] or the Toll-like receptor-2 (TLR2), a receptor modulating the macrophage response [25,47,75,100]. Indeed, it has been shown that depletion or suppression of these genes suppresses MA and hyperalgesia [9,13,25,47]. Notably, these mechanisms causing MA and hyperalgesia related to neuropathic pain are highly conserved in both murine and human DRG neurons [4,9,51] and the modulation of receptors and mechanosensitive channels expressed by peripheral sensory neurons has been demonstrated to be a promising strategy in the treatment of neuropathic pain [13,22,47,90]. Also, RNA-Seq studies on DRG after nerve injuries in rats revealed that these genes are independently expressed from sex [3,24,78].

The selective repeated administration of uniaxial tensions to somatic nerves, also known as neurodynamic treatment (NDT), has shown to be effective in pain modulation and nerve conduction

improvements detected by electrophysiological assessment in long term neuropathic pain patients suffering from carpal tunnel syndrome [81,93–95] and radiculopathies of somatic nerves in the upper or lower limbs [8,31,57,66,98,103]. NDT significantly reduces pain and disability with long-term effects in neuropathic pain patients. Also, hypoalgesic and anti-allodynic short-term effects have been reported respectively in asymptomatic [10,35,58] and neuropathic pain patients [6,37,89]. Despite the effectiveness of NDT in modulating neuropathic pain, the biological mechanisms induced by this treatment remain poorly characterized. Our previous studies have shown that NDT induces dose-dependent pro-regenerative and antiapoptotic effects on sensory and motor neuronal cell lines [20], but, as far as we know, no data on MA and hyperalgesia modulation are available. In the current study, we aimed to investigate *in vivo* if the NDT modulates the neuropathic pain and promotes motor-sensory recovery after a nerve crush injury[26,79]. To further explore the relevance of possible pain modulation processes induced by NDT in the DRG, we adopted an *ex-vivo* model of DRG organotypic explant which was subjected to neurodynamic treatment through a bioreactor previously validated for this purpose [20].

Methods

Animals

In vivo surgical and treatment protocols

Adult female Wistar rats approximately weighting 230-250 gr (Charles River Laboratories, Milan, Italy) were used in the present study and a proper sample size (n= 18; 6 for each experimental group) was calculated based on grasping strength (370 ± 90 gr; η_p^2= 0.14 large) after a median nerve crush injury in rat upper limbs reported by Ronchi and colleagues [68] (effect size f = 0.403; Power [1 − β err prob] = 0.95; α = 0.05; Actual power = 0.97). Animals were housed in large cages at the animal facility of Neuroscience Institute Cavalieri Ottolenghi (NICO; Ministerial authorization DM 182 2010-A 3-

11-2010) in humidity and temperature-controlled room with 12-hour light/dark cycles. Free access to water and standard chow was provided *ad libitum*. Humane endpoint criteria were adopted to adequately measure and minimize any animal pain, discomfort, or distress. The study conditions were conformed to the guidelines of the European Union's Directive EU/2010/63. Also, approval by the Ethic Experimental Committee of the University of Turin (Ministry of Health project number 864/2016) was obtained before the research began. As reported in Figure 1A, animals were randomly divided into 3 experimental groups: (i) 6 rats subjected to crush injury but not treated, used as control (NO NDT); (ii) 6 rats subjected to crush injury and treated with an NDT protocol from the third day after surgery until day 24 (NDT POST; described further below); (iii) 6 rats subjected to crush injury and treated with NDT both 12 days before nerve injury (5 days/week) and for 24 days after injury with the same posology of the NDT POST group (NDT PRE+POST).

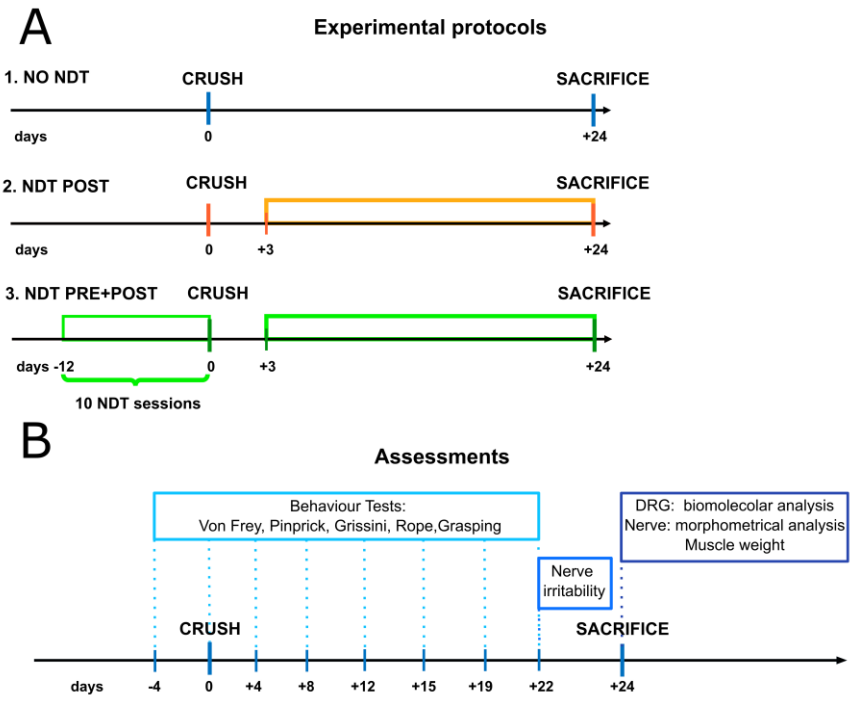


Figure 1 Treatment protocols, and timeline of the assessments. (A) The colored lines of the figure represent the duration of NDT protocols administrated 5 days/week, 30 sessions each day of treatment in orange (NDT POST) and green (NDT PRE+POST). (B) Assessments performed at each time point are reported in the colored frames

The surgical interventions were carried out under deep anesthesia using Tiletamine + Zolazepam (Zoletil) i.m. (40 and 5mg/kg). After trichotomy, the median and ulnar nerves of the left forelimb were exposed from the axillary region of the arm above the elbow (Figure 2A). The nerve crush injury was performed administering a pressure of 17.02MPa for 30 consecutive seconds [68] to the middle third of the nerves using a flat tip clamp as reported in Figures 2B and C.

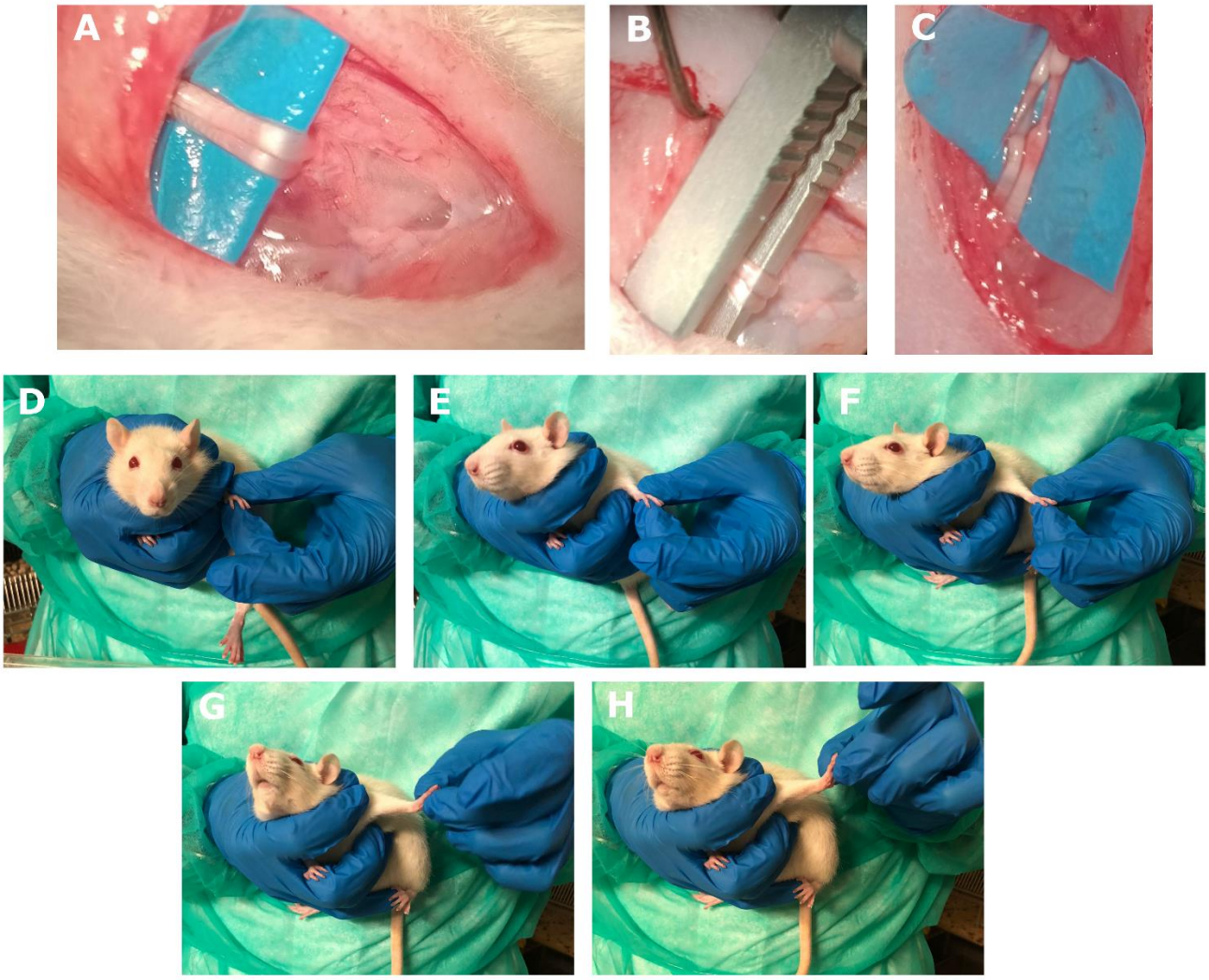


Figure 2. A-C: crush injury protocol. (A) After a 2 cm skin incision along the medial aspect of the long axis of the arm above the elbow, the median and ulnar nerve were isolated and exposed. (B) Median and ulnar nerves were crushed with a non-serrated clamp for 30 sec. (C) The crushed area was checked to make sure there was no loss of continuity of the connective components of the nerves. D-H: Neurodynamic tensioning treatment was administered to the injured side only (left side) to awake animals. The neurodynamic test from the neutral position (D) consisted of contralateral neck side flection (E), shoulder abduction and elbow extension (F), wrist extension (G), and fingers extension (H) performed until the clinician perceived resistance to mobilization.

The protocol of NDT tensioning was administered once a day, with 1 sec of stretch (till resistance was felt by the clinician), and 5 sec to return at the starting position for 30 times [20,91], within the time window 1:00 pm to 3:00 pm 5 days/week until day 24 post-injury (figure 2D-H). The rat neck was kept in contralateral lateral side flexion (right side flexion) by gently and firmly surrounding its neck with the right thumb and index fingers. The clinician gently brought the shoulder into abduction, elbow in extension, writs, and fingers in extension by gently pinching the paw with the left thumb on the back of the paw and abducing the left index finger. The progression of the manoeuvres was performed until resistance was perceived. Indeed, the perception of passive tissue resistance is a standardized and reliable hallmark for NDT administration reported in clinical and preclinical studies [7,17,20,85]. The rats were awake during treatments and were free to withdraw the paw anytime during the manipulations and experimental tests.

Behavioral tests

All reported behavioral tests were administered four days before the nerve crush and 4, 8, 12, 15, 19, and 22 days after the nerve injury with the same chronological order as they are listed below, starting from sensory tests. The scores obtained before nerve injury, from sensory and motor behavior tests, were adopted as a reference to define the physiological normal response.

Sensory assessment

Von Frey Test

The Von Frey [41] calibrated monofilaments were adopted to define non-noxious, noxious pressure threshold before nerve injury, and anesthesia or MA after the injury. The touch threshold (non-noxious pressure threshold) was defined by observing the lack of paw movement or withdrawal

when the filament was bending and the rat was keeping his weight on the assessed paw. The noxious pressure or mechanical pain threshold was defined as the presence of paw withdrawal also associated with licking and flicking behaviors. The anesthesia for pressure or puncture painful stimuli was defined as the lack of reactions related to stimuli administration after the nerve injury. MA was defined by observing paw withdrawals elicited by the pressure that before the nerve injury induced no paw withdrawals. Animals were acclimatized individually for 15 minutes in a Plexiglas box (28x20x14 cm) with a metal grid on the bottom (0.3x0.4 cm). The assessment was started for each side with a filament with 5 grams (gr) bending force applied with uniform pressure to the palmar surface of the paw for 5 seconds. A progressive series of 5, 10, 15, 20, and 25 g was applied till a change in response pattern was elicited and confirmed by 4 additional applications of the previous pressure eliciting no responses and 4 of the reached pressure eliciting responses [42]. When rats did not respond to any filament a score of 45 g was assigned [74].

Pinprick test

A modified pinprick test score on pain [21], assessing the withdrawal responses to 4 consecutive pin administration to the hind paw was adopted as follows: “0” for the absence of withdrawal response, “1” when the rat slowly removes the paw from the pin, “2” when the paw was quickly removed, and “3” when the rat quickly removed the paw flicking and licking it.

Nerve irritability to tension

22 days after the injury, mechanical nerve irritability was assessed at the injured side by recording the number of paw withdrawals during 30 consecutive nerve tensions administered until resistance was perceived by the clinician. Tensions were administered at 0.5Hz frequency (five times higher

rate than treatment protocols; 0.5/sec tension/release) able to induce a windup or temporal summation phenomenon, consisting in transiently increased responsiveness of spinal neurons to nociceptive peripheral fibers repeated stimulations [1,99]. This test assessed the performance of the pain descending inhibitory system and central sensitization to pain, processes that NDT possibly affects as reported in the literature [69,71] (Supplementary Video).

Motor performances

Grissini Test

The forepaws' dexterity was assessed and quantitatively measured through the modified Cappellini handling test described by Tennent and colleagues [82], using “grissini”, typical Italian breadsticks. In brief, a 70mm long grissini piece (Ø 6mm diameter; Roberto S.r.L. TV, Italy), was given to the rat and the time to eat the entire piece and the number of adjustments (defined as any observed replacement or removal of the paw on the grissini after it started eating) were video-recorded and later analyzed by observing slowed videos at ~ 50% of real-time speed.

Rope Test

The global strength of upper limbs and coordination were quantitatively assessed through the Rope Climbing test [19,83]. The rat was positioned at the bottom of the vertical 160cm rope and was persuaded to climb it by gently touching the tail. The time from the climbing of the rat start until the crossing of the entrance on the top platform (40x40cm) was recorded. Rats were gradually trained to climb for 10 days before the surgery, as described by Diogo and co-workers [21], with a daily increment of 20cm rope.

Grasping Test

The grasping test was performed to assess the finger flexors strength as described by Papalia and colleagues [61] by holding the rat by its tail and lowering it towards the grid of the BS-GRIP Grip Meter device (2Biological Instruments, Varese, Italy) till a firm grip was observed, then the rat was pulled upward till the grip was lost and the highest force was recorded by the device. The mathematical mean value obtained by three consecutive attempts was used for statistical analysis.

Morphological and morphometrical analysis of nerves

After 24 days from the nerve crush, animals were sacrificed by an intraperitoneal Zoletil+Xilazina (>60 and >10 mg/kg) injection. The median and ulnar nerves of both sides were isolated and excised 5 mm above and 10 mm below the middle third of their brachial portion (site of injury for the left side), and DRGs of the brachial plexus of both sides were excised and samples were harvested as described below. Then, the flexor digitorum superficialis and profundus tendons were cut, and the entire muscle bellies were excised from the surrounding soft tissues and muscles. Finally, the muscle wet mass was weighted using an electronic analytical balance with a precision of 0.1 gr [91].

Resin embedding and high-resolution light microscopy

As previously described [68], a segment of the median and ulnar nerves distally to the injury site was removed for the high resolution light microscopy. The same nerve portion (middle third of the brachial part) was also removed from the not injured side. As previously described [38], the proximal stumps were marked with a 7/0 stitch, and the nerves were fixed in 2.5% purified glutaraldehyde and 0.5% saccharose in 0.1M Sorensen phosphate buffer for 5-6 h at 4°C. The samples were washed

in a 1.5% saccharose in 0.1M Sorensen phosphate buffer solution and post-fixed in 2% osmium tetroxide for 2h. The dehydration of the samples was performed with cycles of 5 minutes in ethanol from 30° to 100° and two cycles of 7 minutes in propylene oxide.

After 1 hour in a 1: 1 mixture of propylene oxide and Glauerts' mixture of resins overnight, samples were embedded in Glauerts' mixture of resins (made of equal parts of Araldite M and the Araldite Harter, HY 964, Sigma Aldrich). In the resin mixture, 0.5% of the plasticizer dibutyl phthalate (Sigma Aldrich) was added. For the final step, 2% of accelerator 964 was added to the resin to promote the polymerization of the embedding mixture, at 60°C.

A 2.5 µm thick semi-thin transverse section was cut (Leica Microsystems Ultracut UCT ultramicrotome, Wetzlar, Germany) at the distal stump of each sample and stained using Toluidine blue for high-resolution light microscopy and stereological analysis. The following parameters were assessed: (1) total number of myelinated fibers, (2) myelinated fiber density; (3) diameter of myelinated fibers and axons; (4) myelin thickness; (5) axon diameter/fiber diameter ratio (g-*ratio*); and (6) intraneural collagen ratio, that was obtained by subtracting the total cross-sectional nerve area from the total area of nerve fibers assessed. The nerve section images used for the analysis were randomly selected through a 2D dissector probe to obtain unbiased representative samples of myelinated nerve fibers and analyzed by an assessor blinded to the treatment and side of the sample following the method previously described [36].

An estimated sample of 33 random pictures from each protocol, 6 from each sample (n= 99, effect size f = 0.5; Power [1 − β err prob] = 0.95; α = 0.05; Actual power = 0.95) was evaluated based on the effect of crush injury on nerve morphology previously reported [68].

Dorsal root ganglia explants

To confirm that the NDT effects on rat sensory performances were induced by processes modulated by the peripheral sensory neurons, NDT protocol was performed on DRG explants.

Rats (n=6, 250-280 gr) were sacrificed by a lethal anesthetic overdose of Zoletil + Xilazina (>60 and >10 mg/kg) by intraperitoneal injection. The vertebral spine from C3 to L5 was surgically dissected and the vertebral body and spinal processes were removed to gain ventral access to the spinal cord. Under the operative microscope, DRG were localized in the intraforaminal region, removed, isolated, and collected in a 100x20mm Petri dish with 6 mL of F12 medium (Gibco, Carlsbad, CA, USA). The fine forceps and fine blades were used to remove the pre and post-ganglionic branches and to reduce the fibrous capsule surrounding the ganglia to allow the neurites extension. The DRG were seeded on type I collagen pre-coated silicon membranes (Flexcell®) [43] and incubated at 37°C for 150 minutes in a 40 µL drop of Geltrex Matrigel (Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:1 in serum-free medium (SFM) [33] containing 50 ng/ml nerve growth factor (NGF; Invitrogen, Karlsruhe, Germany) and 1 ng/ml Vitamin C (Sigma-Aldrich Co, MO, USA). Finally, 3 ml of differentiation medium (SFM with 50 ng/ml NGF; 1 ng/ml Vitamin C) were added to cover Matrigel/SFM drops on the silicon membranes in 60x15mm Petri dish (BD Falcon); DRG were maintained at 37°C with 5% CO2.

Ex vivo NDT protocols

The DRG seeded on the silicone membranes were incubated 48 h in differentiation, then moved into a previously validated manual bioreactor [20], and treated with the protocol of repeated uniaxial tensions (NDT). After the NDT protocol was administered, the membranes were returned into the

differentiation medium till the experiment ended (48 h after the treatment administrations and 96 h from seeding the DRG on the membranes).

As described in our previous study [20] an NDT protocol with 1 sec of stretch (strain rate of about 0.1 and 1% of the membrane rest length, 0.01-0.36 mm), and 5 sec to return at the starting position for 30 times was adopted. This protocol was the same daily administered for the *in vivo* experiments of this study.

A sham control named "control out" (CTR OUT) was designed to assess the environmental effects of nourishment privation induced by the treatment protocols reported above. It consisted of keeping the membranes with DRG off from the medium for 180 sec, corresponding to the time in which NDT treated membranes with DRG were positioned in the bioreactor [54]. Finally, a "control in" (CTR IN) protocol, was determined leaving the membranes in the medium for the duration of the entire experiment. The changes induced by the NDT linked to pain suppression and nerve regeneration, like modulation of genes linked to MA, neuropathic pain, neurite outgrowth, and cell survival, were defined as "positive effects". On the contrary, the changes linked to pain promotion and cell apoptosis were defined as "negative effects". An estimated sample of 10 membranes for each protocol (effect size f = 0. 835; Power [1 − β err prob] = 0.95; α = 0.05; Actual power = 0.96) was evaluated based on the NDT effects on neurite length of nociceptive sensory neurons [20].

Immunohistochemistry

The DRG explants were fixed in 4% PFA for 15 min and a previously described protocol was adopted [20], incubating overnight with primary antibody anti-βIII-tubulin (mouse, monoclonal, 1:1000, SIGMA-ALDRICH) diluted in PBS and secondary antibody goat anti-mouse Alexa Fluor488 (1:200,

MOLECULAR PROBEC) diluted in PBS. Explants were mounted with a Dako fluorescence mounting medium; the silicon membrane long edges were mounted parallel to the coverslip long edges [20].

The DRG explants neurites were evaluated adopting the Sholl analysis method [84].

Images were acquired using a Leica SP5 confocal microscope (Leica Microsystems) equipped with a 40x oil immersion objective (HCX PL APO lambda blue 40.0x1.25 OIL UV). From the ImageJ plugin *Neurite-J* described by Torres-Espín and colleagues, the parameters considered were: the distance of the longest neurite (Dmax), the maximum number of neurites (Nmax), and the Sholl critical value, defined as the distance from the organotypic culture center where the maximum number of interception was detected [84].

Quantitative real-time PCR (qRT-PCR) analysis

For *in vivo* experiments, a pool of DRG from the brachial plexus of the crushed and of the not injured side (from each animal) was collected immediately after animal sacrifice and immediately frozen in dry ice and stored at -80° C.

For *ex vivo* experiments, the medium was removed from the petri dish, membranes were immediately scraped with TRIzol Reagent (Invitrogen, ThermoFisher) and DRGs were collected in DNA LoBind tube 1.5 ml (Eppendorf). 500 µl TRIzol was used at room temperature for each sample and samples were immediately frozen in dry ice and stored at -80° C.

Total RNA was extracted with TRIzol Reagent according to the manufacturer's instructions, and 0.75 µg RNA/sample was retro-transcribed. For *in vivo* experiments, the calibrator for the relative quantification was the average of the samples obtained from the not injured side in the NO NDT

group, while for *ex vivo* experiments the calibrator was the average of the CTR IN samples. TATA-box binding protein (TBP) was adopted as a reference gene [20].

PCR primers are reported in Table 1

Table 1. Sequences of primers used for quantitative real-time PCR			
Gene	*Sequence*	*Amplicon length (bp)*	*Accession number*
TLR2	forward: 5'-CAAACTGGAGACTCTGGAAGCAGG-3' reverse: 5'-CACACAGGTAGCTGTCTGCC-3'	125	NM_198769.2 NM_011905.3
TACAN	forward: 5'-TGCAGCAGGACTTCCAAGGTATCC-3' reverse: 5'-CGCTTCTTCTGGCGTGTGATAGAG-3'	115	NM_001010945.1

Western Blot

After RNA extraction with TRIzol Reagent, proteins were extracted following manufacturer's instructions [34], and protein pellets were dissolved in Laemli buffer (2.5% sodium dodecyl sulfate, 0.125 M Tris-HCl, pH 6.8) at 100 °C. The Bicinchoninic Acid assay kit (SIGMA) was used to determine protein concentration. 50 µg protein for each sample was resolved onto a 12% polyacrylamide denaturing gel; western blot analysis was performed as previously described [20]. Primary and secondary antibodies are listed in Table 2. Bands were quantified through Image Lab version 6.1.0 build 7 software (2020; Bio-Rad Laboratories, Inc.). For *in vivo* experiments, data were expressed relative to the mean value of the quantified bands belonging to the healthy nerve (obtained from the not injured side of the "NO NDT" samples), while for *ex vivo* experiments data were expressed relative to the mean values of the quantified bands of "CTR IN" samples.

Table 2. Antibodies used for Western Blot analysis				
Primary antibodies				
	Code	**Dilution**	**Host**	**Source**
Actin	A5316	1:4000	Mouse	Sigma-Aldrich
Bax	SC-23959	1:600	Rabbit	Santa Cruz Biotechnology Inc.
Bcl-xL	SC-8392	1:600	Mouse	Santa Cruz Biotechnology Inc.
TACAN	17455-1-ap	1:1000	Rabbit	Proteintech Europe

TLR-2	SC-166900	1:300	Mouse	Santa Cruz Biotechnology Inc.
Secondary antibodies				
	Code	**Dilution**	**Host**	**Source**
HRP conjugated-anti-rabbit	7074	1:15000	Goat	Cell Signaling
HRP conjugated-anti-mouse	7076	1:15000	Goat	Cell Signaling

Statistical analysis

R Statistical Software (Foundation for Statistical Computing, Vienna, Austria) was adopted to perform statistical analyses [64]. The analyses were performed using the *rstatix* [49] and *car* [32] packages, and the *ggpbur* [48] package was used to perform plots reported in the figures.

The data normality was assessed through the Shapiro-Wilk and Levene test, and if normality assumption was detected, data from *in vivo* experiments were assessed using a Two-way Analysis Of Variance (ANOVA) considering the factors treatment X side. For data obtained from the behavioral analysis, the factors time X treatment were considered. One-Way ANOVA was used to assess differences between continuous variables from *ex vivo* experiments, and Tukey post-hoc correction was used for between-group comparisons. The Dunn Kruskal-Wallis multiple comparisons with Bonferroni post-hoc method were adopted for not normally distributed variables to assess between-groups comparisons. The partial eta squared (η_p^2) was adopted to estimate the effect size for normally distributed variables (η_p^2; 0.010= small effect; 0.059= medium, and 0.138= large effect) otherwise epsilon squared (ε_p^2) was used for non-normally distributed variables (effects: small <0.08, medium 0.08–0.26, large >0.26) [52,67,92].

Confidence Intervals (CI) at 95% were calculated and a statistical significance level of 0.05 was adopted. Values were expressed as mean ± SD (standard deviation). The level of significance was set at p <.05 (*), p <.01 (**), p <.001 (***), and p <.000 (****).

Data availability

On reasonable request, the datasets used in this study are available from the corresponding author.

Results

Changes in animal behavior tests induced by the neurodynamic treatment

To assess the possible positive and protective effects of NDT on peripheral nerve after injury, promoting nerve function recovery and pain modulation, different NDT protocols were followed, as described in materials and methods (Figure 1).

4 days before nerve injury, sensory and motor behavior tests were performed and the baseline performance homogeneity among all groups was assessed to detect possible differences (see Table S1). No sensory test scores significantly differed from each experimental group. Among motor tests, only the time spent on eating the 7 cm breadstick (grissini test speed) reported a significant difference between the NDT PRE-POST and the NO NDT group (Table S1; F [2,15] = 4.25; p=.035).

The behavioral tests were then performed 4, 8, 12, 15, 19, and 22 days after surgery. Results are reported in Figure 3 e 4. Results of two-way ANOVA statistical analysis are reported in Table S2.

Sensory changes

The two-way ANOVA revealed that the overall mechanical pain threshold (Figure 3A) at the injured side was significantly lower in the NDT POST and NDT PRE+POST groups compared to the NO NDT group (p=.000; 95% CI: 3.59 – 10.45; p =.003; 95% CI: 1.33– 8.19). In particular, the NDT POST group showed a significant treatment effect at days 8 and 12 with a lower mechanical pain threshold compared to the NO NDT group, which had shown a lack of mechanical pain responses till day 15 after surgery (p=.000; 95% CI: 6.85 – 34.81; p =.000; 95% CI: 18.52 – 46.48). Also, the NDT PRE+POST compared to the NO NDT group showed a significant interaction between the 2 factors time and

treatment effect at 12 days (p=.000; 95% CI: 1.33– 8.19). Interestingly, the NO NDT group reported a significantly lower mechanical pain threshold at 19 days compared to the NDT PRE+POST group (p=.048; 95% CI: 0.43– 14.6), which can be described as mechanical allodynia. No significant changes were detected for the mechanical pain threshold on the not injured side (Table S2).

The overall touch threshold at the injured side (Figure 3B) was significantly lower in the NO NDT group compared to the NDT POST and NDT PRE+POST groups at 8 and 15 days post-injury (p = 0.024; 95% CI: 0.22 – 3.83; p=.0005; 95% CI: 1.17 – 4.78). The touch threshold was also measured at the contralateral not injured side and a significant overall lower threshold (p=.004; 95% CI: 0.56 – 3.49) in the NO NDT compared to the NDT PRE+POST group was detected (data not shown).

The Pain Behaviour Scale (Figure 3C) showed that normal pain behaviors after the nerve injury were restored significantly early in the NDT POST and NDT PRE+POST groups compared to the NO NDT group. Indeed, the NO NDT group lacked a normal pain response until 15 days after the nerve crush. Furthermore, the NDT POST group had a significant effect at 8 and 12 days with higher scores (closer to the normal pre-surgery values) than the NO NDT group (p=.0003; 95% CI: 0.23 – 1.43; p =.000; 95% CI: 0.23 – 1.43). Also, a significant effect was detected for the NDT PRE+POST at 12 days with a higher score than the NO NDT group (p=.0003; 95% CI: 0.23 – 1.43).

Finally, the number of withdrawals for repeated high frequency neurodynamic tests to assess descending pain modulation effects (Figure 3D) measured at 22 days after injury was significantly lower in the NDT POST and NDT PRE+POST groups compared to the NO NDT group ($F[2,15]= 6.894$, p=.008; 95% CI: 0.43– 5.57 and 0.76– 5.90; $\eta^2_p = 0.479$, very large effect).

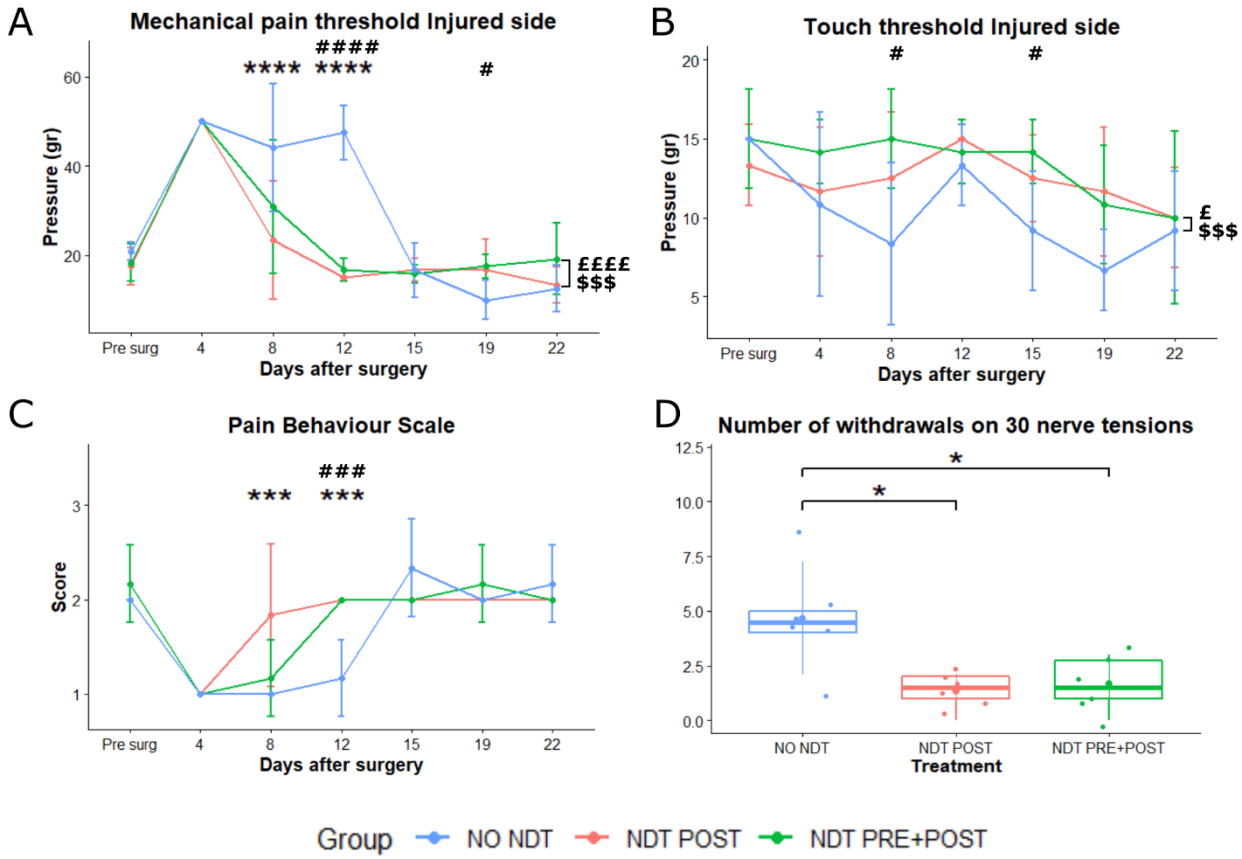


Figure 3. Sensory assays. (A and B) Mechanical pain threshold and touch threshold at the paw of the median and ulnar nerve crush injured side obtained by Von Frey monofilament administration at each experimental time point (x-axis) and expressed in grams (gr). (C) Pain behaviors at the Pinprick test are recorded when four consecutive pin administrations are given to the forepaw. (D) The number of withdrawals, at 22 days after injury, was recorded during 30 consecutive neurodynamic tests of the upper limb. Data are expressed as mean ± SD. The analysis compared all groups to the control group (NO NDT) with ANOVA for repeated measures (data are normally distributed with comparable variances). Differences at each time point between groups are reported * p <.05, *** p <.0001, and **** p <.0000, comparing the group NDT POST to the NO NDT group, or # and ####, p <.05 and p <.000, comparing the NDT PRE+POST to the NO NDT group. Overall differences between groups are reported £ p <.05 ££££ p <.000 comparing the NDT POST to the NO NDT group and $$$ p<.001 comparing the NDT PRE+POST to the NO NDT group.

Motor changes

The speed in eating the 7 cm grissini revealed no significant interaction between time and treatment factors as shown in Figure 4A. An overall significantly lower speed was detected comparing the NDT PRE+POST to the NO NDT group (Table S2; p=.007; 95% CI: 3.34 – 24.83). The number of paw adjustments performed while eating the grissini (Figure 4B) was not significantly different among groups and within time (Table S2).

A significant overall effect for treatment was detected on the climbing speed (Figure 4C and Table S2) between NDT POST and NO NDT group and NDT PRE+POST and NO NDT group (p=.009; 95% CI: 5.46 – 46.97; p=.0005; 95% CI: 13.26 – 54.77). Finally, no significant differences were detectable among groups at the grasping test (Figure 4D), where all animals showed signs of grip strength recovery starting from 12-15 days after the nerve injury.

Finally, the rat total weight and the flexor digitorum superficialis and profundus wet muscle weight were not significantly different among all groups (Table S2).

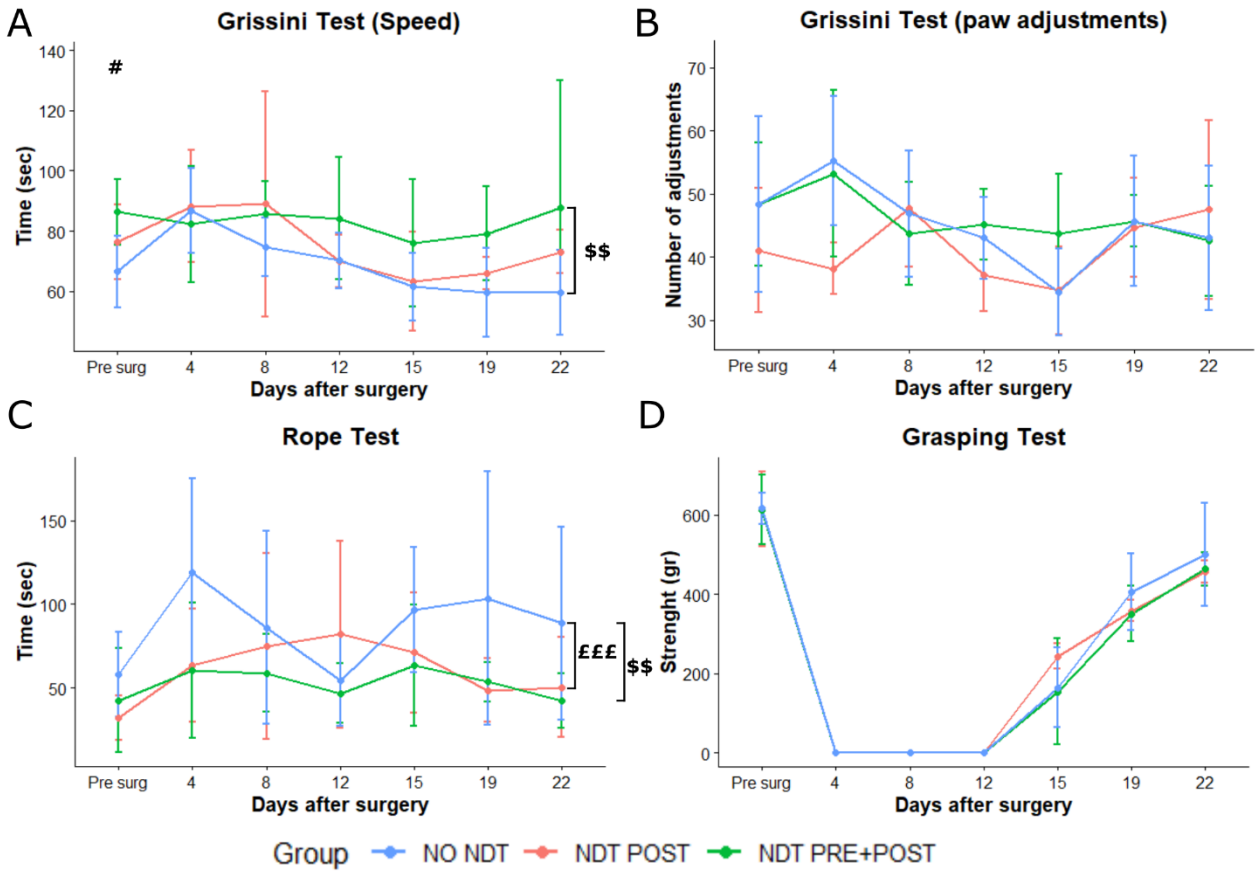


Figure 4. Motor assays. (A and B) Speed and number of paw adjustments recorded while rats are eating 7 cm grissini pieces, at different time points after nerve injury and repair. (C) Speed in climbing a 160 cm vertical rope expressed in seconds (sec). (D) Grasping strength was recorded by the device as maximal resistance to pull the grid of the device with the injured side paw. Data are expressed as mean ± SD. The analysis compared all groups to the control group (NO NDT) with ANOVA for repeated measures (data are normally distributed with comparable variances). Differences at each time point between groups are reported # p <.05, comparing the NDT PRE+POST to the NO NDT group. Overall differences between groups are reported £££ p <.001 comparing the NDT POST to the NO NDT group and $$ p <.01 comparing the NDT PRE+POST to the NO NDT group. Detailed data of the statistical analysis are reported in Table S2.

High-Resolution Light Microscopy

To study the possible effect of NDT on peripheral nerve regeneration, we performed stereological and morphometrical analysis on both the “healthy” median nerves (the contralateral nerve, not injured) and the regenerated nerves, 24 days after the crush lesion.

From a morphological point of view, the regenerated median nerves belonging to the three experimental groups showed the typical aspect of nerve regeneration with smaller nerve fibers and thinner myelin sheath compared to contralateral not injured nerves (Figure 5A). Quantitative analysis showed a significant higher total number of myelinated fibers in the injured side compared to the not injured side (Figure 5B) only for the NO NDT group ($F[1, 156]= -17.67$; $p=.02$; 95% CI: -2214.93 – -371.85; η^2_p =0. 102, medium effect), while no significant effect of side x treatment were detected in the two treated groups ($F[1, 156]= 0.001$; $p=.999$; η^2_p =0.000, very low effect). The axon and fiber diameter were significantly different between the two sides for all the experimental groups (Figure 5D and E), without any significant difference between the treatment groups ($F[1, 156]= 195.95$; $p=.000$; 95% CI: -2.36 – -1.77; $\eta^2_p = 0.557$, very large effect). The same results were observed in myelin thickness evaluation (Figure 5F), with thinner myelin in the injured side compared to the not injured side, for all experimental groups ($F[1, 156]= 266.38$; $p=.000$; 95% CI: 2.69– 3.43; $\eta^2_p = 0.631$, very large effect). A significant difference between sides in the NO NDT group was reported for the g-*ratio* values ($F[1, 156]= 16.68$; $p=.0001$; 95% CI: 0.68– 0.71; $\eta^2_p = 0.097$, small effect; Figure 5G), and a significant higher g-*ratio* between injured NO NDT and NDT POST groups was observed ($F[2, 156]= 6.74$; $p=.0015$; $\eta^2_p = 0.041$, small effect). Finally, a significant difference was detected between the injured and not injured sides in all groups for intraneural fibrosis ($F[1, 156]= 239.27$; $p=.0001$; 95% CI: 41.62 – 52.75; $\eta^2_p = 0.605$, very large effect; Figure 5H). Notably, the NDT POST group reported a significant lower ratio of intraneural collagen compared to the NO NDT group at the injured side ($F[1, 156]= 1.99$; $p=.02$; 95% CI: 0.01 – 0.21; $\eta^2_p = 0.025$, small effect).

The same analyses were performed also on the ulnar nerve, with similar results (data not shown).

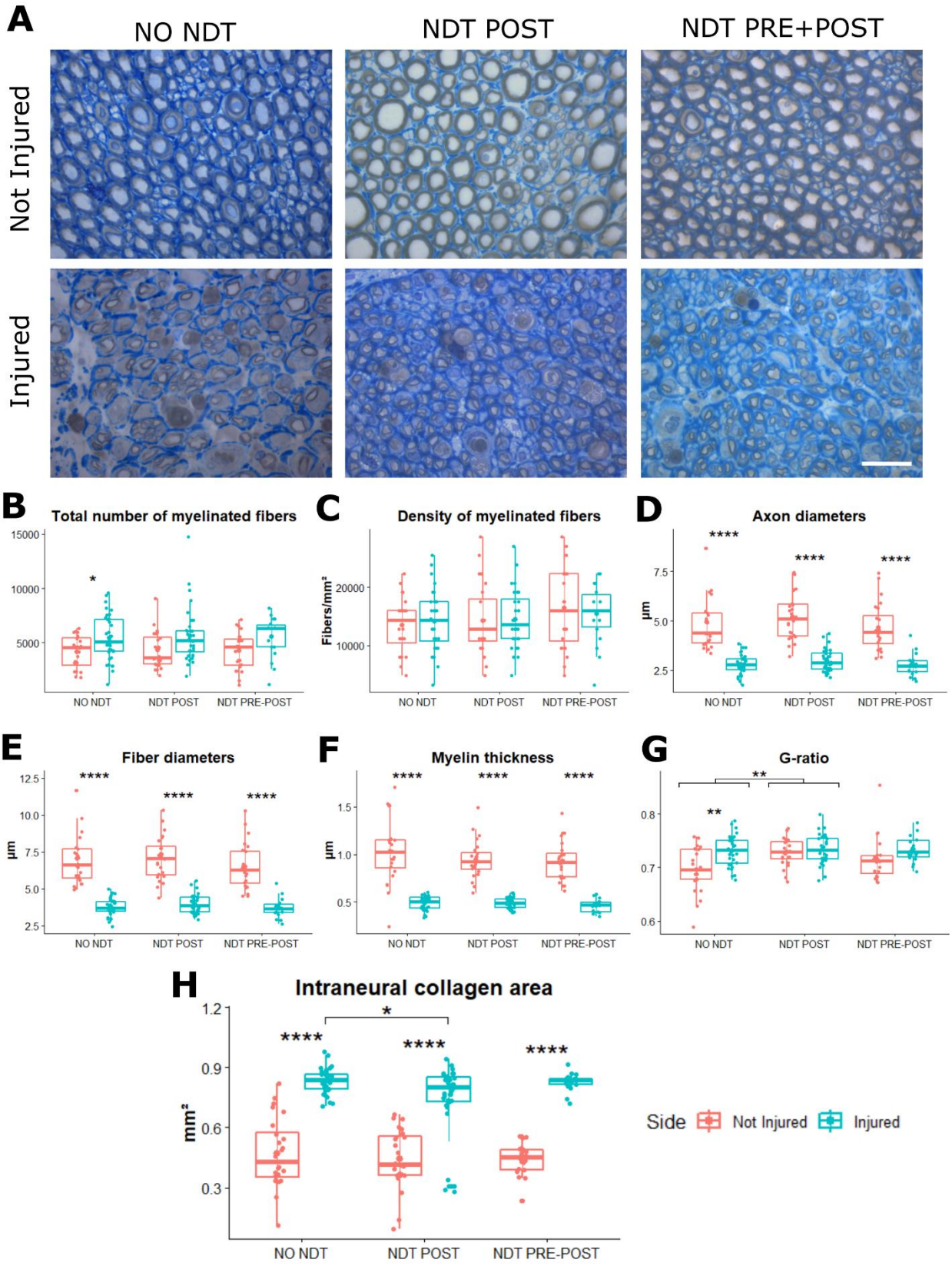


Figure 5. Morphological and morphometric analysis. (A) High magnification light microscopy representative images of toluidine blue-stained semi-thin cross-sections of uninjured median nerves and injured median nerves after 24 days from the lesion, for each treatment. Scale bar: 20 µm. (B–H) Stereological assessment of morphological changes during nerve regeneration. (B) number of myelinated fibers; (C) myelinated fiber density; (D) axon diameters; (E); fiber diameters; (F) myelin thickness; (G) axon diameter/fiber diameter ratio (*g-ratio*); (H) intraneural collagen area. Data are expressed as mean ± SD. The analysis compared all groups and sides to the control group (NO NDT) with a Two-way ANOVA (data are normally distributed with comparable variances). Differences are reported *p <.05, **p <.01, ***p <.001, and ****p <.0000.

Gene and protein expression

Gene expression analysis on DRG of both ipsilateral (injured) and contralateral (not injured) sides was performed 24 days after nerve injury to detect any differences between sides and to assess possible systemic NDT effect. Genes linked to MA mediated by the immune response (TLR2) and by the mechanosensitive channels induced by TNFα (TACAN) were evaluated comparing the treatments and sides from *in vivo* experiments (Figure 6A). No significant treatment effect was detected for the TLR2 gene expression ($F[2,30]= 1.00$; $p=.38$; $\eta_p^2= 0.063$, small effect; $F[2,18]= 0.673$; $p=.418$; $\eta_p^2= 0.022$, small effect). Interestingly, a significant lower TACAN expression was detected between NDT POST and NO NDT groups, both in the injured and not injured sides ($F[2,30]= 1.002$; $p=.02$; 95% CI: 0.01 – 0.18; $\eta_p^2= 0.063$, small effect; and $F[2,30]= 5.08$; $p=.03$; 95% CI: 0.01 – 0.28; $\eta_p^2= 0.022$, small effect). Finally, in the NO NDT group, a significant difference was also observed between the injured and not injured sides (Figure 6A; $F[1,30]= 1.002$; $p=.04$; $\eta_p^2= 0.022$, small effect), while such difference was not observed in the other two experimental groups.

The protein expression of TLR2 and TACAN was also evaluated in the DRG comparing the treatments and sides, as represented in Figure 6B. No significant difference between treatment and sides were detectable at protein level for TLR2 and TACAN ($F[2,18]= 0.087$; $p=.91$; $\eta_p^2= 0.010$, small effect; and $F[2,18]= 0.012$; $p=.99$; $\eta_p^2= 0.001$, very small effect).

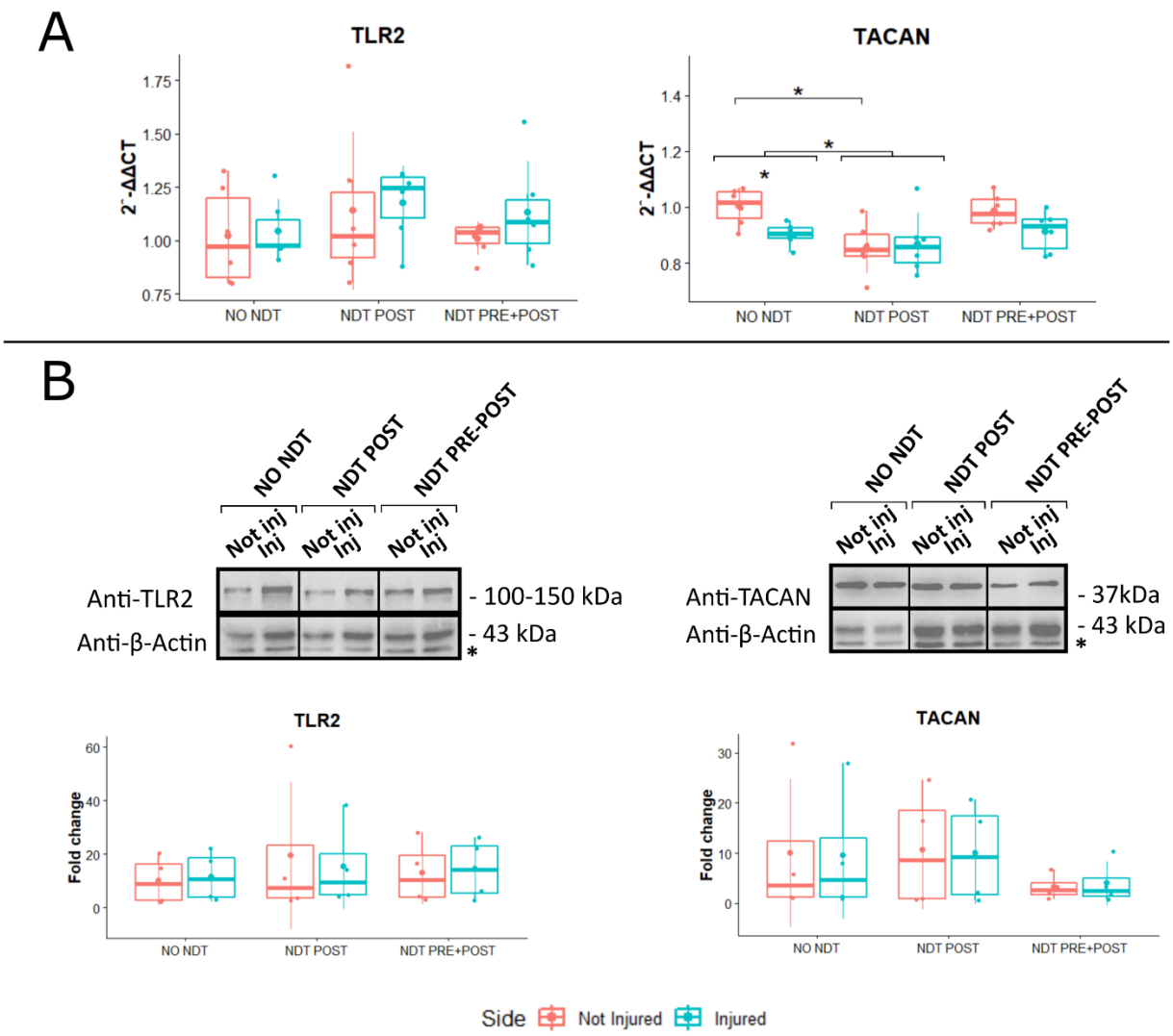


Figure 6. Gene and protein expression analysis of markers linked to mechanical allodynia and apoptosis expressed in the DRG. Panel A: Beneficial or side effects induced by the neurodynamic protocols on DRG were assessed adopting the relative quantification ($2^{-\Delta\Delta Ct}$) of genes by qRT-PCR. Data normalization was performed considering TATA-binding protein (TBP) as a housekeeping gene. All data were calibrated to NO NDT Not Injured samples. Panel B: a representative western blot is shown (original membranes from representative samples were taken from are reported in Figure S1); actin was used as a loading control. Asterisks (*) in panel B identify unspecific bands. Values in the graphics are expressed as mean ± SD. Respectively Two-way and One-way ANOVA were carried out (data are normally distributed with comparable variances). *p <.05

Neurodynamic effects on DRG explant

To further confirm that the NDT protocol induced selective DRG neuron responses, the DRG explants from healthy rats were subjected to NDT (30 repetitions) using a previously validated manual bioreactor [20], and analyses were performed two days after the treatment (Figure 7). A significant NDT effect was observed on the neurite length (Dmax; $F[2, 27]= 5.14$; $p=.005$; $\eta_p^2 =0.3$, large effect).

Indeed, a significantly higher Dmax was detected between NDT and both the CTR OUT and CTR IN groups (Figure 7B and Table S3). No significant differences were detected for the maximum number of neurites (Nmax) and Sholl Critical Value among the three experimental groups (Table S3 and Fig 7B).

Gene expression analysis showed no significant differences for the expression of TLR2 (Figure 7C), ($F[2,26]=2.08$; $p=.145$; 95% CI: 0.46 – 1.01; $\eta^2=0.138$, medium effect). Interestingly, a significant effect of treatment was detected for TACAN expression ($F[2,26]=5.28$; $p=.0132$; 95% CI: 0.66 – 0.91; $\eta^2=0.289$, large effect). In particular, a significant downregulation of TACAN expression was detected in the NDT group compared to sham treatment (CTR OUT) group ($F[2, 26]= 5.28$; $p=.039$; 95% CI: 0.01 – 0.39), and in the CTR IN group compared to CTR OUT group ($F[2, 26]= 5.28$; $p=.017$; 95% CI: 0.04 – 0.44).

Finally, NDT treatment induced a significant anti-apoptotic behavior compared to the sham treatment (CTR OUT) ($F[2,23]= 4.78$; $p=.02$; 95% CI: 2.14 – 20.51; $\eta_p^2= 0.294$, medium effect; Figure 7D), as demonstrated by a lower Bax/Bcl-XL ratio in the NDT group.

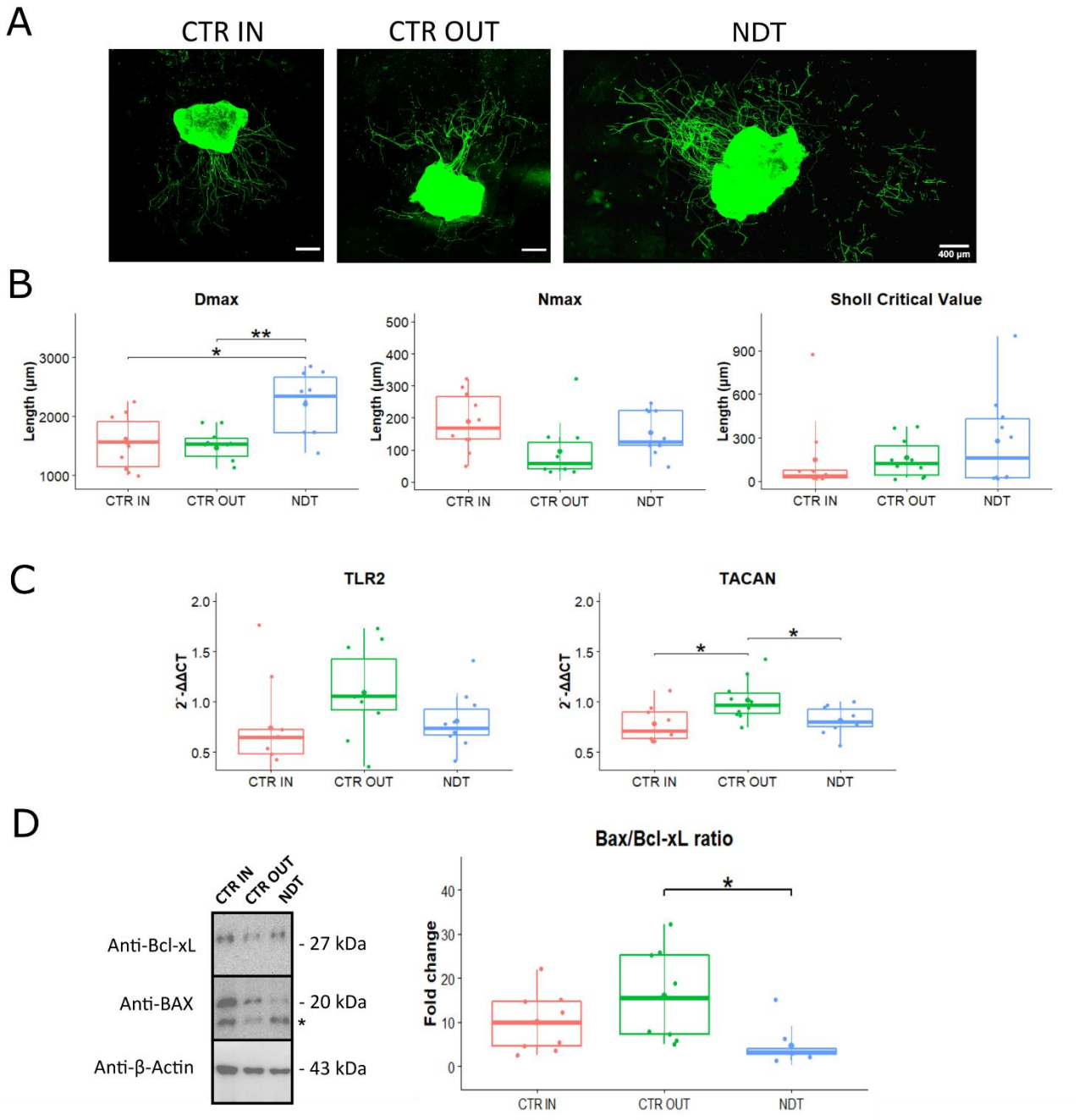


Figure 7. Effects of neurodynamic treatment on cell morphology, gene and protein expressions on dorsal root ganglia (DRG) explants. Panel A: Representative images of rat organotypic dorsal root ganglia neurons stained with βIII-tubulin reported for each type of experimental protocol. Scale bar: 400 µm. Panel B: Quantitative analysis of the distance of the longest neurite (Dmax), the maximum number of neurites (Nmax), and the Sholl critical value, defined as the distance from the organotypic culture center. Values in the graphics are expressed as mean ± SD. Panel C: Gene expression analysis of markers linked to mechanical allodynia and neuropathic pain. Beneficial or side effects induced by the neurodynamic protocols on dorsal root ganglia explants were assessed adopting the relative quantification analysis ($2^{-\Delta\Delta Ct}$) of genes by qRT-PCR. Data normalization was performed considering TATA-binding protein (TBP) as a housekeeping gene. All data were calibrated to CTR OUT sample). Panel D: BAX and Bcl-xL protein expression in DRG explants. Protocols are described as follows: not treated (CTR IN), sham-treated (CTR OUT), and treated (NDT) with 30 repetitions of neurodynamic treatment. Experiments were carried out in a biological octuplicate (n=28). Asterisks (*) in panel D identify unspecific bands. Values in the graphics are expressed as mean ± SD. For normally distributed data with comparable variances One-way ANOVA was carried out, while Kruskal-Wallis-test was used for nonparametric data; asterisks show statistically significant differences with CTR OUT (sham sample); *p <.05, and ** p <.01.

Discussion

The repeated and selective nerve tensioning or flossing techniques, in which NDT consists, are non-pharmacological treatments able to modulate pain and, in particular, mechanical neuropathic pain in humans and animals [8,57,66,81,93–95,98,103]. NDT has been demonstrated to induce a selective response in sensory and motor neurons *in vitro* in a dose-dependent manner [20]. In addition, multilevel changes in the central nervous system have been reported in the literature. In particular, it has been demonstrated that NDT induces significant changes in nerve growth factor (NGF), glial fibrillary acidic protein (GFAP) expression in the homologous DRG and spinal cord [70], and increases the expression of the μ-opioid receptor in the DRG [39]. In a rat neuropathic pain model, NDT affected the expression of GFAP and brain-derived neurotrophic factor (BDNF) proteins in ventral posterolateral thalamus nucleus and periaqueductal gray [71] and the k-opioid receptor expression in the periaqueductal gray [69]. Notably, all these processes are related to pain modulation but none of them has been defined to be selective for mechanical allodynia and nerve regeneration induced by NDT. Our aims were therefore to assess the effects of NDT on sensory-motor recovery and mechanical neuropathic pain modulation and its possible involvement in nerve regeneration after a nerve injury.

The current study shows that the NDT stimulates nerve regeneration, promotes neuronal cell survival, improves behavioral tests, and reduces intraneural fibrosis, following the available evidence reported in the literature on NDT used in clinical and preclinical studies [8,16,39,69–71,89,91,104]. Also, NDT prevents ipsilateral and contralateral mechanical threshold lowering induced by the nerve crush injury which are phenomena sustained by a multilevel PNS plasticity and systemic processes [28,29,45,86].

Contrary to other studies on NDT described in the literature, in which animals under anesthesia are subjected to NDT protocols [39,69–71,91,104], in our study we deliberately kept the animal awake. This

allows the clinician to feel the tissue resistance, including muscle contraction, as it happens in the clinical settings to tailor properly the treatment with real-time feedback. Also, the number of paw withdrawals to repeated high frequency nerve tensions were recorded showing increased irritability of the neural tissue to mechanical stress only in the not treated group. Indeed, at the time in which this test was administered, sensory recovery for touch and pain was restored for all groups (Figures 2 and 3), and the test frequency, five times higher than treatment, was able to elicit temporal summation [1,99]. These data confirm that NDT can promote pain descending modulation effects as confirmed by an increased expression of the μ-opioid receptor in the DRG and k-opioid receptor in the periaqueductal gray detected in neuropathic pain models of rats treated with NDT protocols [39,69].

Our data from *ex vivo* experiments show that the NDT induces an anti-apoptotic effect in the DRG neurons as demonstrated by a lower Bax/BclxL ratio compared to the sham treatment (CTR OUT). Taken together these data confirm that the NDT is well tolerated without any risk for side or negative effects for nerve healing processes or animal discomfort.

Our results show that NDT administered after nerve injury downregulates TACAN expression, but not TLR2 (selectively related to immune-mediated mechanical allodynia) [25], in the ipsilateral and contralateral DRG. Moreover, our results on *ex vivo* DRG explants confirmed TACAN downregulation induced by NDT treatment. These data support the hypothesis that TACAN expression in the DRG gives a direct indication of nociceptive neurons activation after a nerve injury [13] and that NDT is effectively modulating MA and hyperalgesia mechanisms in sensory neurons. As observed by Zhu and colleagues in a rat neuropathic pain model, the NDT induced MA and hyperalgesia suppression with significant changes in IL-1β and TNFα protein expression in the sciatic nerve but not in the DRG [104].

Since Sellheim pioneered the paravertebral block technique in 1905 to suppress local pain before and after surgery [60], the peripheral nerves and the DRG had become a therapeutic target for pain suppression [2,12,44]. Only the recent discovery of gene regulation in the DRG and the studies on intraforaminal lidocaine injection on short-term phantom limb pain suppression [13,25,50,88,101] led to the hypothesis that the DRG can be a pain driver also responsible for pain gating effects. A growing body of evidence shows that DRG stimulation induces a reduction in pain responses for mechanical stimuli [27,65,76,88,97,101]. Indeed, even if the DRG was not included in the pain gate control theory postulated by Melzack and Wall [59], the short and long term effects reported in our *ex vivo* and *in vivo* experiments, in agreement with the literature, confirm that changes observed in the DRG were related to mechanical pain modulation in animals. For these reasons, we suggest that the DRG plays a relevant role in the pathophysiology of pain, and not only nociception, especially when the pain-related phenomenon is stimuli-dependent like allodynia and hyperalgesia. In addition, we have shown that administration of tailored tension to the nerves affects stimuli-dependent pain by affecting receptor expression in the DRG.

In this study, we also pointed out our attention to the possible involvement of NDT in nerve regeneration processes. The morphometric analyses had shown that the number of myelinated fibers is similar between injured and not injured sides only in treated groups, even if they are significantly smaller as normally occurs in regenerating nerves. Notably, only the post-treated group had shown, after injury, a significantly higher maturation of the myelinated fibers (g-*ratio*) and a lower amount of intraneural collagen deposit than the NO NDT group. According to our data, Lima and colleagues [53] have shown that NDT significantly reduces the intraneural scar tissue formation after sciatic nerve crush injury. We suggest that a plausible NDT effect on intraneural collagen deposit possibly leads to lower intraneural pressure that causes aberrant or irregular low rate

nociceptor discharge, as shown by Bove and co-workers [14], and affects positively nerve regeneration.

The NDT positive effects were confirmed also by *ex vivo* experiments. Indeed, one session of 30 repeated strains, administered for 3 minutes, induced a higher neurites length of about one millimeter if compared to sham treatment (CTR OUT). These morphological changes together with our previous *in vitro* studies [20] and behavioral tests from animals suggest that NDT promotes a faster nerve regeneration after a nerve injury with tactile and nociception early recovery.

These pro-regenerative effects may be crucial for the injured nerve to prevent distal atrophic processes and to reach the target tissue to recover normal sensibility or muscular performance in the case of sensory or motor neurons as in the case of the present experiment [5,18,80,91]. Indeed, the results of the motor tests revealed that NDT prevents gross motor function deficits observed at the rope test without compromising the dexterity and strength of the forelimb, at the grasping and grissini test, over time [19,21,82,83].

NDT modulates pain in pre-clinical and clinical studies showing effects only on the treated side [15,104]. Our data provide evidence that NDT prevents sensitization to normal mechanical stimuli that we observed by the significantly lowered mechanical threshold in the untreated rats on both sides, suggesting that NDT may activate metameric or systemic processes avoiding nerve sensitization to mechanical stimuli. Even if some pain behaviors related to contralateral DRG changes after a nerve injury had been described in the literature [28,30,45,86], only a few clinical studies have shown in healthy subjects that NDT improves the mobility of the contralateral untreated limb [62,72]. We provide evidence that NDT administered before nerve injury modulates pain with a significant antiallodynic effect for mechanical stimuli 19 days after nerve injury, with no significant difference in TACAN gene expression in the ipsilateral and contralateral DRG assessed 24 days after the injury when compared to controls. Results obtained have also shown that NDT pretreatment induces a delayed sensory

recovery for mechanical pain stimuli at 12 days instead of 8 days obtained in the NDT POST group. Similar beneficial effects between pre and post NDT treated on motor tasks were detected except for the speed in the grissini piece consumption that was significantly higher in the pretreated than the control group. For these reasons, it is worth further exploring the mechanisms induced by NDT before the injury and studying those that are involved in sensory modulation at the contralateral side to nerve injury.

In conclusion, our study shed light on how the NDT is an intervention able to promote nerve regeneration and mechanical pain suppression, which nowadays is still a very common condition particularly difficult to treat with existing therapies. We have also shown that the NDT affects a specific pathophysiological mechanism responsible for mechanical allodynia and hyperalgesia by modulating the TACAN expression and not TLR2. We provided evidence that NDT is not a time-consuming intervention for neuropathic pain treatment, and we suggest starting the administration early after acute nerve injury (2-4 days after trauma). Our data, together with those available in the literature, showed that NDT is an effective non-pharmacological intervention for nerve injuries and neuropathic pain management. In addition, since it does not require any electronic device or expensive tool it is also a sustainable intervention affordable for any health care system worldwide, with negligible environmental impact.

No data were available in the literature to define the proper dosage and posology of NDT treatment to be adopted in the clinical settings, increasing the risk of under or over-treating the patient. We believe it is worth translating the NDT protocol described in our experiments in the clinical setting to promote nerve recovery after injury or to treat neuropathic pain of the limbs and/or trunk, radiculopathies, and diabetic neuropathy.

References

1. Aby F, Bouali-Benazzouz R, Landry M, Fossat P: Windup of nociceptive flexion reflex depends on synaptic and intrinsic properties of dorsal horn neurons in adult rats. Int J Mol Sci [Internet] Multidisciplinary Digital Publishing Institute; 20:6146, 2019 [cited 2021 Dec 23]. Available from: https://www.mdpi.com/1422-0067/20/24/6146/htm

2. Adriani J: From Koller to Labat: A Historic Resume. Reg Anesth J Neural Blockade Obstet Surgery, Pain Control [Internet] BMJ Publishing Group Ltd; 5:3–7, 1980 [cited 2021 Jul 9]. Available from: https://rapm.bmj.com/content/5/4/3

3. Ahlström FHG, Mätlik K, Viisanen H, Blomqvist KJ, Liu X, Lilius TO, Sidorova Y, Kalso EA, Rauhala P V.: Spared Nerve Injury Causes Sexually Dimorphic Mechanical Allodynia and Differential Gene Expression in Spinal Cords and Dorsal Root Ganglia in Rats. Mol Neurobiol [Internet] Mol Neurobiol; 58:5396–419, 2021 [cited 2022 Jan 25]. Available from: https://pubmed.ncbi.nlm.nih.gov/34331199/

4. Arenas OM, Lumpkin EA: Touching Base with Mechanical Pain. Cell , 2020.

5. Arnaout A, Fontaine C, Chantelot C: Sensory recovery after primary repair of palmar digital nerves using a Revolnerv® collagen conduit: A prospective series of 27 cases. Chir Main , 2014.

6. Arumugam V, Selvam S, MacDermid JC: Radial Nerve Mobilization Reduces Lateral Elbow Pain and Provides Short-Term Relief in Computer Users§. Open Orthop J [Internet] Bentham Science Publishers Ltd.; 8:368–71, 2014 [cited 2021 Apr 14]. Available from: https://pubmed-ncbi-nlm-nih-gov.bibliopass.unito.it/25352930/

7. Ayub A, Osama M, Shakil-Ur-Rehman, Ahmad S: Effects of active versus passive upper extremity neural mobilization combined with mechanical traction and joint mobilization in females with cervical radiculopathy: A randomized controlled trial. J Back Musculoskelet Rehabil IOS Press; 32:725–30, 2019.

8. Basson A, Olivier B, Ellis R, Coppieters M, Stewart A, Mudzi W: The effectiveness of neural mobilizations in the treatment of musculoskeletal conditions: a systematic review protocol. JBI database Syst Rev Implement reports 13:65–75, 2015.

9. Beaulieu-Laroche L, Christin M, Donoghue A, Agosti F, Yousefpour N, Petitjean H, Davidova A, Stanton C, Khan U, Dietz C, Faure E, Fatima T, MacPherson A, Mouchbahani-Constance S, Bisson DG, Haglund L, Ouellet JA, Stone LS, Samson J, Smith MJ, Ask K, Ribeiro-da-Silva A, Blunck R, Poole K, Bourinet E, Sharif-Naeini R: TACAN Is an Ion Channel Involved in Sensing Mechanical Pain. Cell [Internet] Cell; 180:956-967.e17, 2020 [cited 2021 Aug 11]. Available from: https://pubmed-ncbi-nlm-nih-gov.bibliopass.unito.it/32084332/

10. Beltran-Alacreu H, Jiménez-Sanz L, Fernández Carnero J, La Touche R: Comparison of Hypoalgesic Effects of Neural Stretching vs Neural Gliding: A Randomized Controlled Trial. J Manipulative Physiol Ther [Internet] Mosby Inc.; 38:644–52, 2015 [cited 2021 Apr 14]. Available from: https://pubmed-ncbi-nlm-nih-gov.bibliopass.unito.it/26481666/

11. Beth Darnall: To treat pain, study people in all their complexity. , 2018.

12. Boezaart AP, Lucas SD, Elliott CE: Paravertebral block: Cervical, thoracic, lumbar, and sacral. Curr. Opin. Anaesthesiol. 2009.

13. Bonet IJM, Araldi D, Bogen O, Levine JD: Involvement of TACAN, a Mechanotransducing Ion Channel, in Inflammatory But Not Neuropathic Hyperalgesia in the Rat. J Pain [Internet] Elsevier B.V.; 22:498–508, 2021 [cited 2021 Jul 6]. Available from: http://www.jpain.org/article/S152659002030105X/fulltext

14. Bove GM, Delany SP, Hobson L, Cruz GE, Harris MY, Amin M, Chapelle SL, Barbe MF: Manual therapy prevents onset of nociceptor activity, sensorimotor dysfunction, and neural fibrosis induced by a volitional repetitive task. Pain 160:632–44, 2019.

15. Boyd BS, Nee RJ, Smoot B: Safety of lower extremity neurodynamic exercises in adults with diabetes mellitus: a feasibility study. J Man Manip Ther [Internet] J Man Manip Ther; 25:30–8, 2017 [cited 2021 Nov 8]. Available from: https://pubmed.ncbi.nlm.nih.gov/28855790/

16. Boyd BS, Villa PS: Normal inter-limb differences during the straight leg raise neurodynamic test: A cross sectional study. BMC Musculoskelet Disord 13:, 2012.

17. Boyd BS, Wanek L, Gray AT, Topp KS: Mechanosensitivity of the Lower Extremity Nervous System During Straight-Leg Raise Neurodynamic Testing in Healthy Individuals. J Orthop Sport Phys Ther [Internet] 39:780–90, 2009. Available from: http://www.jospt.org/doi/10.2519/jospt.2009.3002

18. Bueno FR, Shah SB: Implications of tensile loading for the tissue engineering of nerves. Tissue Eng. - Part B Rev. 2008.

19. Carlini EA, Teresa M, Silva A, Cesare LC, Endo RM: Effects of chronic administration of β β-(3,4-dimethoxyplienyl)-ethylamine and β-(3,4,5-trimethoxyphenyl)-ethylamine on the climbing rope performance of rats. Pharmacology [Internet] Med Pharmacol Exp Int J Exp Med; 17:534–42, 1967 [cited 2021 Oct 15]. Available from: https://pubmed.ncbi.nlm.nih.gov/5631690/

20. Carta G, Gambarotta G, Fornasari BE, Muratori L, El Soury M, Geuna S, Raimondo S, Fregnan F: The neurodynamic treatment induces biological changes in sensory and motor neurons in vitro. Sci Rep [Internet] Sci Rep; 11:13277, 2021 [cited 2021 Jun 30]. Available from: http://www.nature.com/articles/s41598-021-92682-2

21. Casal D, Mota-Silva E, Iria I, Pais D, Farinho A, Alves S, Pen C, Mascarenhas-Lemos L, Ferreira-Silva J, Ferraz-Oliveira M, Vassilenko V, Videira PA, Goyri-O'neill J: Functional and physiological methods of evaluating median nerve regeneration in the rat. J Vis Exp [Internet] J Vis Exp; 2020:, 2020 [cited 2021 Sep 27]. Available from: https://pubmed.ncbi.nlm.nih.gov/32364547/

22. Castro J, Maddern J, Erickson A, Caldwell A, Grundy L, Harrington AM, Brierley SM: Pharmacological modulation of voltage-gated sodium (NaV) channels alters nociception arising from the female reproductive tract. Pain 162:227–42, 2021.

23. Cavanaugh DJ, Lee H, Lo L, Shields SD, Zylka MJ, Basbaum AI, Anderson DJ: Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. Proc Natl Acad Sci U S A , 2009.

24. Chernov A V., Shubayev VI: Sexual Dimorphism of Early Transcriptional Reprogramming in Dorsal Root Ganglia After Peripheral Nerve Injury. Front Mol Neurosci [Internet] Front Mol

Neurosci; 14:, 2021 [cited 2022 Jan 25]. Available from: https://pubmed.ncbi.nlm.nih.gov/34966260/

25. Cobos EJ, Nickerson CA, Gao F, Chandran V, Bravo-Caparrós I, González-Cano R, Riva P, Andrews NA, Latremoliere A, Seehus CR, Perazzoli G, Nieto FR, Joller N, Painter MW, Ma CHE, Omura T, Chesler EJ, Geschwind DH, Coppola G, Rangachari M, Woolf CJ, Costigan M: Mechanistic Differences in Neuropathic Pain Modalities Revealed by Correlating Behavior with Global Expression Profiling. Cell Rep , 2018.

26. Corso CR, Martins DF, Borges SC, Beltrame OC, Telles JEQ, Buttow NC, Werner MF de P: Effect of simvastatin on sensorial, motor, and morphological parameters in sciatic nerve crush induced-neuropathic pain in rats. Inflammopharmacology [Internet] Inflammopharmacology; 26:793–804, 2018 [cited 2022 Feb 1]. Available from: https://pubmed.ncbi.nlm.nih.gov/29188473/

27. Deer TR, Levy RM, Kramer J, Poree L, Amirdelfan K, Grigsby E, Staats P, Burton AW, Burgher AH, Obray J, Scowcroft J, Golovac S, Kapural L, Paicius R, Kim C, Pope J, Yearwood T, Samuel S, McRoberts WP, Cassim H, Netherton M, Miller N, Schaufele M, Tavel E, Davis T, Davis K, Johnson L, Mekhail N: Dorsal root ganglion stimulation yielded higher treatment success rate for complex regional pain syndrome and causalgia at 3 and 12 months: A randomized comparative trial. Pain , 2017.

28. Dubový P, Brázda V, Klusáková I, Hradilová-Svíženská I: Bilateral elevation of interleukin-6 protein and mRNA in both lumbar and cervical dorsal root ganglia following unilateral chronic compression injury of the sciatic nerve. J Neuroinflammation [Internet] J Neuroinflammation; 10:, 2013 [cited 2021 Nov 10]. Available from: https://pubmed.ncbi.nlm.nih.gov/23634725/

29. Dubový P, Hradilová-Svíženská I, Brázda V, Joukal M: Toll-like receptor 9-mediated neuronal innate immune reaction is associated with initiating a pro-regenerative state in neurons of the dorsal root ganglia non-associated with sciatic nerve lesion. Int J Mol Sci [Internet] Int J Mol Sci; 22:, 2021 [cited 2021 Nov 10]. Available from: https://pubmed.ncbi.nlm.nih.gov/34299065/

30. Dubový P, Klusáková I, Hradilová-Svíženská I, Joukal M, Boadas-Vaello P: Activation of astrocytes and microglial cells and CCL2/CCR2 upregulation in the dorsolateral and ventrolateral nuclei of periaqueductal gray and rostral ventromedial medulla following different types of sciatic nerve injury. Front Cell Neurosci [Internet] Front Cell Neurosci; 12:, 2018 [cited 2021 Sep 3]. Available from: https://pubmed.ncbi.nlm.nih.gov/29515373/

31. Ellis R, Carta G, Andrade RJ, Coppieters MW: Neurodynamics: is tension contentious? J Man Manip Ther [Internet] J Man Manip Ther; , 2021 [cited 2022 Jan 31]. Available from: https://pubmed.ncbi.nlm.nih.gov/34781843/

32. Fox J, Weisberg S: Package 'car'. Companion to Applied Regression, Second Edition. Thousand Oaks CA Sage. 2011.

33. Fueshko S, Wray S: LHRH cellsmigrate on peripherin fibers in embryonic olfactory explant cultures: An in vitro model for neurophilic neuronal migration. Dev Biol [Internet] Dev Biol; 166:331–48, 1994 [cited 2021 Apr 14]. Available from: https://pubmed.ncbi.nlm.nih.gov/7958456/

34. Gambarotta G, Garzotto D, Destro E, Mautino B, Giampietro C, Cutrupi S, Dati C, Cattaneo E,

Fasolo A, Perroteau I: ErbB4 expression in neural progenitor cells (ST14A) is necessary to mediate neuregulin-1β1-induced migration. J Biol Chem , 2004.

35. Gamelas T, Fernandes A, Magalhães I, Ferreira M, Machado S, Silva AG: Neural gliding versus neural tensioning: Effects on heat and cold thresholds, pain thresholds and hand grip strength in asymptomatic individuals. J Bodyw Mov Ther [Internet] Churchill Livingstone; 23:799–804, 2019 [cited 2021 Apr 14]. Available from: https://pubmed-ncbi-nlm-nih-gov.bibliopass.unito.it/31733764/

36. Geuna S: Appreciating the difference between design-based and model-based sampling strategies in quantitative morphology of the nervous system. J Comp Neurol [Internet] 427:333–9, 2000 [cited 2021 Oct 18]. Available from: https://onlinelibrary.wiley.com/doi/epdf/10.1002/1096-9861%2820001120%29427%3A3%3C333%3A%3AAID-CNE1%3E3.0.CO%3B2-T

37. Geuna S, Raimondo S, Fregnan F, Haastert-Talini K, Grothe C: In vitro models for peripheral nerve regeneration. Kirik D, editor. Eur. J. Neurosci. Blackwell Publishing Ltd; page 287–96Feb, 2016.

38. Geuna S, Raimondo S, Ronchi G, Di Scipio F, Tos P, Czaja K, Fornaro M: Chapter 3 Histology of the Peripheral Nerve and Changes Occurring During Nerve Regeneration [Internet]. Int. Rev. Neurobiol. Int Rev Neurobiol; page 27–462009 [cited 2021 Sep 3]. Available from: https://pubmed-ncbi-nlm-nih-gov.bibliopass.unito.it/19682632/

39. Giardini AC, Santos FM Dos, Da Silva JT, De Oliveira ME, Martins DO, Chacur M: Neural Mobilization Treatment Decreases Glial Cells and Brain-Derived Neurotrophic Factor Expression in the Central Nervous System in Rats with Neuropathic Pain Induced by CCI in Rats. Pain Res Manag [Internet] Pain Res Manag; 2017:, 2017 [cited 2021 Sep 3]. Available from: https://pubmed.ncbi.nlm.nih.gov/28420943/

40. Van Hecke O, Austin SK, Khan RA, Smith BH, Torrance N: Neuropathic pain in the general population: A systematic review of epidemiological studies. Pain. 2014.

41. Hestehave S, Abelson KS, Brønnum Pedersen T, Munro G: Stress sensitivity and cutaneous sensory thresholds before and after neuropathic injury in various inbred and outbred rat strains. Behav Brain Res [Internet] Behav Brain Res; 375:, 2019 [cited 2021 Sep 28]. Available from: https://pubmed.ncbi.nlm.nih.gov/31419521/

42. Hestehave S, Abelson KSP, Brønnum Pedersen T, Finn DP, Andersson DR, Munro G: The influence of rat strain on the development of neuropathic pain and comorbid anxio-depressive behaviour after nerve injury. Sci Rep [Internet] Nature Publishing Group UK; 10:, 2020 [cited 2021 Sep 27]. Available from: https://pubmed.ncbi.nlm.nih.gov/33262364/

43. Higgins S, Lee JS, Ha L, Lim JY: Inducing neurite outgrowth by mechanical cell stretch. Biores Open Access , 2013.

44. Hussain N, Shastri U, McCartney CJL, Gilron I, Fillingim RB, Clarke H, Katz J, Juni P, Laupacis A, Wijeysundera D, Abdallah FW: Should thoracic paravertebral blocks be used to prevent chronic postsurgical pain after breast cancer surgery? a systematic analysis of evidence in light of IMMPACT recommendations. Pain. 2018.

45. Jančálek R, Dubový P, Svíženská I, Klusáková I: Bilateral changes of TNF-α and IL-10 protein

in the lumbar and cervical dorsal root ganglia following a unilateral chronic constriction injury of the sciatic nerve. J Neuroinflammation 7:, 2010.

46. Jensen TS, Finnerup NB: Allodynia and hyperalgesia in neuropathic pain: Clinical manifestations and mechanisms. Lancet Neurol [Internet] Lancet Neurol; 13:924–35, 2014 [cited 2021 Aug 11]. Available from: https://pubmed-ncbi-nlm-nih-gov.bibliopass.unito.it/25142459/

47. Jurga AM, Rojewska E, Piotrowska A, Makuch W, Pilat D, Przewlocka B, Mika J: Blockade of toll-like receptors (TLR2, TLR4) attenuates pain and potentiates buprenorphine analgesia in a rat neuropathic pain model. Neural Plast , 2016.

48. Kassambara A: Package 'ggpubr': "ggplot2" Based Publication Ready Plots. R Packag version 040 , 2020.

49. Kassambara A, Jourdan M, Bruyer A, Robert N, Pantesco V, Elemento O, Klein B, Moreaux J: Global miRNA expression analysis identifies novel key regulators of plasma cell differentiation and malignant plasma cell. Nucleic Acids Res , 2017.

50. Koplovitch P, Devor M: Dilute lidocaine suppresses ectopic neuropathic discharge in dorsal root ganglia without blocking axonal propagation: A new approach to selective pain control. Pain [Internet] Pain; 159:1244–56, 2018 [cited 2021 Nov 8]. Available from: https://pubmed.ncbi.nlm.nih.gov/29533387/

51. Kremer M, Yalcin I, Goumon Y, Wurtz X, Nexon L, Daniel D, Megat S, Ceredig RA, Ernst C, Turecki G, Chavant V, Théroux JF, Lacaud A, Joganah LE, Lelievre V, Massotte D, Lutz PE, Gilsbach R, Salvat E, Barrot M: A dual noradrenergic mechanism for the relief of neuropathic allodynia by the antidepressant drugs duloxetine and amitriptyline. J Neurosci , 2018.

52. Lakens D: Calculating and reporting effect sizes to facilitate cumulative science: A practical primer for t-tests and ANOVAs. Front Psychol , 2013.

53. Lima ÊM, Santana HHS, Medrado AP, Martinez AMB, Baptista AF: Neurodynamic Mobilization Reduces Intraneural Fibrosis After Sciatic Crush Lesion in Rats. Brazilian J Med Hum Heal 5:54–62, 2017.

54. Lin MY, Frieboes LS, Forootan M, Palispis WA, Mozaffar T, Jafari M, Steward O, Gall CM, Gupta R: Biophysical stimulation induces demyelination via an integrin-dependent mechanism. Ann Neurol , 2012.

55. Loeser JD, Cahana A: Pain medicine versus pain management: Ethical dilemmas created by contemporary medicine and business. Clin J Pain 29:311–6, 2013.

56. Machado-Duque ME, Gaviria-Mendoza A, Machado-Alba JE, Castaño N: Evaluation of Treatment Patterns and Direct Costs Associated with the Management of Neuropathic Pain. Pain Res Manag , 2020.

57. Mahmoud ELDesoky MT: Efficacy of Neural Mobilization on Low Back Pain with S1 Radiculopathy. Int J Physiother 3:362–70, 2016.

58. Martins C, Pereira R, Fernandes I, Martins J, Lopes T, Ramos L, Pacheco J, Silva AG: Neural gliding and neural tensioning differently impact flexibility, heat and pressure pain thresholds in asymptomatic subjects: A randomized, parallel and double-blind study. Phys Ther Sport [Internet] Churchill Livingstone; 36:101–9, 2019 [cited 2021 Apr 14]. Available

from: https://pubmed-ncbi-nlm-nih-gov.bibliopass.unito.it/30710858/

59. Melzack R, Wall PD: Pain mechanisms: A new theory. Science (80-) , 1965.

60. Nagpal A, Clements N, Duszynski B, Boies B: The Effectiveness of Dorsal Root Ganglion Neurostimulation for the Treatment of Chronic Pelvic Pain and Chronic Neuropathic Pain of the Lower Extremity: A Comprehensive Review of the Published Data. Pain Med [Internet] Pain Med; 22:49–59, 2021 [cited 2021 Nov 8]. Available from: https://pubmed.ncbi.nlm.nih.gov/33260203/

61. Papalia I, Tos P, D'Alcontres FS, Battiston B, Geuna S: On the use of the grasping test in the rat median nerve model: A re-appraisal of its efficacy for quantitative assessment of motor function recovery. J Neurosci Methods [Internet] J Neurosci Methods; 127:43–7, 2003 [cited 2021 Oct 15]. Available from: https://pubmed.ncbi.nlm.nih.gov/12865147/

62. Pietrzak M, Vollaard NBJ: Effects of a Novel Neurodynamic Tension Technique on Muscle Extensibility and Stretch Tolerance: A Counterbalanced Crossover Study. J Sport Rehabil [Internet] J Sport Rehabil; 27:55–65, 2018 [cited 2021 Nov 22]. Available from: https://pubmed.ncbi.nlm.nih.gov/27992294/

63. Prato V, Taberner FJ, Hockley JRF, Callejo G, Arcourt A, Tazir B, Hammer L, Schad P, Heppenstall PA, Smith ES, Lechner SG: Functional and Molecular Characterization of Mechanoinsensitive "Silent" Nociceptors. Cell Rep , 2017.

64. R Development Core Team: R: A language and environment for statistical computing. Vienna, Austria. 2017.

65. Raja SN, Ringkamp M, Guan Y, Campbell JN: John J. Bonica Award Lecture: Peripheral neuronal hyperexcitability: the "low-hanging" target for safe therapeutic strategies in neuropathic pain. Pain [Internet] NLM (Medline); 161:S14–26, 2020 [cited 2021 Jul 9]. Available from: https://journals.lww.com/pain/Fulltext/2020/09001/John_J__Bonica_Award_Lecture__Peripheral_neuronal.3.aspx

66. Rajalaxmi M.V., Lavanya R., Kirupa K., Divya Mary S.M. YM: Efficacy of Neural Mobilization and Cervical Stabilization in Cervicobrachial Pain: A Randomized Controlled Trial. Medico-Legal Updat [Internet] Physical Therapy Rehabilitation Science; 4:1398–403, 2020 [cited 2021 May 17]. Available from: http://www.jptrs.org/journal/view.html?doi=10.14474/ptrs.2020.9.3.149

67. Richardson JTE: Eta squared and partial eta squared as measures of effect size in educational research. Educ. Res. Rev. page 135–472011.

68. Ronchi G, Nicolino S, Raimondo S, Tos P, Battiston B, Papalia I, Varejão ASP, Giacobini-Robecchi MG, Perroteau I, Geuna S: Functional and morphological assessment of a standardized crush injury of the rat median nerve. J Neurosci Methods [Internet] J Neurosci Methods; 179:51–7, 2009 [cited 2021 Mar 30]. Available from: https://pubmed-ncbi-nlm-nih-gov.bibliopass.unito.it/19428511/

69. Santos FM, Grecco LH, Pereira MG, Oliveira ME, Rocha PA, Silva JT, Martins DO, Miyabara EH, Chacur M: The neural mobilization technique modulates the expression of endogenous opioids in the periaqueductal gray and improves muscle strength and mobility in rats with neuropathic pain. Behav Brain Funct [Internet] Behav Brain Funct; 10:1–8, 2014 [cited 2021

Sep 3]. Available from: https://pubmed.ncbi.nlm.nih.gov/24884961/

70. Santos FM, Silva JT, Giardini AC, Rocha PA, Achermann APP, S Alves A, Britto LRG, Chacur M: Neural mobilization reverses behavioral and cellular changes that characterize neuropathic pain in rats. Mol Pain 8:1–9, 2012.

71. Santos FM, Silva JT, Rocha IRC, Martins DO, Chacur M: Non-pharmacological treatment affects neuropeptide expression in neuropathic pain model. Brain Res 1687:60–5, 2018.

72. Shacklock M, Yee B, Van Hoof T, Foley R, Boddie K, Lacey E, Poley JB, Rade M, Kankaanpää M, Kröger H, Airaksinen O: Slump Test: Effect of Contralateral Knee Extension on Response Sensations in Asymptomatic Subjects and Cadaver Study. Spine (Phila Pa 1976) [Internet] Spine (Phila Pa 1976); 41:E205–10, 2016 [cited 2021 Nov 22]. Available from: https://pubmed.ncbi.nlm.nih.gov/26571159/

73. Shi XQ, Zekki H, Zhang J: The role of TLR2 in nerve injury-induced neuropathic pain is essentially mediated through macrophages in peripheral inflammatory response. Glia , 2011.

74. Shin SM, Moehring F, Itson-Zoske B, Fan F, Stucky CL, Hogan QH, Yu H: Piezo2 mechanosensitive ion channel is located to sensory neurons and nonneuronal cells in rat peripheral sensory pathway: implications in pain. Pain 162:2750–68, 2021.

75. Silva CEA, Guimarães RM, Cunha TM: Sensory neuron–associated macrophages as novel modulators of neuropathic pain. PAIN Reports [Internet] Pain Rep; 6:e873, 2021 [cited 2021 Nov 9]. Available from: https://pubmed.ncbi.nlm.nih.gov/33981924/

76. Sindhi V, Jenkins T, Antony A, Hagen J: Successful treatment of peripheral causalgia with DRG stimulation in a complex case of orthopedic trauma. Neuromodulation , 2019.

77. Smith BH, Harry LH, Abirami Veluchamy: Neuropathic pain in the community: prevalence, impact, and risk factors. 161:S127–36, 2020. Available from: https://journals.lww.com/pain/pages/articleviewer.aspx?year=2020&issue=09001&article=00014&type=Fulltext

78. Stephens KE, Zhou W, Ji Z, Chen Z, He S, Ji H, Guan Y, Taverna SD: Sex differences in gene regulation in the dorsal root ganglion after nerve injury. BMC Genomics [Internet] BMC Genomics; 20:, 2019 [cited 2022 Jan 25]. Available from: https://pubmed.ncbi.nlm.nih.gov/30782122/

79. Su HL, Chiang CY, Lu ZH, Cheng FC, Chen CJ, Sheu ML, Sheehan J, Pan HC: Late administration of high-frequency electrical stimulation increases nerve regeneration without aggravating neuropathic pain in a nerve crush injury. BMC Neurosci [Internet] BioMed Central; 19:1–12, 2018. Available from: https://doi.org/10.1186/s12868-018-0437-9

80. Sulaiman W, Gordon T: Neurobiology of peripheral nerve injury, regeneration, and functional recovery: From bench top research to bedside application. Ochsner J , 2013.

81. Talebi GA, Saadat P, Javadian Y, Taghipour M: Comparison of two manual therapy techniques in patients with carpal tunnel syndrome: A randomized clinical trial. Casp J Intern Med [Internet] Babol University of Medical Sciences; 11:163–70, 2020 [cited 2021 May 17]. Available from: https://pubmed-ncbi-nlm-nih-gov.bibliopass.unito.it/32509244/

82. Tennant KA, Asay AL, Allred RP, Ozburn AR, Kleim JA JT: The vermicelli and capellini handling tests: Simple quantitative measures of dexterous forepaw function in rats and mice. J Vis Exp [Internet] MyJoVE Corporation; :2076, 2010 [cited 2021 Oct 15]. Available from: https://pubmed.ncbi.nlm.nih.gov/20689506/

83. Thallmair M, Metz GAS, Z'Graggen WJ, Raineteau O, Kartje GL, Schwab ME: Neurite growth inhibitors restrict plasticity and functional recovery following corticospinal tract lesions. Nat Neurosci 1998 12 [Internet] Nature Publishing Group; 1:124–31, 1998 [cited 2021 Oct 15]. Available from: https://www.nature.com/articles/nn0698_124

84. Torres-Espín A, Santos D, González-Pérez F, del Valle J, Navarro X: Neurite-J: An Image-J plug-in for axonal growth analysis in organotypic cultures. J Neurosci Methods Elsevier; 236:26–39, 2014.

85. Torres JR, Martos IC, Sánchez IT, Rubio AO, Pelegrina AD, Valenza MC: Results of an Active Neurodynamic Mobilization Program in Patients with Fibromyalgia Syndrome: A Randomized Controlled Trial. Arch Phys Med Rehabil 96:1771–8, 2015.

86. Tran EL, Crawford LTK: Revisiting PNS Plasticity: How Uninjured Sensory Afferents Promote Neuropathic Pain. Front Cell Neurosci [Internet] Front Cell Neurosci; 14:1–13, 2020 [cited 2021 Nov 10]. Available from: https://pubmed.ncbi.nlm.nih.gov/33362476/

87. Truini A: A Review of Neuropathic Pain: From Diagnostic Tests to Mechanisms. Pain Ther , 2017.

88. Vaso A, Adahan HM, Gjika A, Zahaj S, Zhurda T, Vyshka G, Devor M: Peripheral nervous system origin of phantom limb pain. Pain , 2014.

89. Villafañe JH, Silva GB, Bishop MD, Fernandez-Carnero J: Radial nerve mobilization decreases pain sensitivity and improves motor performance in patients with thumb carpometacarpal osteoarthritis: A randomized controlled trial. Arch Phys Med Rehabil [Internet] W.B. Saunders; 93:396–403, 2012 [cited 2021 Apr 14]. Available from: https://pubmed-ncbi-nlm-nih-gov.bibliopass.unito.it/22218138/

90. Wang J, Zhao M, Jia P, Liu FF, Chen K, Meng FY, Hong JH, Zhang T, Jin XH, Shi J: The analgesic action of larixyl acetate, a potent TRPC6 inhibitor, in rat neuropathic pain model induced by spared nerve injury. J Neuroinflammation [Internet] J Neuroinflammation; 17:, 2020 [cited 2021 Nov 9]. Available from: https://pubmed.ncbi.nlm.nih.gov/32299452/

91. Wang Y, Ma M, Tang Q, Zhu L, Koleini M, Zou D: The effects of different tensile parameters for the neurodynamic mobilization technique on tricipital muscle wet weight and MuRf-1 expression in rabbits with sciatic nerve injury. J Neuroeng Rehabil 12:1–7, 2015.

92. Watson P: Rules of thumb on magnitudes of effect sizes. MRC Cogn Brain Sci Unit Wiki , 2009.

93. Wolny T, Linek P: Is manual therapy based on neurodynamic techniques effective in the treatment of carpal tunnel syndrome? A randomized controlled trial. Clin Rehabil [Internet] SAGE Publications Ltd; 33:408–17, 2019 [cited 2021 Apr 14]. Available from: https://pubmed-ncbi-nlm-nih-gov.bibliopass.unito.it/30306805/

94. Wolny T, Linek P: Neurodynamic Techniques Versus "Sham" Therapy in the Treatment of Carpal Tunnel Syndrome: A Randomized Placebo-Controlled Trial. Arch Phys Med Rehabil [Internet] W.B. Saunders; 99:843–54, 2018 [cited 2021 Apr 14]. Available from:

https://pubmed-ncbi-nlm-nih-gov.bibliopass.unito.it/29307812/

95. Wolny T, Saulicz E, Linek P, Shacklock M, Myśliwiec A: Efficacy of Manual Therapy Including Neurodynamic Techniques for the Treatment of Carpal Tunnel Syndrome: A Randomized Controlled Trial. J Manipulative Physiol Ther [Internet] Mosby Inc.; 40:263–72, 2017 [cited 2021 Apr 14]. Available from: https://pubmed-ncbi-nlm-nih-gov.bibliopass.unito.it/28395984/

96. Woolf CJ, Salter MW: Neuronal plasticity: Increasing the gain in pain. Science (80-.). 2000.

97. Xu J, Liu A, Cheng J: New advancements in spinal cord stimulation for chronic pain management. Curr. Opin. Anaesthesiol. 2017.

98. Yamin F, Musharraf H, Rehman AU, Aziz S: Efficacy of Sciatic Nerve Mobilization in Lumbar Radiculopathy due to Prolapsed Intervertebral Disc. Indian J Physiother Occup Ther - An Int J , 2016.

99. Yang F, Zhang C, Xu Q, Tiwari V, He SQ, Wang Y, Dong X, Vera-Portocarrero LP, Wacnik PW, Raja SN, Guan Y: Electrical stimulation of dorsal root entry zone attenuates wide-dynamic-range neuronal activity in rats. Neuromodulation [Internet] Neuromodulation; 18:33–40, 2015 [cited 2021 Dec 23]. Available from: https://pubmed.ncbi.nlm.nih.gov/25308522/

100. Yang H, Wu L, Deng H, Chen Y, Zhou H, Liu M, Wang S, Zheng L, Zhu L, Lv X: Anti-inflammatory protein TSG-6 secreted by bone marrow mesenchymal stem cells attenuates neuropathic pain by inhibiting the TLR2/MyD88/NF-κB signaling pathway in spinal microglia. [cited 2021 May 4]. Available from: https://doi.org/10.1186/s12974-020-1731-x

101. Yatziv SL, Devor M: Suppression of neuropathic pain by selective silencing of dorsal root ganglion ectopia using nonblocking concentrations of lidocaine. Pain [Internet] Pain; 160:2105–14, 2019 [cited 2021 Nov 8]. Available from: https://pubmed.ncbi.nlm.nih.gov/31095098/

102. Yeh TY, Luo IW, Hsieh YL, Tseng TJ, Chiang H, Hsieh ST: Peripheral Neuropathic Pain: From Experimental Models to Potential Therapeutic Targets in Dorsal Root Ganglion Neurons. Cells. 2020.

103. Yun Y-H, Lee B-K, Yi J-H, Seo D-K: Effect of nerve mobilization with intermittent cervical segment traction on pain, range of motion, endurance, and disability of cervical radiculopathy. Phys Ther Rehabil Sci Physical Therapy Rehabilitation Science; 9:149–54, 2020.

104. Zhu G-CC, Tsai K-LL, Chen Y-WW, Hung C-HH: Neural mobilization attenuates mechanical allodynia and decreases proinflammatory cytokine concentrations in rats with painful diabetic neuropathy. Phys Ther [Internet] Phys Ther; 98:214–22, 2018 [cited 2021 Apr 27]. Available from: https://pubmed.ncbi.nlm.nih.gov/29309710/

SUPPLEMENTARY

Table S1. Baseline values of animal sensory and motor profiles							
Sensory assessment							
	NO NDT	**NDT POST**	**NDT PRE-POST**	**df**	**F**	**η^2_p**	**P**
Mechanical pain threshold injured side (gr)	20.8 ± 2.04	17.5 ± 4.18	18.3 ± 4.08	2, 15	1.413	.159	.274
Mechanical pain threshold not injured side (gr)	18.3 ± 4.08	20 ± 8.94	19.2 ± 4.92	2, 15	0.103	.014	.902
Touch threshold injured side (gr)	15 ± 0	13.3 ± 2.58	15 ± 3.16	2, 15	1	.118	.391
Touch threshold not injured side (gr)	15 ± 0	13.3 ± 2.58	14.2 ± 2.04	2, 15	1.154	.133	.342
Pain behavior to noxious stimuli	2 ± 0	2 ± 0	2.17 ± 0.408	2, 15	1	.118	.391
Number of paw withdrawals on 30 repeated nerve tensioning	1.83 ± 1.84	3.33 ± 3.08	3.67 ± 2.58	2, 15	0.88	.105	.435
Motor assessment							
Grissini test speed (sec)	66.5 ± 11.8	76.3 ± 12.5	86.3 ± 10.9*	2, 15	4.247	.362	.035
Grissini test number of adjustments	48.3 ± 14.0	41 ± 9.84	48.3 ± 9.81	2, 15	.83	.1	.455
Grasping test (gr)	617 ± 39.5	614 ± 94.1	613 ± 87.1	2, 15	0.004	.0005	.996
Rope test (sec)	57.8 ± 25.2	32.2 ± 13.3	42.3 ± 31.2	2, 15	1.684	.183	.219
Animal weight (gr)	238 ± 9.56	237 ± 5.88	239 ± 6.43	2, 15	0.079	.01	.925

Table S2. ANOVA for repeated measures results from behavioral assays (η^2_p: partial eta squared)

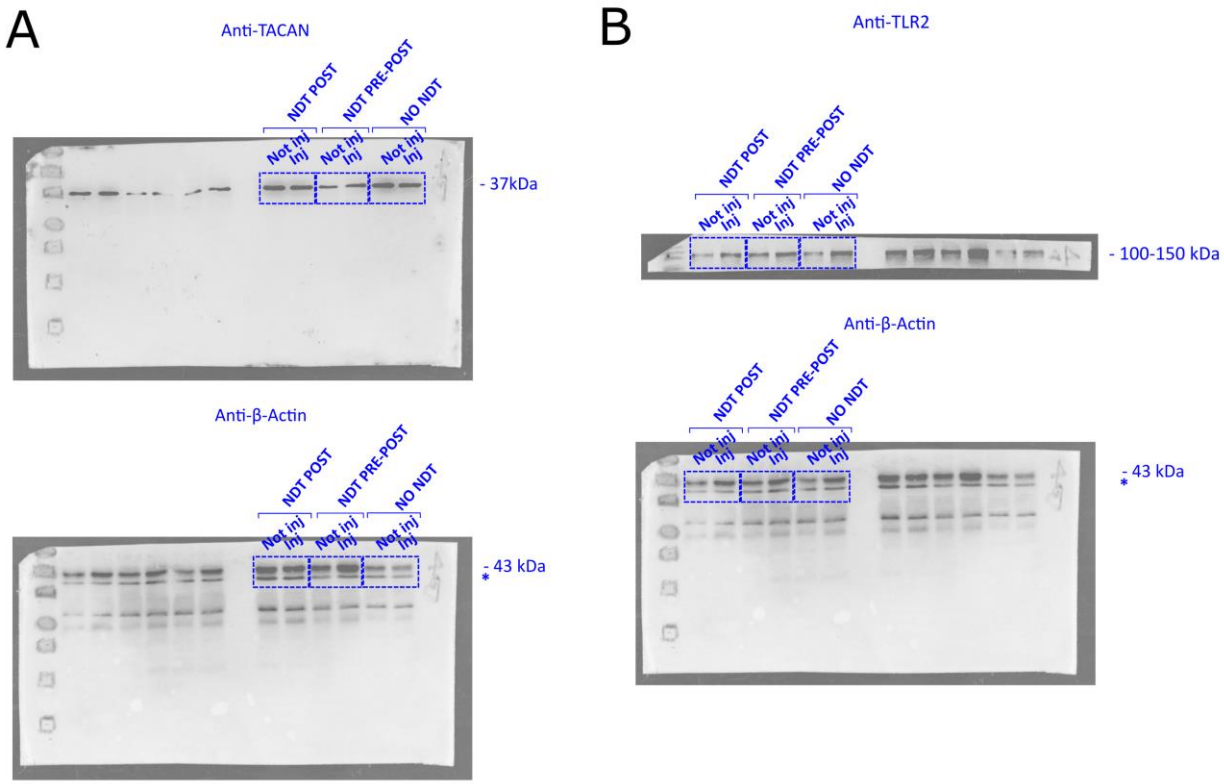
Sensory assays									
	Time			*Treatment*			*Time x Treatment*		
Variable	**F**	**p**	**η^2_p**	**F**	**p**	**η^2_p**	**F**	**p**	**η^2_p**
Mechanical pain threshold injured side (gr)	68.971	**.0000**	.798	12.357	**.0000**	.191	8.918	**.0000**	.505
Touch threshold injured side (gr)	5.23	**.0001**	.23	8.016	**.0006**	.132	1.131	.343	.114
Mechanical pain threshold not injured side (gr)	2.274	.042	.115	1.553	.216	.029	0.797	.652	.084
Touch threshold not injured side (gr)	5.481	**.0001**	.238	5.547	**.005**	.096	0.73	.72	.077
Pain behavior to noxious stimuli	43.333	**.0000**	.712	3.9	**.023**	.069	5.533	**.0000**	.387
Motor assays									
Grissini test speed (sec)	2.405	**.043**	.12	5.081	**.008**	.104	0.637	.779	.067
Grissini test number of adjustments	3.0020	**.015**	.147	2.817	.065	.06	1.651	.105	.158
Grasping test (gr)	300.04	**.000**	.945	0.729	.485	.014	0.858	.591	.089
Rope test (sec)	1.7638	.114	.092	8.3343	**.0004**	.137	0.8732	0.576	.091
Animal weight (gr)	23.395	**.0000**	0.51	1.778	.181	.073	0.522	.720	.044
	Treatment			*Side*			*Treatment x Side*		
Wet muscle weight (gr)	10.309	**.00039**	.407	157.012	**.0000**	.84	1.023	.372	.064

Table S3. Morphological characteristics of DRG explants

	CTR IN	CTR OUT	NDT	F[df]	95% CI	P
Dmax (μm)	1552 ±447	1465 ±368	2200 ±524	7.92 [2,27]	234.85 - 1235.14	.003
Nmax	188 ±92.1	94.2 ±89.6	153 ±69.5	3.15[2,27]	131.74 - 243.46	.06
Sholl Critica Value	148 ±267	160 ±137	278 ±323	0.79 [2,27]	-19.96 - 314.96	.46

Values are reported as means ± Standard Deviation (SD). CTR IN, Control group; CTR OUT, sham treatment group; NDT, neurodynamic protocol (30 repeated tensions); the distance of the longest neurite; Nmax, the maximum number of neurites; One-way ANOVA was carried out (data are normally distributed with comparable variances); df, degree of freedom; 95% CI, 95% Confidence Intervals; P, level of significance (P <.05).

Figure S1. Blots of DRG from *in vivo* experiments from 6 - Chemiluminescence detection was performed with Clarity Western ECL Substrate (BIORAD) Anti-TACAN (A) and Anti-TLR2 (B) with the equivalent β-Actin bands are reported.



3.4 Paper n°4

Validation and Reliability of a Novel Vagus Nerve Neurodynamic Test and Its Effects on Heart Rate in Healthy Subjects: Little Differences Between Sexes

Giacomo Carta[1,2,3], Agnese Seregni[4], Andrea Casamassima[5], Manuela Galli[4] 4, Stefano Geuna[1,2], Pasquale Pagliaro[6], Matteo Zago[7].

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Frontiers in Neuroscience, 2021

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 06 September 2021
doi: 10.3389/fnins.2021.698470

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OPEN ACCESS

Edited by:
Virginia H. Huxley, University of Missouri, United States

Reviewed by:
Rossella Rizzo, Trinity College Dublin, Ireland
Winfried Neuhuber, University of Erlangen Nuremberg, Germany

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Specialty section:
This article was submitted to Autonomic Neuroscience, a section of the journal Frontiers in Neuroscience

Received: *10 June 2021*
Accepted: *21 July 2021*
Published: *06 September 2021*

Citation:
Carta G, Seregni A, Casamassima A, Galli M, Geuna S, Pagliaro P and Zago M (2021) Validation and Reliability of a Novel Vagus Nerve Neurodynamic Test and Its Effects on Heart Rate in Healthy Subjects: Little Differences Between Sexes. Front. Neurosci. 15:698470. doi: 10.3389/fnins.2021.698470

Background: The vagus nerve (VN), also called the pneumogastric nerve, connects the brainstem to organs contained in the chest and abdomen. Physiologically, VN stimulation can rapidly affect cardiac activity and heart rate (HR). VN neuropathy can increase the risk of arrhythmias and sudden death. Therefore, a selective test of VN function may be very useful. Since peripheral neurodynamic tests (NDT) are reliable for the assessment of neuropathies in somatic nerves, we aimed to validate a novel NDT to assess VN activity, namely, the VN-NTD.

Methods: In this cross-sectional double-blind, sex-balanced study, 30 participants (15 females) completed a checklist of autonomic dysfunction symptoms. During the VN-NDT administration, HR and symptoms (i.e., mechanical allodynia) were monitored in parallel to a real-time ultrasonography imaging (USI) and motion capture analysis of the neck. The VN-NDT impact on HR and its accuracy for autonomic symptoms reported in the last 7 days were tested.

Results: The VN-NDT induced a significant HR reduction of about 12 and 8 bpm in males and females [$t(1, 119) = 2.425$; $p < 0.017$; $\eta_p^2 = 0.047$, 95% confidence interval (CI): 0.93–9.18], respectively. No adverse events were observed during VN-NDT. A substantial interexaminer agreement between the evaluators in symptoms induction by VN-NDT was detected [$F(1, 119) = 0.540$; $p = 0.464$; $\eta_p^2 = 0.005$, low effect]. Notably, mechanical allodynia accuracy for gastrointestinal dysfunctions was excellent ($p < 0.05$; 95% CI: 0.52–0.73; $p < 0.001$; 95% CI: 0.81–0.96).

Conclusions: The novel VN-NDT is a valid and accurate test capable of detecting VN activation with high sensitivity. Data provided are suitable for both sexes as a hallmark of

HR variation due to VN normal response. The proposed VN-NDT may be reliable as daily routine neurological examination tests for the evaluation of neuropathic signs related to neuroinflammation of the VN.

Clinical Trial Registration: www.ClinicalTrials.gov, identifier NCT04192877.

Keywords: vagus nerve stimulation, heart rate, diagnostic test, ultrasound, neuropathic pain

INTRODUCTION

Several health-related conditions have prominent and clinically important manifestations including autonomic peripheral neuropathies (APN) (Freeman, 2005). The main causes described in the literature are diabetes, amyloidosis, immune-mediated, neoplastic, paraneoplastic, hereditary, secondary to infectious diseases, and intoxications (Oaklander and Nolano, 2019). The pathophysiology of neurodegenerative disorders often involves a microbiota–gut–brain axis perturbation (Fung et al., 2017). Cranial neuropathies due to acute diseases like Zika virus-associated Guillain–Barré syndrome (Parra et al., 2016), local lesions like in schwannomas of the vagus nerve (VN) (Sunaryo et al., 2012), and iatrogenic damage of the VN can affect nerve function with critical immediate (bradycardia and cardiac asystole) or delayed consequences (Nazir et al., 2017; Aggarwal et al., 2018).

Autonomic neuropathies are conditions difficult to be detected that increase hemodynamic instability (Ang et al., 2020), postsurgery complications (Lankhorst et al., 2015; Suarez-Roca et al., 2019), and sudden death in obese and diabetic patients (Freeman, 2005; Santos Breder and Sposito, 2019; Williams et al., 2019; Malaty et al., 2021). Patients with coronavirus disease 2019 (COVID-19) have an increased prevalence of cardiac arrhythmias (Ho et al., 2020) with an estimated incidence of 15% in post-COVID-19 patients (Malaty et al., 2021). Since APN is a growing health problem, it is of paramount importance to have a reliable clinical tool that investigates selectively VN neuropathies. Ultrasound imaging (USI) is the most reliable and cost-effective imaging tool to assess VN morphology (Anil and Tan, 2011; Kasehagen et al., 2018), but morphological changes alone cannot predict the clinical conditions of patients. A "gold standard" to assess only the VN functioning level excluding its interaction with the sympathetic system is missing, so general autonomic response tests, involving sympathetic and parasympathetic responses, are used with no negligible side effects and risks like retinal detachment, syncope, chest pain, and arrhythmias (Valsalva maneuver, tilt-table protocols, lower body negative pressure, noradrenaline spillover, etc.) (Fujii et al., 2004; Schrezenmaier et al., 2007; Pstras et al., 2016; Fajgenbaum et al., 2018; Ehrman et al., 2020). Therefore, a selective and reliable test to assess VN functions with no or possibly negligible side effects is necessary. Peripheral nerve selective tension tests or neurodynamic tests (NDT) are bedside examinations and reliable clinical tests validated for the detection of neuropathies of the somatic nerves (Taenzer et al., 2000; Wasan et al., 2011; Bueno-Gracia et al., 2016; Verwoerd et al., 2016; Ekedahl et al., 2018; Koulidis et al., 2019). NDTs assess the nerve response to mechanical stimuli which are transduced by stretch-sensitive ion channels in peripheral nerves also present in the VN axons and cell body membranes (Beaulieu-Laroche et al., 2020; Bonet et al., 2021). Therefore, the aim of the present study was threefold: (1) to describe and validate a tool for selective VN assessment as NDT of the VN (VN-NDT), (2) to collect normative data to define a hallmark of physiological spectrum in males and females for heart rate (HR) variations induced by the VN-NDT maneuvers, and (3) to describe the relationship between symptoms induced during the VN-NDT and any autonomic dysfunction-related symptom.

MATERIALS AND METHODS

Study Design

Since no selective test for the VN exists, a validation process was performed *ex novo* taking advantage of the available data reported in the literature. An *a priori* power analysis was performed referring to Cohen's kappa coefficient values reported by Martínez-Payá et al. (2015) ($k = 0.66$; $k = 0.94$) studying the USI during a neurodynamic test. A sample size of 30 subjects provided a statistical power of 0.90 assuming a moderate strength of agreement between two evaluators and correct classification of subjects as positive of 0.50 with an alpha of 0.05. Also, considering an HR reduction induced by the test similar to the one described by Antonino et al. (2017) ($\eta^2 = 1.134$), 13 subjects for each sex were identified to provide a statistical power of 0.96 with an alpha error of 0.05 and $1 - \beta$ error of 0.95. Estimating a 20% dropout rate, we enrolled 36 subjects in the study. An expert (a physical therapist with more than 12 years of experience in neurodynamic test administration) and a novice examiner (a medical doctor with no training in neurodynamic tests) blinded to their judgments performed the maneuver sequences of VN-NDT to every participant on the VNs of participants on both sides.

Participants voluntarily took part in the examination after an explanation of all the risks and benefits, and they all signed the written informed consent form according to the Declaration of Helsinki. Before data collection, the study was approved by the University Bioethics Committee (protocol 139870-14/03/2019) and registered on www.ClinicalTrials.gov (trial registration number: NCT04192877) on December 5, 2019. Subjects were enrolled from December 12, 2019[1]. Participants were asked to not consume tea, caffeine, energy drinks, alcohol, and tobacco within 2 h of the study and avoid them 24 h before the study. Subjects were blinded to the expertise level of the evaluators, and

[1]https://bit.ly/3kkhZQL

the results were communicated only when the assessment was completed. Also, USI during the VN-NDT was performed by an expert medical doctor, currently a licensed USI international instructor in critical and acute care. The test results were available to the participants and evaluators only at the end of the study.

Settings

The study was conducted in the Posture and Movement Analysis Laboratory of the Department of Electronics, Information, and Bioengineering, Politecnico di Milano.

Inclusion/Exclusion Criteria and Motivation

Subjects were included if they were between 18 and 70 years old and sober. Subjects were excluded if they reported significant neck pain or headache [with Numeric Pain Rating Scale (NPRS) greater than 3/10] (Salaffi et al., 2004), pregnancy, recent neck or cardiac surgery or significant trauma in the preceding 3 months, cancer or inflammatory disorders, spinal cord or cauda equina signs, widespread neurological disorders affecting the tone of the upper limb and neck muscles, or underlying diseases, such as diabetes mellitus.

Procedures and Data Collection

Data collection was performed in a standardized order: (1) fulfillment of self-report questionnaires, (2) neurological examination, (3) VN-NDT under USI and motion capture analysis assessment (MCA), and (4) short-term autonomic response (STAR) measured based on HR.

Self-Report Questionnaires

Epidemiological data (**Supplementary Table 1**), diagnosis, medication prescribed, a checklist of AD symptoms, and signs were declared by every participant (Terkelsen et al., 2017). An 11-item Likert scale was also administered to assess the perceived health status (PHS: 100 the best, 0 the worst health status ever).

Neurological Examination

A segmental neurological examination was performed to confirm that the participants had no signs of nerve conduction loss. In short, dermatomes from C2 to C5 were evaluated bilaterally with a 10-g monofilament (Paisley et al., 2002). The presence of mechanical allodynia as a sign of central sensitization (Jensen and Finnerup, 2014) was assessed by asking the participants to keep a clothes peg on the middle fingernail for 5 s and on the middle earlobe (to assess sensitization away from the "assessed area") of both sides (Egloff et al., 2011).

Sensory discrimination was tested by administering a random sequence of 10 nociceptive and tactile stimuli on the skin of the neck (using a Neuropen®, Owen Mumford Ltd., Woodstock, United Kingdom). The upper limb NDT (ULNDT) was administered bilaterally to assess any subclinical neuropathic condition involving the neck or upper limbs (Schmid et al., 2009).

Participants were instructed to verbally stop the test immediately when any type of tension, discomfort, or unpleasant sensation was felt during the sequence of passive movements of the VN-NDT. The location of the symptom and behavior were defined using a pain drawing tool at the end of every single test (Bertilson et al., 2007), and their intensity was rated (NPRS) (Salaffi et al., 2004).

Vagus Nerve Neurodynamic Test

The VN emerges from the medulla of the brainstem and reaches the coeliac and mesenteric plexi in the abdomen passing through the jugular foramen (Verlinden et al., 2016) of the skull, between the internal carotid artery and the jugular vein in the neck and between the cardiac and pulmonary plexi in the thorax. The VN-NDT was developed starting from its morphology, selecting a combination of physiological movements that induce a higher mechanical tension on the nerve (**Figure 1A**). The subjects were assessed supine on an examination table, and evaluators were standing at the cranial short side of the table.

Upper cervical flexion and contralateral lateral flexion were selected for loading the intracranial part (Verlinden et al., 2016). Ipsilateral neck rotation was added to load the cervical tract.

Considering that the VN has afferent endings that are mechanosensitive (Zeng et al., 2018; Besecker et al., 2020), discrimination between VN and other tissues was performed while holding the head of the subject in the final pose, gently pushing the upper abdomen caudally and cranially to load and unload the thoracic tract. The test was positive (indicating abnormal responses) if discrimination maneuvers changed the symptoms of the subject indicating a neurogenic source; otherwise, it was declared negative (Schmid et al., 2009). To standardize the test, all participants were placed in the supine position without a pillow in a room at 25°C for 30 min as described by Fujii et al. (2004).

Short-Term Autonomic Response

Short-term autonomic response was assessed as described by Devalle and coworkers, which is a reliable outcome for the autonomic response to pain even in subjects with disturbances of consciousness, comparing the HR values (fingertip portable pulse oximeter Intermed SAT-200) at rest (10 s after the ULNDT administration) and after a 10-s window holding the end position of the VN-NDT (Devalle et al., 2018). Moreover, we verified that no chances in HR were induced by abdominal compression alone. To avoid any placebo/nocebo response, HR was blinded to the assessors and participants.

Ultrasound Imaging and Motion Capture Analysis Protocols

Protocols defined by Martinoli and coworkers for the detection of the anterior tubercle of C6 (Martinoli et al., 2002) and the cervical tract of the VN (Giovagnorio and Martinoli, 2001) were adopted. Participants were assessed in a supine position on a medical table and real-time USI was performed by the medical doctor standing near to the right long edge of the table, while the assessor performing the neurodynamic test was standing near to the short edge of the table where the head of the participant was (**Figure 1** and **Supplementary Figure 1**). Axial scans were obtained using the inferior margin of the thyroid as an initial reference from which the probe was moved laterally to the region

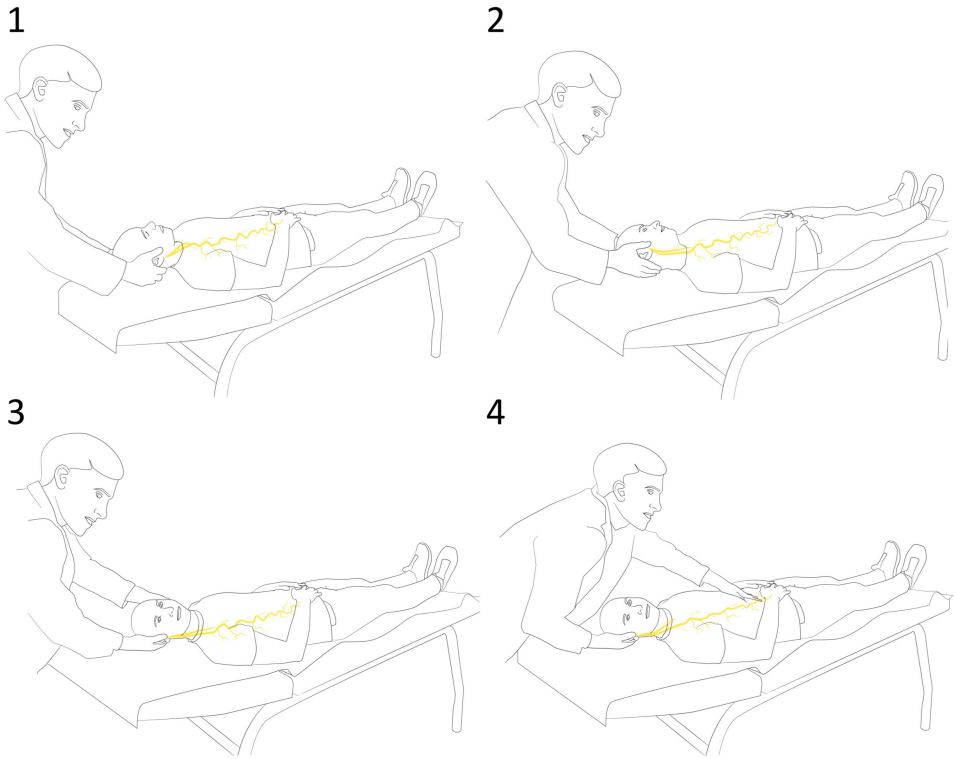

FIGURE 1 | Sequence of the vagus nerve neurodynamic test (VN-NDT) with **(1)** starting position with upper cervical spine in flexion. **(2)** Contralateral lateral flexion. **(3)** Ipsilateral neck rotation. **(4)** The end position of the test with gentle movements of the upper abdomen caudally and cranially as discrimination maneuverers.

of the transverse processes. The probe was moved cranially till the anterior tubercle of C6 was detected. Distance between the VN and C6 anterior tubercle (VN–C6) was measured at rest and at the final position of the VN-NDT to quantify the lateralization or proximalization of the VN induced by the test, suggesting an increased or decreased tension on the wire-like structure of the VN. Esaote® MyLab Alpha (Esaote S.p.A, Genoa, Italy) USI equipment was used with a 5–7-MHz convex array probe (**Figure 1B**). All subjects were screened for thyroid problems at the end of the assessment.

Throughout the whole duration of the VN-NDT and real-time USI assessment, the three-dimensional head orientation of the subjects was recorded at 100 Hz with an optoelectronic motion capture system (Smart-DX, BTS S.p.A., Milan, Italy). A cluster with three retroreflective markers (diameter: 15 mm) was secured on the head of the subject using an elastic band; three additional markers were fixed on the acromion and the sternum (**Supplementary Figure 1**). The rest and final head positions were manually annotated upon explicit communication by the USI operator. System calibration was conducted according to the guidelines of the manufacturers and returned an average error in marker position of 0.35 mm, on a working volume of $2.6 \times 1.8 \times 2.5\ m^3$.

Data Analysis

Differences from baseline were checked, as well as the effects between and within factors among symptoms induced by the test and perceived AD signs and symptoms. Custom routines were developed within Smart Analyzer (version 1.10.465, BTS S.p.A) to extract kinematic data. Three-dimensional coordinates were smoothed with a fourth-order low-pass Butterworth filter with a cutoff frequency of 1 Hz. A local reference system fixed on the head was defined: the x-axis was anteroposterior and pointed forward; the y-axis was craniocaudal and pointed upward; the z-axis was mediolateral and pointed to the right of the subject. The acromial and sternum markers defined a local trunk coordinate system (Zago et al., 2020), with an analogous axes convention, that served as a reference for head orientation (**Supplementary Figure 1**).

Head lateral inclination on the frontal plane (positive to the right), axial rotation on the transverse plane (positive to the left), and flexion (negative)–extension (positive) angles on the sagittal plane were computed as the Euler angles (*XYZ* rotation sequence) between the head and trunk reference frames, respectively. The initial position, i.e., that assumed by the participants laying down on the bed before the test initiation, was taken as neutral (all angles equal to zero). An explanatory representation of head rotations during the test is depicted in **Figure 4A**.

To provide an indirect measure of the vagal strain level, we also measured the distance between the sternum and right (or left) head marker, according to the side the test was performed on. The ratio between final and initial values was termed as head displacement ratio: higher values indicate larger head motion and vagal strain.

TABLE 1 | Differences between sexes at baseline characteristics and reported autonomic signs and symptoms (experienced during the last 7 days).

Variable	*F*	*M*	Total/cases (%)	*p*
Epidemiologic data				
Age, years	31.68 ± 11.08	31.64 ± 13.44	31.7 ± 12.0	0.99
Education	Bachelor's degree	Bachelor's degree	Bachelor's degree	0.15
Smoke	0	0.14 ± 0.36	2 (6.7)	0.13
BMI	22.4 ± 3.37	23.2 ± 2.37	22.8 ± 2.92	0.45
NRS (0–10 points)	0.66 ± 1.19	1.1 ± 1.62	0.87 ± 1.4	0.39
Health status (0–100 points)	82.81 ± 13.9	87.14 ± 12.04	84 ± 13	0.38
HR at rest (bpm)	76 ± 12.22	74.57 ± 11.59	75.3 ± 11.7	0.75
Autonomic checklist				
At least one autonomic symptom	0.56 ± 0.51	0.36 ± 0.51	14 (46.7)	0.28
Nausea	0.12 ± 0.34	0	2 (6.7)	0.18
Orthostatic hypotension	0.31 ± 0.48	0.14 ± 0.36	7 (23.3)	0.29
Digestion alterations	0.32 ± 0.48	0.33 ± 0.48	7 (23.3)	0.29
Breathing alterations (shortness of breath)	0	0	0	–
Voice changes	0	0	0	–
Altered deglutition	0.06 ± 0.25	0	1 (3.3)	0.36
Perceived augmented HR	0.19 ± 0.4	0.07 ± 0.27	4 (13.3)	0.37
Perceived reduced HR	0.06 ± 0.25	0	1 (3.3)	0.36
Burning sensation in the stomach	0.25 ± 0.45	0.28 ± 0.47	8 (26.7)	0.83
Constipation	0.06 ± 0.25	0	1 (3.3)	0.36
Diarrhea	0.06 ± 0.25	0	1 (3.3)	0.36
Vomiting	0.06 ± 0.25	0	1 (3.3)	0.36
Augmented lacrimation	0	0	0 (0)	–
Reduced lacrimation	0.12 ± 0.34	0	2 (6.7)	0.183
Augmented salivation	0	0	0 (0)	–
Reduced salivation	0	0	0 (0)	–
Head and neck sweating attacks	0.06 ± 0.25	0	1 (3.3)	0.36
Head and neck skin dryness	0.06 ± 0.25	0	1 (3.3)	0.36
Sleep alteration	0.34 ± 0.48	0.14 ± 0.36	7 (23.3)	0.29

Numeric values are reported as means ± standard deviation (SD); nominal values are reported as medians. The checklist was administered to all participants and answers are expressed as the number of subjects having symptoms and percentage (%).
HR, heart rate; AD, autonomic dysfunction; APN, peripheral neuropathies; p, level of significance ($p < 0.05$).

Statistics

Statistical analyses were performed within SPSS v.20.0 (IBM Corp., Armonk, NY, United States). Paired Student's *t*-tests were used to detect differences from rest to end position in terms of STAR and VN–C6 distance. As an effect size measure, Cohen's *d* was used. The agreement in reporting test outcomes between the two operators was computed as Cohen's kappa (Martínez-Payá et al., 2015).

Receiver operating characteristic (ROC) curves were adopted to define the sensibility, specificity, and positive and negative likelihood ratios of the VN-NDT-related symptoms to predict VN dysfunctions or neuropathies. The overall diagnostic accuracy of the VN-NDT was defined by the area under the curve (AUC); a value of 0.5 was deemed as no discrimination, a value from 0.7 to 0.8 as acceptable, from 0.8 to 0.9 as excellent, and more than 0.9 as outstanding (Sarkar and Midi, 2010). CI at 95% was calculated and a statistical significance level of 0.05 was implemented throughout.

A two-way analysis of variance (ANOVA) for repeated measures with a 2 × 2 full-interaction design was adopted to test changes on the side (test administered on the right or left of the participant) and operator factors (experienced, not experienced) on the following variables: tests positivity, symptoms location, anatomical and physiological parameters assessed at rest and end of the VN-NDT, angular rotations, and head displacement ratio. The two-way ANOVA for repeated measures was also adopted to define differences between sexes and HR variations induced by the VN-NDT test. The effect size of each factor was computed as partial eta-squared (η_p^2): a value of η_p^2 of 0.010 was considered a small effect, a value of 0.059 a medium effect, and a value of 0.138 a large effect (Richardson, 2011).

RESULTS

As can be seen in **Table 1**, 46.7% of the participants had at least one symptom of the AD checklist (nine females and six males); 23.3% of the subjects had experienced in the previous 7 days an episode of orthostatic hypotension (five females and two males), and one-fourth reported gastrointestinal symptoms (six females and three males).

The STARD flowchart (**Figure 2**) shows that six out of the 36 participants were not able to perform the experiments because of work or family issues. Notably, the sample size actual power was not affected by the loss of participants since 20% of the dropout was calculated as reported above. The age of the participants was not significantly different between males and females [$t(1, 30) = -0.01$; $p = 0.992$; 95% CI: −9.22 to 9.21]. Twenty-one out of 30 subjects were pain-free, five reported low back pain, three leg pain, and one facial pain. Five participants had a medical diagnosis with drug prescriptions: two for asthma, one for hyperthyroidism, one for gastric reflux, and one for hypertension and gastric reflux. No alteration was detected at the neurological examination for all participants. Four subjects reported mechanical allodynia of the right ear lobe. The ULNDT was positive on both sides in two subjects and on one side in four subjects. Cohen's kappa of 0.67 (95% CI: 0.49–0.85; $p < 0.001$) defined that VN-NDT reliability was significantly substantial.

No significant differences were detected between the sides and positive or negative tests between the two evaluators (**Supplementary Table 1**) nor the type and location of symptoms provoked [$F(1, 119) = 0.540$; $p = 0.464$; $\eta_p^2 = 0.005$, low effect]. Tension or mechanical allodynia in the suboccipital ipsilateral neck portion was reported in 66.7 and 5% of the cases, respectively. No adverse events (nausea, vomiting, hypotension, or neurological symptoms) were recorded during and after the VN-NDT administration.

The HR of the participants (**Figure 3A**) at rest (75.33 ± 11.61 bpm, $n = 30$) displayed no significant differences between females and males [$t(1, 119) = -0.672$; $p = 0.502$; $\eta_p^2 = 0.004$, 95% CI: −5.64 to 2.78]. Intriguingly, the VN-NDT induced a significant HR reduction in all participants [$F(1, 119) = 89.919$; $p < 0.000$; $\eta_p^2 = 0.432$, very high effect]. The HR drop was of 8 (±12.13) in females and 11.63 (±10.02) bpm in males and resulted statistically different between females and males [$t(1, 119) = 2.425$; $p = 0.017$; $\eta_p^2 = 0.047$, 95% CI: 0.93–9.18]. Notably, even when mild pain was provoked, an HR reduction was recorded confirming a selective VN stimulation by the VN-NDT (Devalle et al., 2018).

Anatomical and Biological Variables

The USI revealed no variations nor pathologies of the cervical portion of the VN (Giovagnorio and Martinoli, 2001). The VN-NDT induced a significant overall reduction of the VN–C6 distance (**Figure 3B**) of about 0.1 mm [$t(1, 119) = 2.48$; $p < 0.01$; $d = 0.2$; 95% CI: 0.03–0.3]. The VN–C6 distance was significantly higher on the right side at rest and in the VN-NDT end position of 0.30 and 0.34 mm, respectively [$t(1, 118) = 3.24$; $p < 0.002$; $d = 0.592$; $t(1, 118) = 3.83$; $p < 0.000$; $d = 0.699$, respectively]. USI identified a significant interaction (**Supplementary Table 1**) for side factor [$F(1, 119) = 14.98$; $p < 0.000$; $\eta_p^2 = 0.114$] and between operator but not for side factor and VN–C6 distance [$F(1, 119) = 0.032$; $p = 0.571$; $\eta_p^2 = 0.003$]. Also, no significant interaction between sexes of the participants and VN–C6 changes before and after the test was detectable [$F(1, 119) = 0.378$; $p = 0.540$; $\eta_p^2 = 0.003$]. These data indicate a higher distance on the right side between C6 and VN, but the degree of tension induced by the VN-NDT is similar to each side and not dependent on the sex of the participants.

Head Kinematics

To reach the VN-NDT final position, the neck of the subject was moved to stretch one VN each time, from the anatomical position of rest, of about 52° (±11°) of ipsilateral to the tested side rotation, 12° (±8.5°) of contralateral lateral flexion, and 12° (±8.5°) of flexion (**Figure 4A**), which indicates that the test was performed in a normal cervical range of motion not able to overstress muscle ligaments and joints of this anatomical region. Neither head inclination nor head flexion–extension significantly changed between sides relative to the assessor factor (**Supplementary Table 3**). Conversely, the head

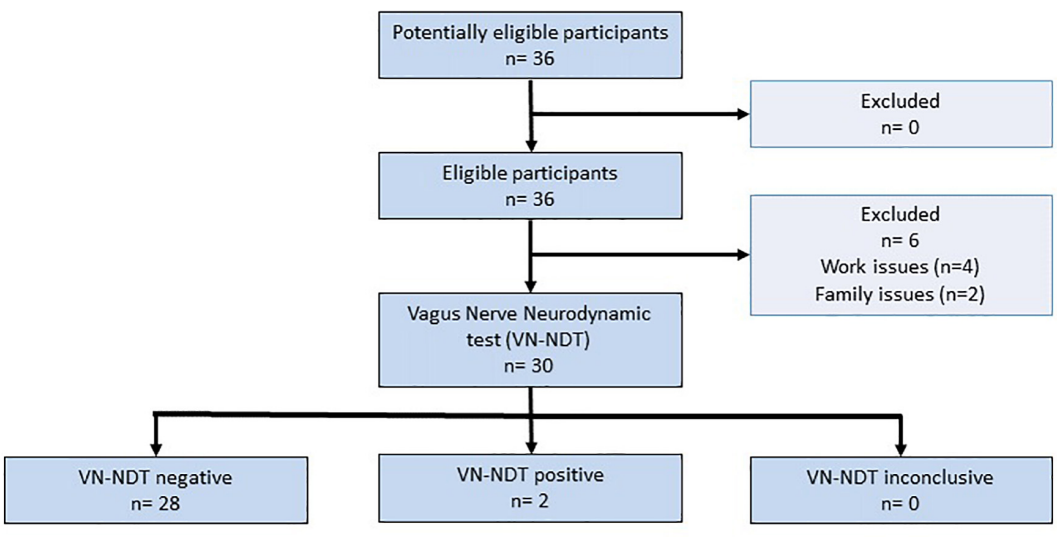

FIGURE 2 | STARD flowchart of the vagus nerve neurodynamic test (VN-NDT).

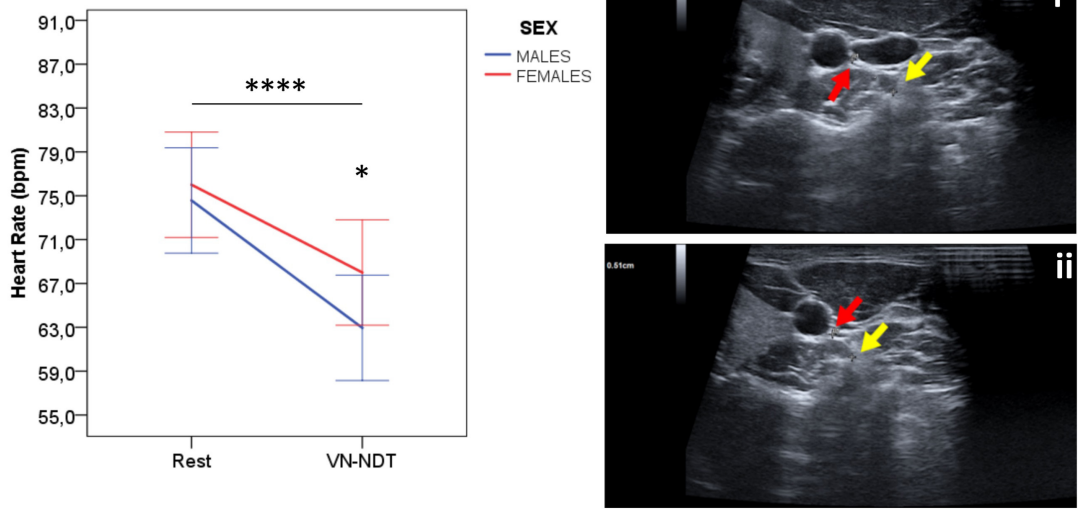

FIGURE 3 | (A) Effect of the VN-NDT on HR in males and females. Values in the graph are expressed as mean ± SD. Two-way ANOVA was carried out (data are normally distributed with comparable variances); asterisk shows the statistically significant difference between sexes (*$p \leq 0.05$ and ****$p \leq 0.0001$). **(B)** Ultrasound imaging axial scans of the (i) right vagus nerve at rest and (ii) the final position of the neurodynamic test. The red arrow indicates the vagus nerve and the yellow arrow indicates the anterior tubercle of C6 in male or female participants (no differences between sexes were detected by USI, $p = 0.54$).

was more laterally rotated by about 4° [$F(1, 119) = 6.29$; $p = 0.015$; $\eta_p^2 = 0.101$] when the left side was tested, as prompted in **Supplementary Figure 2** and **Supplementary Table 3**. Consistently, a slightly but significantly higher head displacement ratio was observed on the left side [**Supplementary Figure 2D**, $F(1, 119) = 6.211$; $p = 0.016$; $\eta_p^2 = 0.1$, medium-to-large effect], especially when the novice assessor performed the test [$F(1, 119) = 6.969$; $p = 0.011$; $\eta_p^2 = 0.111$, medium-to-large effect]. No significant side-by-operator interaction was found.

Autonomic Symptoms Detection Accuracy

The onset of tension or mechanical allodynia in the suboccipital ipsilateral region during the VN-NDT showed a significant ability to detect AD-related symptoms (**Figure 4B** and **Supplementary Table 3**). In particular, burning sensation in the stomach was significantly detected by tension and mechanical allodynia in the neck with an accuracy of 0.62 and 0.89, respectively ($p < 0.026$; 95% CI: 0.52–0.73; $p < 0.001$; 95% CI: 0.81–0.96; **Table 2**). Levels of PHS inferior or equal to 80 on 100 were significantly detected by neck tension (**Figure 4B**) with an accuracy of 0.61 ($p < 0.045$; 95% CI: 0.51–0.72).

DISCUSSION

This study indicates that the proposed VN-NDT induces a consistently moderate HR reduction in subjects of both sexes. Therefore, we propose it as a sensitive, fast, and riskless screening test for vagal function assessment which could be useful in the assessment of autonomic nervous system neuropathies.

Our data validate the proposed VN-NDT as a selective tool for VN function assessment. The collected normative data define the hallmark of physiological spectrum in males and females for HR variations induced by the VN-NDT and suggest a relationship between symptoms induced during the test and some autonomic dysfunction-related symptoms.

As described by Velten et al. (2020), autonomic symptoms related to orthostatic hypotension are commonly reported in 20% of the healthy population. Indeed, none of the participants had a diagnosis related to an autonomic disease, but many had experienced 1 week before the test at least one symptom related to autonomic dysregulation. In particular, orthostatic hypotension and altered digestion were the more prevalent conditions. The VN-NDT induces an HR reduction greater than those reported with Valsalva maneuver (VM) (Schrezenmaier et al., 2007), VN transcutaneous, or direct electrical stimulation (Clancy et al., 2014; Anand et al., 2020). Indeed, the VN-NTD induces a consistent and significant HR reduction of about 8 bpm in females and 12 bpm in males, respectively, likely triggered by the stretch-sensitive baroreceptor fibers traveling in the nodose and petrosal sensory ganglia of the VN (Berthoud and Neuhuber, 2000; Zeng et al., 2018; Norcliffe-Kaufmann, 2019; Besecker et al., 2020). Although neck torsion during the test was performed in a physiological mid-range of motion and the hands of the assessor were positioned on the head and upper cervical spine of the participant, we cannot exclude a role for the esophageal intraganglionic laminar endings in mechanical stress transduction (Zagorodnyuk and Brookes, 2000;

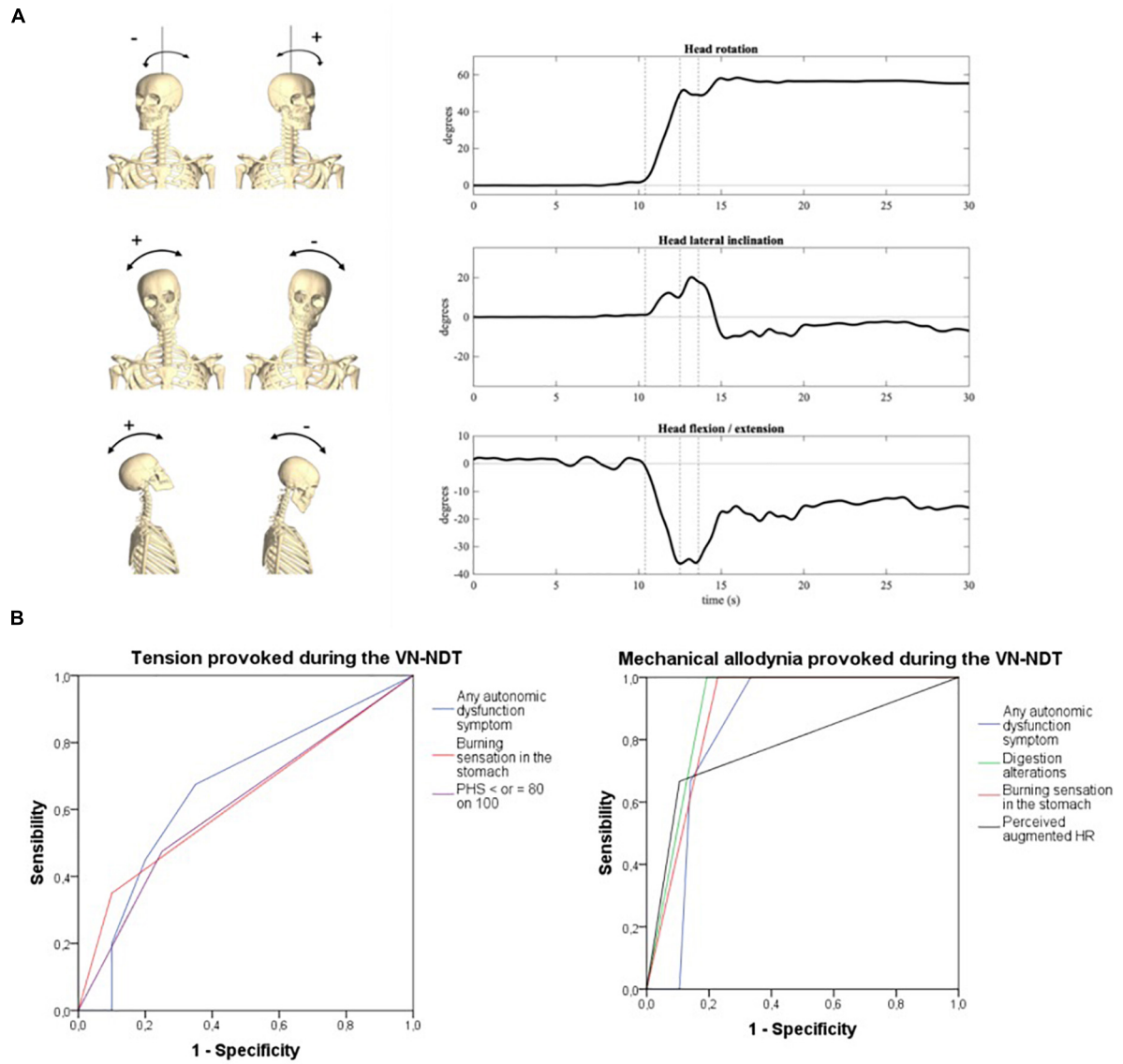

FIGURE 4 | (A) Head orientation at the end of the neurodynamic test (R: head inclined on the right of the subject; L: head inclined on the left of the subject). (i) Inclination, (ii) rotation, and (iii) flexion/extension. The three dotted vertical lines in the graphs represent the three movements of the VN-NDT combined from the left to the right: upper cervical flexion, contralateral later flexion, and ipsilateral rotation, respectively. **(B)** ROC curves of symptoms related to vagal dysfunctions or autonomic peripheral neuropathies detected by the onset of tension (left) and pain (right) in the neck during the vagus nerve neurodynamic test. PHS, perceived health status; HR, heart rate.

Brookes et al., 2013). A somewhat similar effect on HR has been found in normotensive humans during prolonged submaximal mandibular extension (60% of the maximal interincisal distance), prevented by minimal mandibular extension keeping a wooden tongue depressor between the incisors (Del Seppia et al., 2016, 2017). We cannot definitively rule out that similar effects are triggered by the two maneuvers, but the VN-NDT maneuvers did not induce any remarkable changes in the temporomandibular joint, prevented by the upper cervical flexion. Also, the effects on HR were detected at a short latency of 10 s of test administration, while the effects of the prolonged mandibular extension were recorded after 10 min of submaximal mandibular extension (Del Seppia et al., 2016; Devalle et al., 2018). Considering those data, we can reasonably hypothesize a marginal role of the glossopharyngeal nerve stretch reflex enrolment in the VN-NDT cardiac effects.

The VN-NDT is less invasive than the VM and other neural provocative tests (Schrezenmaier et al., 2007; Ehrman et al., 2020), since no side events were recorded, and no stress is applied to the cardiocirculatory system (Pstras et al., 2016). Also, no active participation of the tested

TABLE 2 | The vagus nerve neurodynamic test accuracy.

	Tension				Pain (mechanical allodynia)			
Variable	**Sensitivity**	**Specificity**	**+LR**	**−LR**	**Sensitivity**	**Specificity**	**+LR**	**−LR**
Digestion alterations					1	0.81	5.26	0
Perceived augmented HR					0.67	0.90	6.7	0.37
Burning sensation in the stomach	0.35	0.90	3.5	0.72	1	0.77	4.35	0
Any APN symptoms	0.67	0.65	1.91	0.51	1	0.46	1.85	0
Number of symptoms								
More than 1	0.45	0.80	2.2	0.7	1	0.67	3.03	0
More than 7	0	0.90	0	1.1	0	0.96	0	1.04
PHS (80 < on 100)	0.47	0.75	1.88	0.71				

Results about symptoms-induced diagnostic features in detecting vagal impairment and APN-related symptoms of the vagus nerve neurodynamic test are reported above (only significant predicted symptoms using ROC curves were reported). Sensitivity, specificity, and positive and negative likelihood ratios are reported (+LR, −LR). HR, heart rate; PHS, perceived health status.

subjects is required, which is particularly useful in subjects with communication problems like in the case of intensive care patients with COVID-19 and with disturbances of consciousness. The USI and the motion capture analysis confirmed that the VN-NDT induces a standardized anatomical reduction of the bone–nerve distance, which can stretch the VN and provoke symptoms related to autonomic dysfunctions.

The test accuracy and interrater agreement are comparable or higher than other clinical tests commonly used in the neurological assessment for neuropathic conditions like sensory testing, manual muscle testing, and nerve mechanosensitivity (Schmid et al., 2009; Terkelsen et al., 2017; Reshef et al., 2019).

Notably, mechanical allodynia—which is a common symptom when nerves receive prolonged exposure to inflammatory cytokines (Jensen and Finnerup, 2014; Beaulieu-Laroche et al., 2020; Bonet et al., 2021)—provoked by the VN-NDT had the best test accuracy in detecting digestion alterations and burning sensation in the stomach. Indeed, gastrointestinal dysfunctions are very common in acute and chronic APNs (Freeman, 2005; Oaklander and Nolano, 2019; Gutierrez et al., 2020; Marathe et al., 2020). Since the perioperative and postsurgery risks of cardiovascular side events (Lankhorst et al., 2015; Ho et al., 2020; Malaty et al., 2021) are higher in post-COVID-19 patients and patients with APN, which are difficult to be studied, it is possible to adopt the VN-NDT as a sensitive, faster, and riskless screening test. Yet, the test does not require other instruments than a finger pulse oximeter and a medical examination table, which makes it usable in low- and high-income countries.

Here, we report for the first time that a sequence of neck movements can systematically affect HR, both in males and females, suggesting a key role of the stretch on the neck portion of the VN in HR modulation. Gutierrez and coworkers reported that a patient with acute sensory and autonomic neuropathy had her symptoms relieved by neck movements (Gutierrez et al., 2020) which are included in the VN-NDT. Therefore, we can argue that studying the VN-NDT effects can be helpful in diagnosis and symptoms management in autonomic dysfunctions. Indeed, neurodynamic tests had been adopted successfully as treatment interventions for peripheral neuropathies. For instance, it has been established that invasive and non-invasive stimulation on the cervical tract of the VN ameliorates survival rates in sepsis models (Huston et al., 2007) and promotes heart and lung regeneration in preclinical models (Brandt et al., 2019; Chen et al., 2020), HR variability in cardiological patients (Kobayashi et al., 2013), and symptoms improvement in people with pharmacoresistant problems such as acute and chronic pain, dementia, psychiatric illness, consciousness disorder, and epilepsy (Kirchner et al., 2000; Schachter, 2006; Corazzol et al., 2017; Breit et al., 2018; Dong and Feng, 2018; Johnson and Wilson, 2018).

Since the VN-NDT can induce an effective VN stimulation, it would be useful to investigate its effects on these pathophysiological conditions and other conditions like diabetes-related gastrointestinal alterations, cardiac neuropathies, and arrhythmias secondary to coronavirus infection (Garamendi-Ruiz and Gómez-Esteban, 2019; Santos Breder and Sposito, 2019; Malaty et al., 2021).

CONCLUSION

The tests currently available for APN are neither selective nor sex-specific for evaluating the parasympathetic nervous system and can have troubling side effects. The proposed VN-NDT is a reliable, sensible, and sustainable screening test to assess parasympathetic activity and VN alterations also in patients with verbal/communication problems. The physiological HR changes induced by the VN-NDT are provided for healthy males and females. The VN-NDT can be safely incorporated into bedside assessment routines and pretreatment routine tests for all conditions in which APN is suspected and to discriminate APN from neck musculoskeletal problems.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University Bioethics Committee (protocol 139870-14/03/2019). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GC and MZ devised the concept of the study. GC, MZ, and AS designed the study. GC, MZ, AS, AC, MG, and SG participated in the acquisition of data. GC, MZ, AS, and PP analyzed and interpreted the data. GC, PP, and MZ wrote the initial draft. All authors critically revised the manuscript and approved the final version.

ACKNOWLEDGMENTS

We would like to thank Filippo Carta for the drawings.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.698470/full#supplementary-material

REFERENCES

Aggarwal, P., Zaveri, J. S., Goepfert, R. P., Shi, Q., Du, X. L., Swartz, M., et al. (2018). Symptom burden associated with late lower cranial neuropathy in long-term oropharyngeal cancer survivors. *JAMA Otolaryngol. Head Neck Surg.* 144, 1066–1076. doi: 10.1001/jamaoto.2018.1791

Anand, I. S., Konstam, M. A., Klein, H. U., Mann, D. L., Ardell, J. L., Gregory, D. D., et al. (2020). Comparison of symptomatic and functional responses to vagus nerve stimulation in ANTHEM-HF, INOVATE-HF, and NECTAR-HF. *ESC Hear. Fail.* 7, 75–83. doi: 10.1002/ehf2.12592

Ang, L., Dillon, B., Mizokami-Stout, K., and Pop-Busui, R. (2020). Cardiovascular autonomic neuropathy: A silent killer with long reach. *Auton. Neurosci. Basic Clin.* 225, 1–9. doi: 10.1016/j.autneu.2020.102646

Anil, G., and Tan, T. Y. (2011). CT and MRI evaluation of nerve sheath tumors of the cervical vagus nerve. *Am. J. Roentgenol.* 197, 195–201. doi: 10.2214/AJR.10.5734

Antonino, D., Teixeira, A. L., Maia-Lopes, P. M., Souza, M. C., Sabino-Carvalho, J. L., Murray, A. R., et al. (2017). Non-invasive vagus nerve stimulation acutely improves spontaneous cardiac baroreflex sensitivity in healthy young men: A randomized placebo-controlled trial. *Brain Stimul.* 10, 875–881. doi: 10.1016/j.brs.2017.05.006

Beaulieu-Laroche, L., Christin, M., Donoghue, A., Agosti, F., Yousefpour, N., Petitjean, H., et al. (2020). TACAN Is an Ion Channel Involved in Sensing Mechanical Pain. *Cell* 180, 956–967.e17. doi: 10.1016/j.cell.2020.01.033

Berthoud, H. R., and Neuhuber, W. L. (2000). Functional and chemical anatomy of the afferent vagal system. *Auton. Neurosci.* 85, 1–17. doi: 10.1016/S1566-0702(00)00215-0

Bertilson, B. C., Grunnesjö, M., Johansson, S. E., and Strender, L. E. (2007). Pain drawing in the assessment of neurogenic pain and dysfunction in the neck/shoulder Region: Inter-Examiner reliability and concordance with clinical examination. *Pain Med.* 8, 134–146. doi: 10.1111/j.1526-4637.2006.00145.x

Besecker, E. M., Blanke, E. N., Deiter, G. M., and Holmes, G. M. (2020). Gastric vagal afferent neuropathy following experimental spinal cord injury. *Exp. Neurol.* 323:113092. doi: 10.1016/j.expneurol.2019.113092

Bonet, I. J. M., Araldi, D., Bogen, O., and Levine, J. D. (2021). Involvement of TACAN, a Mechanotransducing Ion Channel, in Inflammatory But Not Neuropathic Hyperalgesia in the Rat. *J. Pain* 22, 498–508. doi: 10.1016/j.jpain.2020.11.004

Brandt, E. B., Bashar, S. J., and Mahmoud, A. I. (2019). Stimulating ideas for heart regeneration: the future of nerve-directed heart therapy. *Bioelectron. Med.* 5:8. doi: 10.1186/s42234-019-0024-0

Breit, S., Kupferberg, A., Rogler, G., and Hasler, G. (2018). Vagus nerve as modulator of the brain-gut axis in psychiatric and inflammatory disorders. *Front. Psychiatry* 9:44. doi: 10.3389/fpsyt.2018.00044

Brookes, S. J. H., Spencer, N. J., Costa, M., and Zagorodnyuk, V. P. (2013). Extrinsic primary afferent signalling in the gut. *Nat. Rev. Gastroenterol. Hepatol.* 10, 286–296. doi: 10.1038/nrgastro.2013.29

Bueno-Gracia, E., Tricás-Moreno, J. M., Fanlo-Mazas, P., Malo-Urriés, M., Haddad-Garay, M., Estébanez-de-Miguel, E., et al. (2016). Validity of the Upper Limb Neurodynamic Test 1 for the diagnosis of Carpal Tunnel Syndrome. The role of structural differentiation. *Man. Ther.* 22, 190–195. doi: 10.1016/j.math.2015.12.007

Chen, X., Zhao, C., Zhang, C., Li, Q., Chen, J., Cheng, L., et al. (2020). Vagal-α7nAChR signaling promotes lung stem cells regeneration via fibroblast growth factor 10 during lung injury repair. *Stem Cell Res. Ther.* 11:230. doi: 10.1186/s13287-020-01757-w

Clancy, J. A., Mary, D. A., Witte, K. K., Greenwood, J. P., Deuchars, S. A., and Deuchars, J. (2014). Non-invasive Vagus nerve stimulation in healthy humans reduces sympathetic nerve activity. *Brain Stimul.* 7, 871–877. doi: 10.1016/j.brs.2014.07.031

Corazzol, M., Lio, G., Lefevre, A., Deiana, G., Tell, L., André-Obadia, N., et al. (2017). Restoring consciousness with vagus nerve stimulation. *Curr. Biol.* 27, R994–R996. doi: 10.1016/j.cub.2017.07.060

Del Seppia, C., Ghione, S., Foresi, P., Fommei, E., Lapi, D., Colantuoni, A., et al. (2016). Further evidence of a prolonged hypotensive and a bradycardic effect after mandibular extension in normal volunteers. *Arch. Ital. Biol.* 154, 143–150. doi: 10.12871/00039829201645

Del Seppia, C., Ghione, S., Foresi, P., Lapi, D., Fommei, E., Colantuoni, A., et al. (2017). Evidence in the human of a hypotensive and a bradycardic effect after mouth opening maintained for 10 min. *Eur. J. Appl. Physiol.* 117, 1485–1491. doi: 10.1007/s00421-017-3643-8

Devalle, G., Castiglioni, P., Arienti, C., Abbate, C., Mazzucchi, A., Agnello, L., et al. (2018). Cardio-respiratory autonomic responses to nociceptive stimuli in patients with disorders of consciousness. *PLoS One.* 13:e0201921. doi: 10.1371/journal.pone.0201921

Dong, X. Y., and Feng, Z. (2018). Wake-promoting effects of vagus nerve stimulation after traumatic brain injury: Upregulation of orexin-A and orexin receptor type 1 expression in the prefrontal cortex. *Neural Regen. Res.* 13, 244–251. doi: 10.4103/1673-5374.226395

Egloff, N., Klingler, N., Von Känel, R., Cámara, R., Curatolo, M., Wegmann, B., et al. (2011). Algometry with a clothes peg compared to an electronic pressure algometer: A randomized cross-sectional study in pain patients. *BMC Musculoskelet. Disord.* 12:174. doi: 10.1186/1471-2474-12-174

Ehrman, J. K., Fernandez, A. B., Myers, J., Oh, P., Thompson, P. D., and Keteyian, S. J. (2020). Aortic Aneurysm: DIAGNOSIS, MANAGEMENT, EXERCISE TESTING, and TRAINING. *J. Cardiopulm. Rehabil. Prev.* 40, 215–223. doi: 10.1097/HCR.0000000000000521

Ekedahl, H., Jönsson, B., Annertz, M., and Frobell, R. B. (2018). Accuracy of Clinical Tests in Detecting Disk Herniation and Nerve Root Compression in Subjects With Lumbar Radicular Symptoms. *Arch. Phys. Med. Rehabil.* 99, 726–735. doi: 10.1016/j.apmr.2017.11.006

Fajgenbaum, M. A. P., Antonakis, S. N., Membrey, L., and Laidlaw, D. A. (2018). Acute retinal detachment induced by the Valsalva manoeuvre in morning glory disc anomaly. *BMJ Case Rep.* 2018:bcr2017223131. doi: 10.1136/bcr-2017-223131

Freeman, R. (2005). Autonomic peripheral neuropathy. *Lancet* 365, 1259–1270. doi: 10.1016/S0140-6736(05)74815-7

Fujii, K., Yamaguchi, S., Egawa, H., Hamaguchi, S., Kitajima, T., and Minami, J. (2004). Effects of head-up tilt after stellate ganglion block on QT interval and QT dispersion. *Reg. Anesth. Pain Med.* 29, 317–322. doi: 10.1016/j.rapm.2004.03.009

Fung, T. C., Olson, C. A., and Hsiao, E. Y. (2017). Interactions between the microbiota, immune and nervous systems in health and disease. *Nat. Neurosci.* 20, 145–155. doi: 10.1038/nn.4476

Garamendi-Ruiz, I., and Gómez-Esteban, J. C. (2019). Cardiovascular autonomic effects of vagus nerve stimulation. *Clin. Auton. Res.* 29, 183–194. doi: 10.1007/s10286-017-0477-8

Giovagnorio, F., and Martinoli, C. (2001). Sonography of the cervical vagus nerve: Normal appearance and abnormal findings. *Am. J. Roentgenol.* 176, 745–749. doi: 10.2214/ajr.176.3.1760745

Gutierrez, J., Palma, J. A., and Kaufmann, H. (2020). Acute sensory and autonomic neuronopathy: A devastating disorder affecting sensory and autonomic ganglia. *Semin. Neurol.* 40, 580–590. doi: 10.1055/s-0040-1713843

Ho, J. S. Y., Tambyah, P. A., Ho, A. F. W., Chan, M. Y. Y., and Sia, C. H. (2020). Effect of coronavirus infection on the human heart: A scoping review. *Eur. J. Prev. Cardiol.* 27, 1136–1148. doi: 10.1177/2047487320925965

Huston, J. M., Gallowitsch-Puerta, M., Ochani, M., Ochani, K., Yuan, R., Rosas-Ballina, M., et al. (2007). Transcutaneous vagus nerve stimulation reduces serum high mobility group box 1 levels and improves survival in murine sepsis. *Crit. Care Med.* 35, 2762–2768. doi: 10.1097/01.CCM.0000288102.15975.BA

Jensen, T. S., and Finnerup, N. B. (2014). Allodynia and hyperalgesia in neuropathic pain: Clinical manifestations and mechanisms. *Lancet Neurol.* 13, 924–935. doi: 10.1016/S1474-4422(14)70102-4

Johnson, R. L., and Wilson, C. G. (2018). A review of vagus nerve stimulation as a therapeutic intervention. *J. Inflamm. Res.* 11, 203–213. doi: 10.2147/JIR.S163248

Kasehagen, B., Ellis, R., Pope, R., Russell, N., and Hing, W. (2018). Assessing the Reliability of Ultrasound Imaging to Examine Peripheral Nerve Excursion: A Systematic Literature Review. *Ultrasound Med. Biol.* 44, 1–13. doi: 10.1016/j.ultrasmedbio.2017.08.1886

Kirchner, A., Birklein, F., Stefan, H., and Handwerker, H. O. (2000). Left vagus nerve stimulation suppresses experimentally induced pain. *Neurology* 55, 1167–1171. doi: 10.1212/WNL.55.8.1167

Kobayashi, M., Massiello, A., Karimov, J. H., Van Wagoner, D. R., and Fukamachi, K. (2013). Cardiac autonomic nerve stimulation in the treatment of heart failure. *Ann. Thorac. Surg.* 96, 339–345. doi: 10.1016/j.athoracsur.2012.12.060

Koulidis, K., Veremis, Y., Anderson, C., and Heneghan, N. R. (2019). Diagnostic accuracy of upper limb neurodynamic tests for the assessment of peripheral neuropathic pain: A systematic review. *Musculoskelet. Sci. Pract.* 40, 21–33. doi: 10.1016/j.msksp.2019.01.001

Lankhorst, S., Keet, S. W. M., Bulte, C. S. E., and Boer, C. (2015). The impact of autonomic dysfunction on peri-operative cardiovascular complications. *Anaesthesia* 70, 336–343. doi: 10.1111/anae.12904

Malaty, M., Kayes, T., Amarasekera, A. T., Kodsi, M., MacIntyre, C. R., and Tan, T. C. (2021). Incidence and treatment of arrhythmias secondary to coronavirus infection in humans: A systematic review. *Eur. J. Clin. Invest.* 51:e13428.. doi: 10.1111/eci.13428

Marathe, C. S., Jones, K. L., Wu, T., Rayner, C. K., and Horowitz, M. (2020). Gastrointestinal autonomic neuropathy in diabetes. *Auton. Neurosci. Basic Clin.* 229:102718. doi: 10.1016/j.autneu.2020.102718

Martínez-Payá, J. J., Ríos-Díaz, J., Del Baño-Aledo, M. E., García-Martínez, D., De Groot-Ferrando, A., and Meroño-Gallut, J. (2015). Biomechanics of the median nerve during stretching as assessed by ultrasonography. *J. Appl. Biomech.* 31, 439–444. doi: 10.1123/jab.2015-0026

Martinoli, C., Bianchi, S., Santacroce, E., Pugliese, F., Graif, M., and Derchi, L. E. (2002). Brachial plexus sonography: A technique for assessing the root level. *Am. J. Roentgenol.* 179, 699–702. doi: 10.2214/ajr.179.3.1790699

Nazir, H. F., AlFutaisi, A., Zacharia, M., Elshinawy, M., Mevada, S. T., Alrawas, A., et al. (2017). Vincristine-induced neuropathy in pediatric patients with acute lymphoblastic leukemia in Oman: Frequent autonomic and more severe cranial nerve involvement. *Pediatr. Blood Cancer* 64:12. doi: 10.1002/pbc.26677

Norcliffe-Kaufmann, L. (2019). The Vagus and Glossopharyngeal Nerves in Two Autonomic Disorders. *J. Clin. Neurophysiol.* 36, 443–451. doi: 10.1097/WNP.0000000000000604

Oaklander, A. L., and Nolano, M. (2019). Scientific Advances in and Clinical Approaches to Small-Fiber Polyneuropathy: A Review. *JAMA Neurol.* 76, 1240–1251. doi: 10.1001/jamaneurol.2019.2917

Paisley, A. N., Abbott, C. A., Van Schie, C. H. M., and Boulton, A. J. M. (2002). A comparison of the Neuropen against standard quantitative sensory-threshold measures for assessing peripheral nerve function. *Diabet. Med.* 19, 400–405. doi: 10.1046/j.1464-5491.2002.00706.x

Parra, B., Lizarazo, J., Jiménez-Arango, J. A., Zea-Vera, A. F., González-Manrique, G., Vargas, J., et al. (2016). Guillain–Barré Syndrome Associated with Zika Virus Infection in Colombia. *N. Engl. J. Med.* 375, 1513–1523. doi: 10.1056/nejmoa1605564

Pstras, L., Thomaseth, K., Waniewski, J., Balzani, I., and Bellavere, F. (2016). The Valsalva manoeuvre: Physiology and clinical examples. *Acta Physiol.* 217, 103–119. doi: 10.1111/apha.12639

Reshef, E. R., Schiff, N. D., and Brown, E. N. (2019). A Neurologic Examination for AnesthesiologistsAssessing Arousal Level during Induction, Maintenance, and Emergence. *Anesthesiology* 130, 462–471. doi: 10.1097/ALN.0000000000002559

Richardson, J. T. E. (2011). Eta squared and partial eta squared as measures of effect size in educational research. *Educ. Res. Rev.* 6, 135–147. doi: 10.1016/j.edurev.2010.12.001

Salaffi, F., Stancati, A., Silvestri, C. A., Ciapetti, A., and Grassi, W. (2004). Minimal clinically important changes in chronic musculoskeletal pain intensity measured on a numerical rating scale. *Eur. J. Pain* 8, 283–291. doi: 10.1016/j.ejpain.2003.09.004

Santos Breder, I. S., and Sposito, A. C. (2019). Cardiovascular autonomic neuropathy in type 2 diabetic patients. *Rev. Assoc. Med. Bras.* 65, 56–60. doi: 10.1590/1806-9282.65.1.56

Sarkar, S. K., and Midi, H. (2010). Importance of assessing the model adequacy of binary logistic regression. *J. Appl. Sci.* 10, 479–486. doi: 10.3923/jas.2010.479.486

Schachter, S. C. (2006). Therapeutic effects of vagus nerve stimulation in epilepsy and implications for sudden unexpected death in epilepsy. *Clin. Auton. Res.* 16, 29–32. doi: 10.1007/s10286-006-0275-1

Schmid, A. B., Brunner, F., Luomajoki, H., Held, U., Bachmann, L. M., Künzer, S., et al. (2009). Reliability of clinical tests to evaluate nerve function and mechanosensitivity of the upper limb peripheral nervous system. *BMC Musculoskelet. Disord.* 10:11. doi: 10.1186/1471-2474-10-11

Schrezenmaier, C., Singer, W., Swift, N. M., Sletten, D., Tanabe, J., and Low, P. A. (2007). Adrenergic and vagal baroreflex sensitivity in autonomic failure. *Arch. Neurol.* 64, 381–386. doi: 10.1001/archneur.64.3.381

Suarez-Roca, H., Klinger, R. Y., Podgoreanu, M. V., Ji, R. R., Sigurdsson, M. I., Waldron, N., et al. (2019). Contribution of Baroreceptor Function to Pain Perception and Perioperative Outcomes. *Anesthesiology* 130, 634–650. doi: 10.1097/ALN.0000000000002510

Sunaryo, P., Choudhry, O., Sharer, L., Eloy, J., and Liu, J. (2012). Schwannomas of the Anterior Skull Base: Analysis of 41 Cases in the Literature. *J. Neurol. Surg. Part B Skull Base* 73:56. doi: 10.1055/s-0032-1312208

Taenzer, A. H., Clark, C., and Curry, C. S. (2000). Gender affects report of pain and function after arthroscopic anterior cruciate ligament reconstruction. *Anesthesiology* 93, 670–675. doi: 10.1097/00000542-200009000-00015

Terkelsen, A. J., Karlsson, P., Lauria, G., Freeman, R., Finnerup, N. B., and Jensen, T. S. (2017). The diagnostic challenge of small fibre neuropathy: clinical presentations, evaluations, and causes. *Lancet Neurol.* 16, 934–944. doi: 10.1016/S1474-4422(17)30329-0

Velten, A. P. C., Bensenor, I., Lotufo, P., and Mill, J. G. (2020). Prevalence of orthostatic hypotension and the distribution of pressure variation in the longitudinal study of adult health. *Arq. Bras. Cardiol.* 114, 1040–1048. doi: 10.36660/abc.20180354

Verlinden, T. J. M., Rijkers, K., Hoogland, G., and Herrler, A. (2016). Morphology of the human cervical vagus nerve: Implications for vagus nerve stimulation treatment. *Acta Neurol. Scand.* 133, 173–182. doi: 10.1111/ane.12462

Verwoerd, A. J. H., Mens, J., el Barzouhi, A., Peul, W. C., Koes, B. W., and Verhagen, A. P. (2016). A diagnostic study in patients with sciatica establishing the importance of localization of worsening of pain during coughing, sneezing and straining to assess nerve root compression on MRI. *Eur. Spine J.* 25, 1389–1392. doi: 10.1007/s00586-016-4393-8

Wasan, A. D., Loggia, M. L., Chen, L. Q., Napadow, V., Kong, J., and Gollub, R. L. (2011). Neural correlates of chronic low back pain measured by arterial spin labeling. *Anesthesiology* 115, 364–374. doi: 10.1097/ALN.0b013e318220e880

Williams, S. M., Eleftheriadou, A., Alam, U., Cuthbertson, D. J., and Wilding, J. P. H. (2019). Cardiac Autonomic Neuropathy in Obesity, the Metabolic Syndrome and Prediabetes: A Narrative Review. *Diabetes Ther.* 10, 1995–2021. doi: 10.1007/s13300-019-00693-0

Zago, M., Sforza, C., Mariani, D., Marconi, M., Biloslavo, A., Greca, A., et al. (2020). Educational impact of hand motion analysis in the evaluation of FAST examination skills. *Eur. J. Trauma Emerg. Surg.* 46, 1421–1428. doi: 10.1007/s00068-019-01112-6

Zagorodnyuk, V. P., and Brookes, S. J. H. (2000). Transduction sites of vagal mechanoreceptors in the guinea pig esophagus. *J. Neurosci.* 20, 6249–6255. doi: 10.1523/jneurosci.20-16-06249.2000

Zeng, W. Z., Marshall, K. L., Min, S., Daou, I., Chapleau, M. W., Abboud, F. M., et al. (2018). PIEZOs mediate neuronal sensing of blood pressure and the baroreceptor reflex. *Science* 362, 464–467. doi: 10.1126/science.aau6324

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CHAPTER 4

DISCUSSION

4.1 Effects of the neurodynamic treatment at the cell level

The novelty brought by our *in vitro* experiments are many, and one of the most relevant is that we have described for the first time that a manual treatment, such as the NDT, can induce a dose-dependent response in target cells defining the beneficial effects reported by patients are not dependent on placebo effects and that specific mechanism are involved. Our data shows that NDT induces selective changes in neurons confirming that neurons are the target cells of this treatment. In particular, our results from *in vitro* experiments have confirmed that not only sensory but also motor neurons are mechanosensitive and mechanoreactive in a dose-dependent manner. The cell responses to tailored mechanical stimuli, with the same features as those administered in the clinical settings, are related to neural plasticity and regeneration processes.

The obtained results have also shown that NDT can regulate the gene expression of PIEZO1 (and not PIEZO2) mechanosensitive ion channels responsible for the transduction of light stretch and compressions related to the perception of touch in sensory neurons and it has been hypothesized to be involved in pain modulation (Coste et al., 2010; Zhao et al., 2016; Wang et al., 2019a; Marshall and Patapoutian, 2020; Roh et al., 2020; Lewis and Grandl, 2021). The *ex vivo* and *in vivo* experiments had shown that NDT regulates the expression in sensory neurons of TACAN and TLR2 which are receptors involved in mechanical allodynia

and hyperalgesia with an antiallodynic and anti-hyperalgesic effect (Jurga et al., 2016; Cobos et al., 2018; Arenas and Lumpkin, 2020; Bogen et al., 2020).

A significant anti-apoptotic effect had been reported from *in vitro* and *ex vivo* experiments in sensory neurons two days after a single session of NDT protocol administration without any pro-apoptotic effect. It is indeed fundamental for nerve recovery after an injury that cell death of neurons is prevented by the activation of antiapoptotic processes since the lack of this event is responsible for impaired regeneration with increased loss of nerve function (Kotulska et al., 2005).

Also, from *in vitro* experiments we have observed that NDT in both sensory and motor neurons increased significantly the number of differentiated cells compared to the untreated. The reactivation of neurons after a nerve lesion is observed by a dendritic arbor restoration and axonal regrowth of those neurons that got their axons injured is a key process for nerve regeneration (Navarro, 2016; Dubový et al., 2018a).

Another remarkable effect we have observed from *in vivo* and *ex vivo* experiments is that NDT induces a significant neurite outgrowth which is a fundamental therapeutic effect promoting neural plasticity and nerve regeneration (Wang et al., 2019b; Gordon, 2020). Indeed, the increased axonal regeneration speed is a useful factor to reach the target tissue in a short amount of time avoiding atrophy and restoring faster the nerve functions lost or impaired after the nerve injury. Our experiments have shown that not only the sensory neurons are affected by the NDT, as this treatment had been largely used for pain management, but also motor neurons regeneration processes are induced.

As shown by Wang and colleagues muscular atrophy prevention after a sciatic nerve crush in rabbits was obtained, by the same NDT protocol adopted in our experiments, by regulating the expression of Murf-1 in the innervated muscle, but a clear effect on motor neurons of NDT was lacking in the literature (Wang et al., 2015).

Notably, our experiments have shown that the direction of the neurite outgrowth is not significantly affected by the NDT. These data are supported by results obtained by Kampanis and coworkers who demonstrated that a single session of repeated mechanical tensions applied to DRG explants by a robotic bioreactor does not affect the orientation of the neurites (Kampanis et al., 2021). Other experiments performed on DRG explants treated with progressive tensions, increased day by day for weeks, have shown that the orientation of the neurites follows the force direction applied by the bioreactor (Pfister et al., 2006b, 2006a, 2008; Loverde et al., 2011; Feng et al., 2015; Magou et al., 2015). Despite the latter studies, in which the orientation of the fiber was significantly affected by the progressive elongations during their regrowth, the treatment protocol performed with a robotic bioreactor cannot be translated into clinical practice since no progressive nerve tension is possible to be applied day by day and the parameters of the stretches required a 24 hours intervention for weeks. On the contrary, the same parameters adopted in our *in vitro* and *ex vivo* experiments were easily adopted for *in vivo* experiments being also suitable to be translated in the clinical settings.

4.2 Effects of the neurodynamic treatment at the tissue level

Evidence on rat neuropathic pain models reported in the literature has shown that NDT with tensioning technique induces local effects in the site of the nerve injury. In particular, De Silva and coworkers, adopting a chronic constriction injury neuropathic pain model, described that 10 sessions of NDT, administered 14 days after surgery, induces an NGF and myelin protein zero increased protein expression in the injured nerve observed 24 days after the nerve injury (Da Silva et al., 2015). Zhu and colleagues, adopting a rat diabetic neuropathic pain model, have shown that NDT significantly reduces the IL-1β and TNFα protein expression only in the treated nerve (Zhu et al., 2018). Lima and coworkers reported that NDT administered after a nerve crush injury of the sciatic nerve in rats significantly prevented the intraneural scar formation (Lima et al., 2017) and the results we have obtained have shown the same responses. Notably, the dosage of the NDT we adopted in our *in vivo* experiments compared to this study was six times less in terms of the number of tensions (30 tension compared to 180 tensions), and it had provided significant beneficial results on animal behaviors, nerve morphology, and gene expression related to mechanical pain. Increased intraneural fibrosis forms a physical obstacle for axonal regeneration causing a lack or impaired nerve regeneration (Wang et al., 2019b). Indeed, the prevention of intraneural scar tissue formation is a fundamental aspect for nerve function and regeneration essential to maintain its normal elasticity and blood supply (Lundborg, 1988; Tos et al., 2015; Bove et al., 2019). We

confirmed that NDT prevents intraneural fibrosis providing a minimal dosage able to induce this fundamental beneficial effect.

4.3 Effects of the neurodynamic treatment on animal behaviors

NDT has shown to be effective in pain modulation and nerve conduction improvements detected by electrophysiological assessment in long term neuropathic pain patients suffering from carpal tunnel syndrome (Wolny et al., 2017; Wolny and Linek, 2018, 2019; Talebi et al., 2020) and radiculopathies of somatic nerves in the upper or lower limbs (Basson et al., 2015; Mahmoud ELDesoky, 2016; Yamin et al., 2016; Rajalaxmi M.V., Lavanya R., Kirupa K., Divya Mary S.M., 2020; Yun et al., 2020). Also, animal studies provided evidence that NDT promotes the upregulation of molecules related to neural plasticity in the PNS and CNS (Santos et al., 2012, 2014; Da Silva et al., 2015; Giardini et al., 2017; Martinez et al., 2018), but no data on dose-response and gross and fine motor performances were available yet. After defining the optimal NDT dosage based on in vitro experiments, we aimed therefore to assess the effects of NDT on sensory-motor recovery and its possible involvement in nerve regeneration after a nerve injury.

In our experiments, animals treated with NDT had reported a significant early restoration of pressure mechanical pain and normal pain behaviors compared to untreated animals. As reported above from *in vitro* and *ex vivo* experiments an increased neurite length coupled with antiapoptosis induced by the NDT protocol may reduce the recovery time for sensory neurons. We observed in rats that

contrary to the mechanical pain sensibility, the responsiveness to non-noxious mechanical pressure in the forepaw was not lost 4 and 12 days after the nerve injury. However, increased responsiveness to non-noxious pressure was only observed, on both injured and not injured sides, in untreated rats, while NDT had shown to protect against this pressure hypersensitivity, by maintaining the threshold to non-noxious stimuli same as pre-injury values. Increased activity of unmyelinated and myelinated primary afferent neurons is related to predominantly peripheral neuropathic pain process with stimuli dependent aberrant responses like dysesthesia, hyperalgesia, and allodynia, and is a common condition in most of the neuropathic pain conditions (Woolf and Salter, 2000; Campbell and Meyer, 2006; Jensen and Finnerup, 2014; Truini, 2017; Shin et al., 2021). In particular, these processes are linked to changes in neurochemical, anatomical, electrophysiological properties or protein and gene expression in the DRG neurons (Shi et al., 2011; Kim et al., 2012; Xu et al., 2016; Prato et al., 2017; Truini, 2017; Beaulieu-Laroche et al., 2020; Bogen et al., 2020; Yeh et al., 2020).

Notably, we observed that NDT affects muscles performance in high demanding tasks like climbing a rope but not fine finger movements like eating a grissini piece. In a rabbit sciatic nerve crush injury model, it was observed that calf muscle atrophy was prevented, by an NDT protocol with the same parameters used in our experiments but with a lower dosage (10 tensions 6 days/week) for a longer treatment time (4 weeks), and this effect was associated to a significant lower MuRf-1 expression in the denervated muscles (Wang et al., 2015). To be noted, our *in vitro* experiments have shown that NDT induces neurotrophic

effects also in motor neurons promoting the differentiation and the neurites outgrowth, suggesting a regenerative effect of NDT also on the motor component of the PNS.

To summarize, our experiments have shown that pain modulation and early nerve recovery is promoted by NDT for both sensory and motor components, and this is a crucial aspect for atrophy prevention and functional recovery after a nerve injury that most of the cases are not generally totally restored (Jia et al., 2014; Van Hecke et al., 2014; Smith et al., 2020).

4.4 Translatability of the neurodynamic treatment

Many studies have been published on the neuroplastic effects induced by mechanical repeated or progressive stimulation *in vitro* or *ex vivo*. However, none of them can be translated in clinical settings for therapeutic intervention for several reasons. The main issue is the duration of the protocols described (from hours to weeks of repeated stimuli) impossible to be incorporated in a clinical context and also the amount of elongation varying from 1-5mm/day is not a clear reproducible condition (Bray, 1979; Loverde et al., 2011; Higgins et al., 2013; Loverde and Pfister, 2015; Kampanis et al., 2021).

To promote the clinical translatability of the NDT protocols defined in our experiments we have also adopted the tension perceived by the clinician during the maneuvers as a hallmark to administer the interventions. Indeed, this reference, which is used in clinical practice to tailor the tension parameters and not overstretch the nerve (Basson et al., 2017, 2020; Calvo-Lobo et al., 2018), had shown to induce similar beneficial effects on cell culture, DRG explants, and

animals. Also, we took advantage of our *in vitro* experiments results and selected the more effective dosage in promoting neural plasticity and ion channels regulation for *in vivo* and *ex vivo* experiments. The cycles of tension and relaxation (1/5sec) and dosage of our NDT protocol consisting of 30 tensions administered in 3 minutes makes it very suitable to be adopted in all clinical conditions this intervention is required.

Some studies on manual therapy effects on neuropathic pain administered the interventions to awake rats (Bove et al., 2019; Wang et al., 2020a), but none of them administered NDT protocols (Bertolini et al., 2009; Santos et al., 2012; Da Silva et al., 2015; Wang et al., 2015; Giardini et al., 2017; Lima et al., 2017; Martinez et al., 2018; Zhu et al., 2018). To our knowledge, we have for the first time administered NDT on awake animals, and it led us to observe the absence of pain behaviors or sensitization to nerve tensions during the treatment of the injured nerves in these animals.

Notably, the posology adopted on *in vivo* experiments, consisting in 3 minutes 5 days/week for 3 weeks of treatment, is similar to the one used for both hospitalized and ambulatory patients.

Finally, the regulation of the receptors' expression in the DRG and the processes related to cell survival and neural plasticity we have observed in our studies, are well conserved and shared mechanisms in humans (Kotulska et al., 2005; Kremer et al., 2018; Arenas and Lumpkin, 2020; Beaulieu-Laroche et al., 2020). For these reasons, we truly believe that the standardized NDT protocol we adopted for *in vivo* and *ex vivo* experiments is suitable to be translated into

clinical practice supported by strong evidence of its effects on cell survival, neural repair, and pain modulation processes.

4.5 Neurodynamic to treat neuropathic pain

The NDT is an effective non-pharmacological intervention commonly used in rehabilitation settings to reduce pain and disability in neuropathic pain patients and clinical and preclinical studies have shown that it can promote nerve functions recovery by restoring the proper nerve conduction (Wolny et al., 2017; Wolny and Linek, 2018). Also, hypoalgesic and anti-allodynic short-term effects have been reported respectively in asymptomatic (Beltran-Alacreu et al., 2015; Gamelas et al., 2019; Martins et al., 2019) and neuropathic pain patients (Villafañe et al., 2012; Arumugam et al., 2014). Previous studies on animals provided evidence that NDT promotes the regulation of molecules related to neural plasticity and receptor expression involved in the pain modulation at several levels in the PNS and CNS (Santos et al., 2012, 2014; Da Silva et al., 2015; Giardini et al., 2017; Martinez et al., 2018), but no data on specific effects of NDT on mechanical allodynia and hyperalgesia mechanisms were available yet. In particular, we focused on the effects of receptors modulation expressed in sensory neurons since it is emerging as a promising neuropathic pain target (Broad et al., 2009; Borbiro and Rohacs, 2017; Cobos et al., 2018; Mikhailov et al., 2019; Zhang et al., 2019; Beaulieu-Laroche et al., 2020; Bogen et al., 2020; Roh et al., 2020). Mechanical allodynia is a result of several processes like changes in receptors expression in the DRG like for example TACAN, a

mechanosensitive channel linked to neuroinflammation, or TLR2 linked immune response (Jurga et al., 2016; Cobos et al., 2018). Notably, our experiments on the median and ulnar nerves injury model have revealed that the mechanical pain modulation effect induced by NDT is due to the selective modulation of the TACAN channel in the DRG (Arenas and Lumpkin, 2020; Bogen et al., 2020). Also, we confirmed that NDT prevented sensitization phenomena related to the descending pain modulation system by showing a significantly lower number of paw withdrawals only in treated animals for repeated nerve tensions 22 days after nerve injury. Indeed, is reported in the literature that NDT protocols in rats significantly regulated the expression of k- and m-opioid receptors in the DRG and periaqueductal grey, which are fundamental for pain suppression mechanisms (Santos et al., 2014; Da Silva et al., 2015; Giardini et al., 2017; Martinez et al., 2018).

Considering that the NDT antiallodynic and anti-sensitization effects we have observed in our experiments are shared mechanisms between humans and rats and that none of the drugs available for neuropathic pain management is effective for all patients (Finnerup et al., 2015, 2018), it is fair to consider NDT as a useful time effective clinical tool for those patients suffering for neuropathic mechanical pain.

4.6 Reliability of the neurodynamic tests and vagus nerve test validation

Tension tests of peripheral nerves or neurodynamic tests are bed-side examination clinical tests validated for somatic nerves neuropathies and reliable

for radiculopathies detection (Schmid et al., 2009; Urban and Macneil, 2015; Verwoerd et al., 2016; Koulidis et al., 2019). These tests consisting of a combination of physiological movements can progressively load the nerve tested assessing the normal nerve mechanosensitivity that in case of neuropathic conditions is impaired.

The VN neurodynamic test we have developed stressing the cervical thoracic and upper abdominal portion of the VN is biomechanical plausible since it has induced a significant anatomical displacement in the neck of this structure. Notably, we took advantage of the VN mechanical stimulation of the test to monitor not only subjective reported symptoms, but also the effects on the heart which is a VN target organ. We have discovered that the VN tension test induces a sensation of tension in the ipsilateral side of the upper neck and a remarkable and transient negative dromotrophic effect (causing a heart rate reduction) significantly higher in males. Notably, the test accuracy and inter-rater agreement between an expert and a novel assessor are comparable or higher than other clinical tests commonly used in the neurological assessment for neuropathic conditions like sensory testing, manual muscle testing, and reflexes testing (Schmid et al., 2009; Tawa et al., 2017; Terkelsen et al., 2017; Ekedahl et al., 2018). The great advantage brought by this test is that it is possible to assess the symptoms described by the patient but also the signs of heart rate reduction to establish a VN alteration or sensitization. The VN is the principal component of the parasympathetic nervous system and the main responsible for interoception (Bonaz et al., 2018), and brain-gut axis dysfunctions are prodromal disorders associated with neurological diseases, such as Alzheimer's disease, Parkinson's

disease, multiple sclerosis, and amyotrophic lateral sclerosis (ALS)(Tremlett et al., 2017).

Moreover, several health-related conditions have prominent and clinically relevant manifestations linked to a VN and autonomic system impairment (Freeman, 2005; Lyon, 2018). Diabetes, amyloidosis, immune-mediated, neoplastic, paraneoplastic, hereditary, secondary to infectious diseases, and intoxications (Oaklander and Nolano, 2019) are the leading causes described in the literature. Many invasive and non-invasive interventions stimulating the cervical tract of the VN reported positive results on symptom improvement in people with high social impact problems such as acute and chronic pain, dementia, psychiatric illness, stroke, cranial dysfunctions, consciousness disorder, and epilepsy (Johnson and Wilson, 2018; Möller et al., 2018; Darrow et al., 2020). For these reasons the VN is not only a time-effective test to assess pathological conditions in which an autonomic neuropathy is suspected but also as shown by our experiments on somatic nerve injury, it is fair to explore its effectiveness as a therapeutic tool for the diseases reported above, after an appropriate standardization of the treatment protocol.

4.7 Limitations

We have considered only adult animals in our experiments, making the results obtained not suitable for developmental ages in which the observed processes could be modulated differently. Our data are supported by clinical and preclinical effects of NDT on pain and nerve conduction changes however it is desirable to perform future clinical studies to assess if the NDT protocol we have defined is effective in all acquired neuropathic pain conditions and if children have different responses from adults. Like for drugs administration, it is clinically plausible to consider a lower dosage in children than the one adopted in our experiments but no proper indications are possible to be defined. The VN tension test we have validated in healthy subjects revealed a different HR reduction between males and females, however, no data are available on its effects on other innervated organs in the thorax or abdominal cavity. Since VN electrical stimulation reported remarkable effects of increased activation of the CNS functioning, and on a large number of degenerative or drug-resistant diseases, it is also suitable in the future to evaluate the possible entities of these effects also induced by the test.

CHAPTER 5

CONCLUSIONS

The results obtained in the experiments performed for the present Ph.D. thesis have significantly increased the knowledge of the NDT effects on peripheral nerve healing processes and pain modulation. In particular, we have observed that NDT promotes cell differentiation, neurites outgrowth in sensory and motor neurons, and antiapoptosis in sensory neurons. These are all fundamental processes for neuroplastic changes inducing nerve regeneration and proper functional recovery, which are highly impacting problems common after nerve injuries, and in neuropathic pain conditions. Our results had also shown that NDT activates specific processes promoting mechanical allodynia and hyperalgesia modulation modulating the expression of the mechanosensitive receptor TACAN in the DRG.

NDT is a non-pharmacological treatment and a cost-effective intervention and we have defined a proper dosage that can be safely adopted requiring at least 3 minutes/day to be effective. The NDT protocol that was developed and refined for our *in vitro*, *ex vivo*, and *in vivo* experiments, was designed to be easily translated in clinical practice to treat neuropathic pain patients with dosage and posology of one session for 5 days/week, very suitable for clinical settings.

Also, we have validated in humans the VN tension test that is a non-invasive and selective test to assess autonomic neuropathies and normal vagal function on heart rate modulation. To our knowledge, this is the first clinical tool that selectively assesses the parasympathetic function in humans. Based on the

encouraging data obtained from *in vitro*, *ex vivo*, and *in vivo* studies on NDT in somatic nerves it is also useful to explore the VN tension test as a therapeutic tool for those conditions involving autonomic neuropathies and all conditions in which VN stimulation is reported to have beneficial effects, with an appropriate previous protocol validation.

REFERENCES

Abajobir, A. A., Abate, K. H., Abbafati, C., Abbas, K. M., Abd-Allah, F., Abdulkader, R. S., et al. (2017). Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet* 390, 1211–1259. doi:10.1016/S0140-6736(17)32154-2.

Adriani, J. (1980). From Koller to Labat: A Historic Resume. *Reg. Anesth. J. Neural Blockade Obstet. Surgery, Pain Control* 5, 3–7. doi:10.1136/RAPM-00115550-198010000-00002.

Ang, L., Dillon, B., Mizokami-Stout, K., and Pop-Busui, R., Ang, L., Dillon, B., Mizokami-Stout, K., and Pop-Busui, R. (2020). Cardiovascular autonomic neuropathy: A silent killer with long reach. *Auton. Neurosci. Basic Clin.* 225, 1–9. doi:10.1016/j.autneu.2020.102646.

Arenas, O. M., and Lumpkin, E. A. (2020). Touching Base with Mechanical Pain. *Cell*. doi:10.1016/j.cell.2020.02.022.

Arumugam, V., Selvam, S., and MacDermid, J. C. (2014). Radial Nerve Mobilization Reduces Lateral Elbow Pain and Provides Short-Term Relief in Computer Users§. *Open Orthop. J.* 8, 368–371. doi:10.2174/1874325001408010368.

Attal, N., Martinez, V., and Bouhassira, D. (2021). Potential for increased prevalence of neuropathic pain after the COVID-19 pandemic. *Pain Reports*. doi:10.1097/PR9.0000000000000884.

Balius, R., Pujol, M., Pérez-Cuenca, D., Morros, C., Susín, A., Corominas, H., et al. (2021). Sciatic nerve movement in the deep gluteal space during hip rotations maneuvers. *Clin. Anat.* doi:10.1002/ca.23828.

Basson, A., Olivier, B., Ellis, R., Coppieters, M., Stewart, A., and Mudzi, W. (2015). The effectiveness of neural mobilizations in the treatment of musculoskeletal conditions: a systematic review protocol. *JBI database Syst. Rev. Implement. reports* 13, 65–75. doi:10.11124/jbisrir-2015-1401.

Basson, A., Olivier, B., Ellis, R., Coppieters, M., Stewart, A., and Mudzi, W. (2017). The effectiveness of neural mobilization for neuromusculoskeletal conditions: A systematic review and meta-Analysis. *J. Orthop. Sports Phys. Ther.* doi:10.2519/jospt.2017.7117.

Basson, C. A., Stewart, A., Mudzi, W., and Musenge, E. (2020). Effect of neural mobilization on nerve-related neck and arm pain: A randomized controlled trial. *Physiother. Canada*. doi:10.3138/ptc-2018-0056.

Beaulieu-Laroche, L., Christin, M., Donoghue, A., Agosti, F., Yousefpour, N., Petitjean, H., et al. (2020). TACAN Is an Ion Channel Involved in Sensing Mechanical Pain. *Cell* 180, 956-967.e17. doi:10.1016/j.cell.2020.01.033.

Bekar, E., Altunkaynak, B. Z., Balc, K., Aslan, G., Ayyldz, M., and Kaplan, S. (2014). Effects of high fat diet induced obesity on peripheral nerve regeneration and levels of GAP 43 and TGF-β in rats. *Biotech. Histochem.* 89, 446–456. doi:10.3109/10520295.2014.894575.

Beltran-Alacreu, H., Jiménez-Sanz, L., Fernández Carnero, J., and La Touche, R. (2015). Comparison of Hypoalgesic Effects of Neural Stretching vs Neural Gliding: A Randomized Controlled Trial. *J. Manipulative Physiol. Ther.* 38, 644–652. doi:10.1016/j.jmpt.2015.09.002.

Bendayan, R., Ramírez-Maestre, C., Ferrer, E., López, A., and Esteve, R. (2017). From acute to chronic back pain: Using linear mixed models to explore changes in pain intensity, disability, and depression. *Scand. J. Pain* 16, 45–51. doi:10.1016/j.sjpain.2017.02.009.

Bertolini, G. R. F., Silva, T. S., Trindade, D. L., Ciena, A. P., and Carvalho, A. R. (2009). Neural mobilization and static stretching in an experimental sciatica model: an experimental study. *Brazilian J. Phys. Ther.* doi:10.1590/s1413-35552009005000062.

Beth Darnall (2018). To treat pain, study people in all their complexity. doi:10.1038/d41586-018-04994-5.

Blyth, F. M. (2018). Global burden of neuropathic pain. *Pain*. doi:10.1097/j.pain.0000000000001127.

Boezaart, A. P., Lucas, S. D., and Elliott, C. E. (2009). Paravertebral block: Cervical, thoracic, lumbar, and sacral. *Curr. Opin. Anaesthesiol.* doi:10.1097/ACO.0b013e32832f3277.

Bogen, O., Levine, J. D., Bonet, I. J. M. M., Araldi, D., Bogen, O., and Levine, J. D. (2020). Involvement of TACAN, a mechanotransducing ion channel, in inflammatory but not neuropathic hyperalgesia in the rat. *J. Pain* 22, 498–508. doi:10.1016/j.jpain.2020.11.004.

Bonaz, B., Bazin, T., and Pellissier, S. (2018). The vagus nerve at the interface of the microbiota-gut-brain axis. *Front. Neurosci.* doi:10.3389/fnins.2018.00049.

Borbiro, I., and Rohacs, T. (2017). Regulation of Piezo Channels by Cellular Signaling Pathways. *Curr. Top. Membr.* doi:10.1016/bs.ctm.2016.10.002.

Boudier-Revéret, M., Gilbert, K. K., Allégue, D. R., Moussadyk, M., Brismée, J. M., Sizer, P. S., et al. (2017). Effect of neurodynamic mobilization on fluid dispersion in median nerve at the level of the carpal tunnel: A cadaveric study. *Musculoskelet. Sci. Pract.* 31, 45–51. doi:10.1016/j.msksp.2017.07.004.

Bove, G. M., Delany, S. P., Hobson, L., Cruz, G. E., Harris, M. Y., Amin, M., et al. (2019). Manual therapy prevents onset of nociceptor activity, sensorimotor dysfunction, and neural fibrosis induced by a volitional repetitive task. *Pain* 160, 632–644. doi:10.1097/j.pain.0000000000001443.

Brandt, E. B., Bashar, S. J., and Mahmoud, A. I. (2019). Stimulating ideas for heart regeneration: the future of nerve-directed heart therapy. *Bioelectron. Med.* 5, 1–8. doi:10.1186/s42234-019-0024-0.

Bray, D. (1979). Mechanical tension produced by nerve cells in tissue culture. *J. Cell Sci.* VOL.37, 391–410. doi:10.1242/jcs.37.1.391.

Bray, D. (1984). Axonal growth in response to experimentally applied mechanical tension. *Dev. Biol.* 102, 379–389. doi:10.1016/0012-1606(84)90202-1.

Breit, S., Kupferberg, A., Rogler, G., and Hasler, G. (2018). Vagus nerve as modulator of the brain-gut axis in psychiatric and inflammatory disorders. *Front. Psychiatry* 9, 1. doi:10.3389/fpsyt.2018.00044.

Broad, L. M., Mogg, A. J., Beattie, R. E., Ogden, A. M., Blanco, M. J., and Bleakman, D. (2009). TRP channels as emerging targets for pain therapeutics. *Expert Opin. Ther. Targets* 13, 69–81. doi:10.1517/14728220802616620.

Brown, C. L., Gilbert, K. K., Brismee, J. M., Sizer, P. S., James, C. R., and Smith, M. P. (2011). The effects of neurodynamic mobilization on fluid dispersion within the tibial nerve at the ankle: An unembalmed cadaveric study. *J. Man. Manip. Ther.* 19, 26–34. doi:10.1179/2042618610Y.0000000003.

Bueno-Gracia, E., Tricás-Moreno, J. M., Fanlo-Mazas, P., Malo-Urriés, M., Haddad-Garay, M., Estébanez-de-Miguel, E., et al. (2016). Validity of the Upper Limb Neurodynamic Test 1 for the diagnosis of Carpal Tunnel Syndrome. The role of structural differentiation. *Man. Ther.* 22, 190–195. doi:10.1016/j.math.2015.12.007.

Bueno-Gracia, E., Tricás-Moreno, J. M., Fanlo-Mazas, P., Malo-Urriés, M., Haddad-Garay, M., Estébanez de Miguel, E., et al. (2017). Validity of the upper limb neurodynamic test 1 for the diagnosis of carpal tunnel syndrome. The role of structural differentiation. *Musculoskelet. Sci. Pract.* 28, e26. doi:10.1016/j.math.2016.10.063.

Bueno, F. R., and Shah, S. B. (2008). Implications of tensile loading for the tissue engineering of nerves. *Tissue Eng. - Part B Rev.* doi:10.1089/ten.teb.2008.0020.

Calvo-Lobo, C., Unda-Solano, F., López-López, D., Sanz-Corbalán, I., Romero-Morales, C., Palomo-López, P., et al. (2018). Is pharmacologic treatment better than neural mobilization for cervicobrachial pain? A randomized clinical trial. *Int. J. Med. Sci.* 15, 456–465. doi:10.7150/ijms.23525.

Campbell, J. N., and Meyer, R. A. (2006). Mechanisms of Neuropathic Pain. *Neuron*. doi:10.1016/j.neuron.2006.09.021.

Cavanaugh, D. J., Lee, H., Lo, L., Shields, S. D., Zylka, M. J., Basbaum, A. I., et al. (2009). Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. *Proc. Natl. Acad. Sci. U. S. A.* doi:10.1073/pnas.0901507106.

Chen, X., Zhao, C., Zhang, C., Li, Q., Chen, J., Cheng, L., et al. (2020). Vagal-α7nAChR signaling promotes lung stem cells regeneration via fibroblast growth factor 10 during lung injury repair. *Stem Cell Res. Ther.* 11, 1–13. doi:10.1186/s13287-020-01757-w.

Cintron-Colon, A., Almeida-Alves, G., VanGyseghem, J., and Spitsbergen, J. (2022). GDNF to the rescue: GDNF delivery effects on motor neurons and nerves, and muscle re-innervation after peripheral nerve injuries. *Neural Regen. Res.* 17, 748. doi:10.4103/1673-5374.322446.

Cobos, E. J., Nickerson, C. A., Gao, F., Chandran, V., Bravo-Caparrós, I., González-Cano, R., et al. (2018). Mechanistic Differences in Neuropathic Pain Modalities Revealed by Correlating Behavior with Global Expression Profiling. *Cell Rep.* doi:10.1016/j.celrep.2018.01.006.

Coppieters, M. W., Alshami, A. M., Babri, A. S., Souvlis, T., Kippers, V., and Hodges, P. W. (2006). Strain and excursion of the sciatic, tibial and plantar nerves during a modified straight leg raising test. *J. Orthop. Res.* doi:10.1002/jor.20210.

Coppieters, M. W., and Butler, D. S. (2008). Do "sliders" slide and "tensioners"

tension? An analysis of neurodynamic techniques and considerations regarding their application. *Man. Ther.* 13, 213–221. doi:10.1016/j.math.2006.12.008.
Coppieters, M. W., Hough, A. D., and Dilley, A. (2009). Different nerve-gliding exercises induce different magnitudes of median nerve longitudinal excursion: An in vivo study using dynamic ultrasound imaging. *J. Orthop. Sports Phys. Ther.* 39, 164–171. doi:10.2519/jospt.2009.2913.
Corazzol, M., Lio, G., Lefevre, A., Deiana, G., Tell, L., André-Obadia, N., et al. (2017). Restoring consciousness with vagus nerve stimulation. *Curr. Biol.* 27, R994–R996. doi:10.1016/j.cub.2017.07.060.
Coste, B., Mathur, J., Schmidt, M., Earley, T. J., Ranade, S., Petrus, M. J., et al. (2010). Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. *Science (80-.).* 330, 55–60. doi:10.1126/science.1193270.
Cunningham, C. J., and Martínez, J. L. (2021). The Wandering Nerve: Positional Variations of the Cervical Vagus Nerve and Neurosurgical Implications. *World Neurosurg.* 156, 105–110. doi:10.1016/j.wneu.2021.09.090.
Da Silva, J. T., Dos Santos, F. M., Giardini, A. C., De Oliveira Martins, D., De Oliveira, M. E., Ciena, A. P., et al. (2015). Neural mobilization promotes nerve regeneration by nerve growth factor and myelin protein zero increased after sciatic nerve injury. *Growth Factors* 33, 8–13. doi:10.3109/08977194.2014.953630.
Dagenais, S., Caro, J., and Haldeman, S. (2008). A systematic review of low back pain cost of illness studies in the United States and internationally. *Spine J.* 8, 8–20. doi:10.1016/j.spinee.2007.10.005.
Dai, W. L., Yan, B., Bao, Y. N., Fan, J. F., and Liu, J. H. (2020). Suppression of peripheral NGF attenuates neuropathic pain induced by chronic constriction injury through the TAK1-MAPK/NF-κB signaling pathways. *Cell Commun. Signal.* 18. doi:10.1186/s12964-020-00556-3.
Darrow, M. J., Mian, T. M., Torres, M., Haider, Z., Danaphongse, T., Rennaker, R. L., et al. (2020). Restoration of Somatosensory Function by Pairing Vagus Nerve Stimulation with Tactile Rehabilitation. *Ann. Neurol.* 87, 194–205. doi:10.1002/ana.25664.
Dong, X. yang, and Feng, Z. (2018). Wake-promoting effects of vagus nerve stimulation after traumatic brain injury: Upregulation of orexin-A and orexin receptor type 1 expression in the prefrontal cortex. *Neural Regen. Res.* 13, 244–251. doi:10.4103/1673-5374.226395.
Driscoll, P. J., Glasby, M. A., and Lawson, G. M. (2002). An in vivo study of peripheral nerves in continuity: Biomechanical and physiological responses to elongation. *J. Orthop. Res.* doi:10.1016/S0736-0266(01)00104-8.
Dubový, P., Klusáková, I., Hradilová-Svíženská, I., and Joukal, M. (2018a). Expression of Regeneration-Associated Proteins in Primary Sensory Neurons and Regenerating Axons After Nerve Injury—An Overview. *Anat. Rec.* 301, 1618–1627. doi:10.1002/ar.23843.
Dubový, P., Klusáková, I., Hradilová-Svíženská, I., Joukal, M., and Boadas-Vaello, P. (2018b). Activation of astrocytes and microglial cells and CCL2/CCR2 upregulation in the dorsolateral and ventrolateral nuclei of periaqueductal gray and rostral ventromedial medulla following different

types of sciatic nerve injury. *Front. Cell. Neurosci.* 12. doi:10.3389/fncel.2018.00040.
Ducic, I., Safa, B., and DeVinney, E. (2017). Refinements of nerve repair with connector-assisted coaptation. *Microsurgery* 37, 256–263. doi:10.1002/micr.30151.
Edit, V., Eva, S., Maria, K., Istvan, R., Agnes, C., Zsolt, N., et al. (2013). Psychosocial, educational, and somatic factors in chronic nonspecific low back pain. *Rheumatol. Int.* 33, 587–592. doi:10.1007/s00296-012-2398-0.
Ekedahl, H., Jönsson, B., Annertz, M., and Frobell, R. B. (2018). Accuracy of Clinical Tests in Detecting Disk Herniation and Nerve Root Compression in Subjects With Lumbar Radicular Symptoms. *Arch. Phys. Med. Rehabil.* 99, 726–735. doi:10.1016/j.apmr.2017.11.006.
Ellis, R. F., Hing, W. A., and McNair, P. J. (2012). Comparison of longitudinal sciatic nerve movement with different mobilization exercises: An in vivo study utilizing ultrasound imaging. *J. Orthop. Sports Phys. Ther.* 42, 667–675. doi:10.2519/jospt.2012.3854.
Ellis, R., Rohan, M., Fox, J., Hitt, J., Langevin, H., and Henry, S. (2018). Ultrasound Elastographic Measurement of Sciatic Nerve Displacement and Shear Strain During Active and Passive Knee Extension. *J. Ultrasound Med.* 37, 2091–2103. doi:10.1002/JUM.14560.
Feng, Z. Q., Franz, E. W., Leach, M. K., Winterroth, F., White, C. M., Rastogi, A., et al. (2015). Live imaging of axon stretch growth in embryonic and adult neurons. *J. Biomed. Mater. Res. - Part A*. doi:10.1002/jbm.a.35627.
Finnerup, N. B., Attal, N., Haroutounian, S., McNicol, E., Baron, R., Dworkin, R. H., et al. (2015). Pharmacotherapy for neuropathic pain in adults: A systematic review and meta-analysis. *Lancet Neurol.* 14, 162–173. doi:10.1016/S1474-4422(14)70251-0.
Finnerup, N. B., Haroutounian, S., Baron, R., Dworkin, R. H., Gilron, I., Haanpaa, M., et al. (2018). Neuropathic pain clinical trials: Factors associated with decreases in estimated drug efficacy. *Pain* 159, 2339–2346. doi:10.1097/j.pain.0000000000001340.
Freeman, R. (2005). Autonomic peripheral neuropathy. in *Lancet*, 365: 1259–70. doi:10.1016/S0140-6736(05)74815-7.
Fregnan, F., Muratori, L., Bassani, G. A., Crosio, A., Biagiotti, M., Vincoli, V., et al. (2020). Preclinical Validation of SilkBridgeTM for Peripheral Nerve Regeneration. *Front. Bioeng. Biotechnol.* 8, 835. doi:10.3389/fbioe.2020.00835.
Fregnan, F., Muratori, L., Simões, A. R., Giacobini-Robecchi, M. G., and Raimondo, S. (2012). Role of inflammatory cytokines in peripheral nerve injury. *Neural Regen. Res.* 7, 2259–2266. doi:10.3969/j.issn.1673-5374.2012.29.003.
Fung, T. C., Olson, C. A., and Hsiao, E. Y. (2017). Interactions between the microbiota, immune and nervous systems in health and disease. *Nat. Neurosci.* 20, 145–155. doi:10.1038/nn.4476.
Gamelas, T., Fernandes, A., Magalhães, I., Ferreira, M., Machado, S., and Silva, A. G. (2019). Neural gliding versus neural tensioning: Effects on heat and cold thresholds, pain thresholds and hand grip strength in asymptomatic individuals. *J. Bodyw. Mov. Ther.* 23, 799–804.

doi:10.1016/j.jbmt.2019.04.011.
Gao, Y. M., Weng, C. S., and Wang, X. L. (2013). Changes in nerve microcirculation following peripheral nerve compression. *Neural Regen. Res.* doi:10.3969/j.issn.1673-5374.2013.11.010.
Garamendi-Ruiz, I., and Gómez-Esteban, J. C. (2019). Cardiovascular autonomic effects of vagus nerve stimulation. *Clin. Auton. Res.* 29, 183–194. doi:10.1007/s10286-017-0477-8.
Garrett, A., Rakhilin, N., Wang, N., McKey, J., Cofer, G., Anderson, R. B. J., et al. (2021). Mapping the peripheral nervous system in the whole mouse via compressed sensing tractography. *J. Neural Eng.* 18. doi:10.1088/1741-2552/ac0089.
Geuna, S., Raimondo, S., Fregnan, F., Haastert-Talini, K., and Grothe, C. (2016). In vitro models for peripheral nerve regeneration. , ed. D. Kirik Blackwell Publishing Ltd doi:10.1111/ejn.13054.
Geuna, S., Raimondo, S., Ronchi, G., Di Scipio, F., Tos, P., Czaja, K., et al. (2009). Chapter 3 Histology of the Peripheral Nerve and Changes Occurring During Nerve Regeneration. *Int. Rev. Neurobiol.* 87, 27–46. doi:10.1016/S0074-7742(09)87003-7.
Giardini, A. C., Santos, F. M. Dos, Da Silva, J. T., De Oliveira, M. E., Martins, D. O., and Chacur, M. (2017). Neural Mobilization Treatment Decreases Glial Cells and Brain-Derived Neurotrophic Factor Expression in the Central Nervous System in Rats with Neuropathic Pain Induced by CCI in Rats. *Pain Res. Manag.* 2017. doi:10.1155/2017/7429761.
Gilbert, K. K., Roger James, C., Apte, G., Brown, C., Sizer, P. S., Brismeé, J. M., et al. (2015a). Effects of simulated neural mobilization on fluid movement in cadaveric peripheral nerve sections: Implications for the treatment of neuropathic pain and dysfunction. *J. Man. Manip. Ther.* 23, 219–225. doi:10.1179/2042618614Y.0000000094.
Gilbert, K. K., Smith, M. P., Sobczak, S., James, C. R., Sizer, P. S., Brismée, J.-M. M., et al. (2015b). Effects of lower limb neurodynamic mobilization on intraneural fluid dispersion of the fourth lumbar nerve root: An unembalmed cadaveric investigation. *J. Man. Manip. Ther.* 23, 239–245. doi:10.1179/2042618615Y.0000000009.
Gordon, T. (2020). Peripheral nerve regeneration and muscle reinnervation. *Int. J. Mol. Sci.* 21, 1–24. doi:10.3390/ijms21228652.
Harrisson, S. A., Ogollah, R., Dunn, K. M., Foster, N. E., and Konstantinou, K. (2020). Prevalence, Characteristics, and Clinical Course of Neuropathic Pain in Primary Care Patients Consulting with Low Back-related Leg Pain. *Clin. J. Pain*. doi:10.1097/AJP.0000000000000879.
Higgins, S., Lee, J. S., Ha, L., and Lim, J. Y. (2013). Inducing neurite outgrowth by mechanical cell stretch. *Biores. Open Access*. doi:10.1089/biores.2013.0008.
Ho, J. S. Y., Tambyah, P. A., Ho, A. F. W., Chan, M. Y. Y., and Sia, C. H. (2020). Effect of coronavirus infection on the human heart: A scoping review. *Eur. J. Prev. Cardiol.* 27, 1136–1148. doi:10.1177/2047487320925965.
Hogans, B. B., Siaton, B. C., Taylor, M. N., Katzel, L. I., and Sorkin, J. D. (2021). Low Back Pain and Substance Use: Diagnostic and Administrative Coding for Opioid Use and Dependence Increased in U.S. Older Adults with Low Back

Pain. *Pain Med.* 2021, 1–12. doi:10.1093/pm/pnaa428.
Hoot, M. R., Sim-Selley, L. J., Selley, D. E., Scoggins, K. L., and Dewey, W. L. (2011). Chronic neuropathic pain in mice reduces μ-opioid receptor-mediated G-protein activity in the thalamus. *Brain Res.* 1406, 1–7. doi:10.1016/j.brainres.2011.06.023.
Hoy, D., March, L., Brooks, P., Blyth, F., Woolf, A., Bain, C., et al. (2014). The global burden of low back pain: estimates from the Global Burden of Disease 2010 study. *Ann. Rheum. Dis.* 73, 968–974. doi:10.1136/annrheumdis-2013-204428.
Huo, L., Gao, Y., Zhang, D., Wang, S., Han, Y., Men, H., et al. (2021). Piezo2 channel in nodose ganglia neurons is essential in controlling hypertension in a pathway regulated directly by Nedd4-2. *Pharmacol. Res.* 164. doi:10.1016/j.phrs.2020.105391.
Hussain, N., Shastri, U., McCartney, C. J. L., Gilron, I., Fillingim, R. B., Clarke, H., et al. (2018). Should thoracic paravertebral blocks be used to prevent chronic postsurgical pain after breast cancer surgery? a systematic analysis of evidence in light of IMMPACT recommendations. *Pain*. doi:10.1097/j.pain.0000000000001292.
Huston, J. M., Gallowitsch-Puerta, M., Ochani, M., Ochani, K., Yuan, R., Rosas-Ballina, M., et al. (2007). Transcutaneous vagus nerve stimulation reduces serum high mobility group box 1 levels and improves survival in murine sepsis. *Crit. Care Med.* 35, 2762–2768. doi:10.1097/01.CCM.0000288102.15975.BA.
Jang, J. H., Nam, T. S., Paik, K. S., and Leem, J. W. (2004). Involvement of peripherally released substance P and calcitonin gene-related peptide in mediating mechanical hyperalgesia in a traumatic neuropathy model of the rat. *Neurosci. Lett.* 360, 129–132. doi:10.1016/j.neulet.2004.02.043.
Jara, J. S., Agger, S., and Hollis, E. R. (2020). Functional Electrical Stimulation and the Modulation of the Axon Regeneration Program. *Front. Cell Dev. Biol.* 8, 736. doi:10.3389/fcell.2020.00736.
Jensen, T. S., and Finnerup, N. B. (2014). Allodynia and hyperalgesia in neuropathic pain: Clinical manifestations and mechanisms. *Lancet Neurol.* 13, 924–935. doi:10.1016/S1474-4422(14)70102-4.
Jia, X., Romero-Ortega, M. I., and Teng, Y. D. (2014). Peripheral nerve regeneration: mechanism, cell biology, and therapies. *Biomed Res. Int.* 2014. doi:10.1155/2014/145304.
Jiman, A. A., Ratze, D. C., Welle, E. J., Patel, P. R., Richie, J. M., Bottorff, E. C., et al. (2020). Multi-channel intraneural vagus nerve recordings with a novel high-density carbon fiber microelectrode array. *Sci. Rep.* 10. doi:10.1038/s41598-020-72512-7.
Johnson, R. L., and Wilson, C. G. (2018). A review of vagus nerve stimulation as a therapeutic intervention. *J. Inflamm. Res.* 11, 203–213. doi:10.2147/JIR.S163248.
Jonsdottir, S., Ahmed, H., Tómasson, K., and Carter, B. (2019). Factors associated with chronic and acute back pain in Wales, a cross-sectional study. *BMC Musculoskelet. Disord.* 20, 1–8. doi:10.1186/s12891-019-2477-4.
Juniper, M., Le, T. K., and Mladsi, D. (2009). The epidemiology, economic

burden, and pharmacological treatment of chronic low back pain in France, Germany, Italy, Spain and the UK: a literature-based review. *Expert Opin. Pharmacother.* 10, 2581–2592. doi:10.1517/14656560903304063.
Jurga, A. M., Rojewska, E., Piotrowska, A., Makuch, W., Pilat, D., Przewlocka, B., et al. (2016). Blockade of toll-like receptors (TLR2, TLR4) attenuates pain and potentiates buprenorphine analgesia in a rat neuropathic pain model. *Neural Plast.* doi:10.1155/2016/5238730.
Kampanis, V., Tolou-Dabbaghian, B., Zhou, L., Roth, W., and Puttagunta, R. (2021). Cyclic stretch of either pns or cns located nerves can stimulate neurite outgrowth. *Cells* 10, 1–23. doi:10.3390/cells10010032.
Keene, D. J., Knight, R., Bruce, J., Dutton, S. J., Tutton, E., Achten, J., et al. (2021). Chronic pain with neuropathic characteristics after surgery for major trauma to the lower limb: prevalence, predictors, and association with pain severity, disability, and quality of life in the UK WHiST trial. *Bone Joint J.* doi:10.1302/0301-620X.103B.BJJ-2020-2204.R1.
Kerns, J., Piponov, H., Helder, C., Amirouche, F., Solitro, G., and Gonzalez, M. (2019). Mechanical Properties of the Human Tibial and Peroneal Nerves Following Stretch With Histological Correlations. *Anat. Rec.* 302, 2030–2039. doi:10.1002/ar.24250.
Kim, S. E., Coste, B., Chadha, A., Cook, B., and Patapoutian, A. (2012). Supplemental Information The role of Drosophila Piezo in mechanical nociception. *Nature*.
Kirchner, A., Birklein, F., Stefan, H., and Handwerker, H. O. (2000). Left vagus nerve stimulation suppresses experimentally induced pain. *Neurology* 55, 1167–1171. doi:10.1212/WNL.55.8.1167.
Kobayashi, M., Massiello, A., Karimov, J. H., Van Wagoner, D. R., and Fukamachi, K. (2013). Cardiac autonomic nerve stimulation in the treatment of heart failure. *Ann. Thorac. Surg.* 96, 339–345. doi:10.1016/j.athoracsur.2012.12.060.
Kotulska, K., Marcol, W., Larysz-Brysz, M., Barski, J. J., Fus, Z., and Lewin-Kowalik, J. (2005). Impaired regeneration of bcl-2 lacking peripheral nerves. *Neurol. Res.* 27, 843–849. doi:10.1179/016164105X48815.
Koulidis, K., Veremis, Y., Anderson, C., and Heneghan, N. R. (2019). Diagnostic accuracy of upper limb neurodynamic tests for the assessment of peripheral neuropathic pain: A systematic review. *Musculoskelet. Sci. Pract.* 40, 21–33. doi:10.1016/j.msksp.2019.01.001.
Kreddig, N., and Hasenbring, M. I. (2017). Pain anxiety and fear of (re)injury in patients with chronic back pain: Sex as a moderator. *Scand. J. Pain* 16, 105–111. doi:10.1016/j.sjpain.2017.03.009.
Kremer, M., Yalcin, I., Goumon, Y., Wurtz, X., Nexon, L., Daniel, D., et al. (2018). A dual noradrenergic mechanism for the relief of neuropathic allodynia by the antidepressant drugs duloxetine and amitriptyline. *J. Neurosci.* doi:10.1523/JNEUROSCI.1004-18.2018.
Kumar, R., Lim, J., Mekary, R. A., Rattani, A., Dewan, M. C., Sharif, S. Y., et al. (2018). Traumatic Spinal Injury: Global Epidemiology and Worldwide Volume. *World Neurosurg.* 113, e345–e363. doi:10.1016/j.wneu.2018.02.033.
Lai, W. H., Shih, Y. F., Lin, P. L., Chen, W. Y., and Ma, H. L. (2012). Normal

neurodynamic responses of the femoral slump test. *Man. Ther.* doi:10.1016/j.math.2011.10.003.
Lallukka, T., Viikari-Juntura, E., Raitakari, O. T., Kähönen, M., Lehtimäki, T., Viikari, J., et al. (2014). Childhood and adult socio-economic position and social mobility as determinants of low back pain outcomes. *Eur. J. Pain (United Kingdom)*. doi:10.1002/j.1532-2149.2013.00351.x.
Lankhorst, S., Keet, S. W. M., Bulte, C. S. E., and Boer, C. (2015). The impact of autonomic dysfunction on peri-operative cardiovascular complications. *Anaesthesia* 70, 336–343. doi:10.1111/anae.12904.
Lasegue, C. (1864). Considerations sur la sciatique. *Arch. Gen. Med.* 1, 558–580.
Leoni, D., Storer, D., Gatti, R., Egloff, M., and Barbero, M. (2016). Upper limb neurodynamic test 1 on healthy individuals: Intra-and intersession reliability of the angle between pain onset and submaximal pain. *Pain Res. Manag.* 2016. doi:10.1155/2016/9607262.
Lewis, A. H., and Grandl, J. (2021). "Stretch and poke stimulation for characterizing mechanically activated ion channels," in *Methods in Enzymology* (Academic Press Inc.). doi:10.1016/bs.mie.2020.12.024.
Liedgens, H., Obradovic, M., De Courcy, J., Holbrook, T., and Jakubanis, R. (2016). A burden of illness study for neuropathic pain in Europe. *Clin. Outcomes Res.* doi:10.2147/CEOR.S81396.
Lima, Ê. M., Santana, H. H. S., Medrado, A. P., Martinez, A. M. B., and Baptista, A. F. (2017). Neurodynamic Mobilization Reduces Intraneural Fibrosis After Sciatic Crush Lesion in Rats. *Brazilian J. Med. Hum. Heal.* 5, 54–62. doi:10.17267/2317-3386bjmhh.v5i2.1303.
Liu, G., Zhang, Q., Jin, Y., and Gao, Z. (2012). Stress and strain analysis on the anastomosis site sutured with either epineurial or perineurial sutures after simulation of sciatic nerve injury. *Neural Regen. Res.* 7, 2299–2304. doi:10.3969/j.issn.1673-5374.2012.29.009.
Loeser, J. D., and Cahana, A. (2013a). Pain medicine versus pain management: Ethical dilemmas created by contemporary medicine and business. *Clin. J. Pain* 29, 311–316. doi:10.1097/AJP.0b013e3182516e64.
Loeser, J. D., and Cahana, A. (2013b). Pain medicine versus pain management: Ethical dilemmas created by contemporary medicine and business. *Clin. J. Pain* 29, 311–316. doi:10.1097/AJP.0b013e3182516e64.
Lolignier, S., Eijkelkamp, N., and Wood, J. N. (2014). Mechanical allodynia. *Pflugers Arch. Eur. J. Physiol.* 467, 133–139. doi:10.1007/s00424-014-1532-0.
Loverde, J. R., and Pfister, B. J. (2015). Developmental axon stretch stimulates neuron growth while maintaining normal electrical activity, intracellular calcium flux, and somatic morphology. *Front. Cell. Neurosci.* doi:10.3389/fncel.2015.00308.
Loverde, J. R., Tolentino, R. E., and Pfister, B. J. (2011). Axon stretch growth: The mechanotransduction of neuronal growth. *J. Vis. Exp.* doi:10.3791/2753.
Lundborg, G. (1988). Intraneural microcirculation. *Orthop. Clin. North Am.*
Lundborg, G., and Dahlin, L. B. (1996). Anatomy, function, and pathophysiology of peripheral nerves and nerve compression. *Hand Clin.*

Lutke Schipholt, I. J., Coppieters, M. W., Meijer, O. G., Tompra, N., de Vries, R. B. M., and Scholten-Peeters, G. G. M. (2021). Effects of joint and nerve mobilisation on neuroimmune responses in animals and humans with neuromusculoskeletal conditions: a systematic review and meta-analysis. *PAIN Reports* 6, e927. doi:10.1097/pr9.0000000000000927.

Lyon, L. (2018). "All disease begins in the gut": Was Hippocrates right? *Brain* 141, e20. doi:10.1093/brain/awy017.

Macfarlane, A. W. (1878). CASE OF SCIATICA, TREATED BY NERVE-STRETCHING. *Lancet*. doi:10.1016/S0140-6736(02)40843-4.

Machado-Duque, M. E., Gaviria-Mendoza, A., Machado-Alba, J. E., and Castaño, N. (2020). Evaluation of Treatment Patterns and Direct Costs Associated with the Management of Neuropathic Pain. *Pain Res. Manag.* doi:10.1155/2020/9353940.

Magou, G. C., Guo, Y., Choudhury, M., Chen, L., Hususan, N., Masotti, S., et al. (2011). Engineering a high throughput axon injury system. *J. Neurotrauma*. doi:10.1089/neu.2010.1596.

Magou, G. C., Pfister, B. J., and Berlin, J. R. (2015). Effect of acute stretch injury on action potential and network activity of rat neocortical neurons in culture. *Brain Res.* doi:10.1016/j.brainres.2015.07.056.

Mahmoud ELDesoky, M. T. (2016). Efficacy of Neural Mobilization on Low Back Pain with S1 Radiculopathy. *Int. J. Physiother.* 3, 362–370. doi:10.15621/ijphy/2016/v3i3/100847.

Malaty, M., Kayes, T., Amarasekera, A. T., Kodsi, M., MacIntyre, C. R., and Tan, T. C. (2021). Incidence and treatment of arrhythmias secondary to coronavirus infection in humans: A systematic review. *Eur. J. Clin. Invest.* 51, 1–19. doi:10.1111/eci.13428.

Manvell, N., Manvell, J. J., Snodgrass, S. J., and Reid, S. A. (2015). Tension of the Ulnar, Median, and Radial Nerves During Ulnar Nerve Neurodynamic Testing: Observational Cadaveric Study. *Phys. Ther.* doi:10.2522/ptj.20130536.

Marshall, J. (1883). Bradshaw lecture on nerve-stretching for the relief or cure of pain. *Br. Med. J.* doi:10.1136/bmj.2.1198.1173.

Marshall, K., and Patapoutian, A. (2020). Getting a grip on touch receptors. *Science (80-.).* 368, 1311–1312. doi:10.1126/science.abc7610.

Martinez, F., Teixeira, J., Rafael, I., Rocha, C., Oliveira, D., Chacur, M., et al. (2018). Non-pharmacological treatment affects neuropeptide expression in neuropathic pain model. *Brain Res.* 1687, 60–65. doi:10.1016/j.brainres.2018.02.034.

Martins, C., Pereira, R., Fernandes, I., Martins, J., Lopes, T., Ramos, L., et al. (2019). Neural gliding and neural tensioning differently impact flexibility, heat and pressure pain thresholds in asymptomatic subjects: A randomized, parallel and double-blind study. *Phys. Ther. Sport* 36, 101–109. doi:10.1016/j.ptsp.2019.01.008.

Meyer-Frießem, C. H., Gierthmühlen, J., Baron, R., Sommer, C., Üçeyler, N., and Enax-Krumova, E. K. (2021). Pain during and after COVID-19 in Germany and worldwide: A narrative review of current knowledge. *Pain Reports*. doi:10.1097/PR9.0000000000000893.

Miclescu, A., Straatmann, A., Gkatziani, P., Butler, S., Karlsten, R., and Gordh,

T. (2019). Chronic neuropathic pain after traumatic peripheral nerve injuries in the upper extremity: Prevalence, demographic and surgical determinants, impact on health and on pain medication. *Scand. J. Pain*. doi:10.1515/sjpain-2019-0111.

Mikhailov, N., Leskinen, J., Fagerlund, I., Poguzhelskaya, E., Giniatullina, R., Gafurov, O., et al. (2019). Mechanosensitive meningeal nociception via Piezo channels: Implications for pulsatile pain in migraine? *Neuropharmacology* 149, 113–123. doi:10.1016/j.neuropharm.2019.02.015.

Möller, M., Schroeder, C. F., and May, A. (2018). Vagus nerve stimulation modulates the cranial trigeminal autonomic reflex. *Ann. Neurol.* doi:10.1002/ana.25366.

Mueller, M. J., and Maluf, K. S. (2002). Tissue adaptation to physical stress: A proposed "Physical Stress Theory" to guide physical therapist practice, education, and research. *Phys. Ther.* doi:10.1093/ptj/82.4.383.

Mutubuki, E. N., Luitjens, M. A., Maas, E. T., Huygen, F. J. P. M., Ostelo, R. W. J. G., van Tulder, M. W., et al. (2020). Predictive factors of high societal costs among chronic low back pain patients. *Eur. J. Pain (United Kingdom)*. doi:10.1002/ejp.1488.

Nadi, M., Ramachandran, S., Islam, A., Forden, J., Guo, G. F., and Midha, R. (2019). Testing the effectiveness and the contribution of experimental supercharge (reversed) end-to-side nerve transfer. *J. Neurosurg.* 130, 702–711. doi:10.3171/2017.12.JNS171570.

Navarro, X. (2016). Functional evaluation of peripheral nerve regeneration and target reinnervation in animal models: A critical overview. *Eur. J. Neurosci.* 43, 271–286. doi:10.1111/ejn.13033.

Navarro, X., Vivó, M., and Valero-Cabré, A. (2007). Neural plasticity after peripheral nerve injury and regeneration. *Prog. Neurobiol.* 82, 163–201. doi:10.1016/j.pneurobio.2007.06.005.

Nee, R. J., Jull, G. A., Vicenzino, B., and Coppieters, M. W. (2012a). The validity of upper-limb neurodynamic tests for detecting peripheral neuropathic pain. *J. Orthop. Sports Phys. Ther.* 42, 413–424. doi:10.2519/jospt.2012.3988.

Nee, R. J., Vicenzino, B., Jull, G. A., Cleland, J. A., and Coppieters, M. W. (2012b). Neural tissue management provides immediate clinically relevant benefits without harmful effects for patients with nerve-related neck and arm pain: A randomised trial. *J. Physiother.* doi:10.1016/S1836-9553(12)70069-3.

Neto, T., Freitas, S. R., Marques, M., Gomes, L., Andrade, R., and Oliveira, R. (2017). Effects of lower body quadrant neural mobilization in healthy and low back pain populations: A systematic review and meta-analysis. *Musculoskelet. Sci. Pract.* 27, 14–22. doi:10.1016/j.msksp.2016.11.014.

Niedermair, T., Straub, R. H., Brochhausen, C., and Grässel, S. (2020). Impact of the sensory and sympathetic nervous system on fracture healing in ovariectomized mice. *Int. J. Mol. Sci.* 21, 1–18. doi:10.3390/ijms21020405.

Núñez de Arenas-Arroyo, S., Cavero-Redondo, I., Torres-Costoso, A., Reina-Gutiérrez, S., Álvarez-Bueno, C., and Martínez-Vizcaíno, V. (2021). Short-term Effects of Neurodynamic Techniques for Treating Carpal Tunnel Syndrome: A Systematic Review With Meta-analysis. *J. Orthop. Sport. Phys. Ther.* 51, 1–67. doi:10.2519/jospt.2021.10533.

Nuttall, F. Q. (2015). Body mass index: Obesity, BMI, and health: A critical review. *Nutr. Today* 50, 117–128. doi:10.1097/NT.0000000000000092.
Oaklander, A. L., and Nolano, M. (2019). Scientific Advances in and Clinical Approaches to Small-Fiber Polyneuropathy: A Review. *JAMA Neurol.* 76, 1240–1251. doi:10.1001/jamaneurol.2019.2917.
Odozor, C. U., Roles, K., Burk, C., Kannampallil, T., Clifford, D. B., Piccirillo, J. F., et al. (2021). Cohort study protocol to characterize the incidence and severity of neuropathic pain in patients with severe acute respiratory syndrome coronavirus 2 infection. *PAIN Reports*. doi:10.1097/pr9.0000000000000925.
Padovano, W. M., Dengler, J., Patterson, M. M., Yee, A., Snyder-Warwick, A. K., Wood, M. D., et al. (2020). Incidence of Nerve Injury After Extremity Trauma in the United States. *Hand (N. Y).* doi:10.1177/1558944720963895.
Pfister, B. J., Bonislawski, D. P., Smith, D. H., and Cohen, A. S. (2006a). Stretch-grown axons retain the ability to transmit active electrical signals. *FEBS Lett.* doi:10.1016/j.febslet.2006.05.030.
Pfister, B. J., Iwata, A., Taylor, A. G., Wolf, J. A., Meaney, D. F., and Smith, D. H. (2006b). Development of transplantable nervous tissue constructs comprised of stretch-grown axons. *J. Neurosci. Methods*. doi:10.1016/j.jneumeth.2005.10.012.
Pfister, L. A., Alther, E., Papaloïzos, M., Merkle, H. P., and Gander, B. (2008). Controlled nerve growth factor release from multi-ply alginate/chitosan-based nerve conduits. *Eur. J. Pharm. Biopharm.* 69, 563–572. doi:10.1016/j.ejpb.2008.01.014.
Plaza-Manzano, G., Cancela-Cilleruelo, I., Fernández-De-Las-Penãs, C., Cleland, J. A., Arias-Buriá, J. L., Thoomes-De-Graaf, M., et al. (2020). Effects of Adding a Neurodynamic Mobilization to Motor Control Training in Patients with Lumbar Radiculopathy Due to Disc Herniation: A Randomized Clinical Trial. *Am. J. Phys. Med. Rehabil.* doi:10.1097/PHM.0000000000001295.
Prato, V., Taberner, F. J., Hockley, J. R. F., Callejo, G., Arcourt, A., Tazir, B., et al. (2017). Functional and Molecular Characterization of Mechanoinsensitive "Silent" Nociceptors. *Cell Rep.* doi:10.1016/j.celrep.2017.11.066.
Qi, Y., Andolfi, L., Frattini, F., Mayer, F., Lazzarino, M., and Hu, J. (2015). Membrane stiffening by STOML3 facilitates mechanosensation in sensory neurons. *Nat. Commun.* doi:10.1038/ncomms9512.
Rajalaxmi M.V., Lavanya R., Kirupa K., Divya Mary S.M., Y. M. (2020). Efficacy of Neural Mobilization and Cervical Stabilization in Cervicobrachial Pain: A Randomized Controlled Trial. *Medico-Legal Updat.* 4, 1398–1403. doi:10.37506/mlu.v20i4.2025.
Ramos, M., Cruz, C. A. H., Laurentino, M. F., Ashmawi, H. A., Santos, F. M., and Chacur, M. (2020). Effects of neural mobilization on individuals with chronic low back pain. *Brazilian J. Pain* 3, 1–8. doi:10.5935/2595-0118.20200041.
Ridehalgh, C., Moore, A., and Hough, A. (2015). Sciatic nerve excursion during a modified passive straight leg raise test in asymptomatic participants and participants with spinally referred leg pain. *Man. Ther.* 20, 564–569. doi:10.1016/j.math.2015.01.003.
Rodriguez-Fontan, F., Reeves, B., Tuaño, K., Colakoglu, S., D'Agostino, L., and Banegas, R. (2020). Tobacco use and neurogenesis: A theoretical review of

pathophysiological mechanism affecting the outcome of peripheral nerve regeneration. *J. Orthop.* 22, 59–63. doi:10.1016/j.jor.2020.03.026.
Roh, J., Hwang, S. M., Lee, S. H., Lee, K., Kim, Y. H., and Park, C. K. (2020). Functional expression of piezo1 in dorsal root ganglion (DRG) neurons. *Int. J. Mol. Sci.* 21. doi:10.3390/ijms21113834.
Sanna, M. D., Manassero, G., Vercelli, A., Herdegen, T., and Galeotti, N. (2020). The isoform-specific functions of the c-Jun N-terminal kinase (JNK) in a mouse model of antiretroviral-induced painful peripheral neuropathy. *Eur. J. Pharmacol.* 880, 173161. doi:10.1016/j.ejphar.2020.173161.
Santos Breder, I. S., and Sposito, A. C. (2019). Cardiovascular autonomic neuropathy in type 2 diabetic patients. *Rev. Assoc. Med. Bras.* 65, 56–60. doi:10.1590/1806-9282.65.1.56.
Santos, F. M., Grecco, L. H., Pereira, M. G., Oliveira, M. E., Rocha, P. A., Silva, J. T., et al. (2014). The neural mobilization technique modulates the expression of endogenous opioids in the periaqueductal gray and improves muscle strength and mobility in rats with neuropathic pain. *Behav. Brain Funct.* 10, 1–8. doi:10.1186/1744-9081-10-19.
Santos, F. M., Silva, J. T., Giardini, A. C., Rocha, P. A., Achermann, A. P. P., S Alves, A., et al. (2012). Neural mobilization reverses behavioral and cellular changes that characterize neuropathic pain in rats. *Mol. Pain* 8, 1–9. doi:10.1186/1744-8069-8-57.
Schachter, S. C. (2006). Therapeutic effects of vagus nerve stimulation in epilepsy and implications for sudden unexpected death in epilepsy. *Clin. Auton. Res.* 16, 29–32. doi:10.1007/s10286-006-0275-1.
Schmid, A. B., Brunner, F., Luomajoki, H., Held, U., Bachmann, L. M., Künzer, S., et al. (2009). Reliability of clinical tests to evaluate nerve function and mechanosensitivity of the upper limb peripheral nervous system. *BMC Musculoskelet. Disord.* 10, 1–9. doi:10.1186/1471-2474-10-11.
Schofield, D. J., Shrestha, R. N., Passey, M. E., Earnest, a, and Fletcher, S. L. (2008). Chronic disease and labour force participation among older Australians. *Med. J. Aust.* 189, 447–450. doi:sch11012_fm [pii].
Shacklock, M., Yee, B., Van Hoof, T., Foley, R., Boddie, K., Lacey, E., et al. (2016). Slump Test: Effect of Contralateral Knee Extension on Response Sensations in Asymptomatic Subjects and Cadaver Study. *Spine (Phila. Pa. 1976).* 41, E205–E210. doi:10.1097/BRS.0000000000001218.
Sharma, S., Balthillaya, G., Rao, R., and Mani, R. (2016). Short term effectiveness of neural sliders and neural tensioners as an adjunct to static stretching of hamstrings on knee extension angle in healthy individuals: A randomized controlled trial. *Phys. Ther. Sport*. doi:10.1016/j.ptsp.2015.03.003.
Shi, X. Q., Zekki, H., and Zhang, J. (2011). The role of TLR2 in nerve injury-induced neuropathic pain is essentially mediated through macrophages in peripheral inflammatory response. *Glia*. doi:10.1002/glia.21093.
Shin, S. M., Moehring, F., Itson-Zoske, B., Fan, F., Stucky, C. L., Hogan, Q. H., et al. (2021). Piezo2 mechanosensitive ion channel is located to sensory neurons and nonneuronal cells in rat peripheral sensory pathway: implications in pain. *Pain* 162, 2750–2768. doi:10.1097/j.pain.0000000000002356.

Smith, B. H., Harry, L. H., Abirami Veluchamy, Hébert, H. L., and Veluchamy, A. (2020). Neuropathic pain in the community: prevalence, impact, and risk factors. *Pain* 161, S127–S136. doi:10.1097/j.pain.0000000000001824.

Song, Y., Li, D., Farrelly, O., Miles, L., Li, F., Kim, S. E., et al. (2019). The Mechanosensitive Ion Channel Piezo Inhibits Axon Regeneration. *Neuron*. doi:10.1016/j.neuron.2019.01.050.

Stephens, K. E., Zhou, W., Ji, Z., Chen, Z., He, S., Ji, H., et al. (2019). Sex differences in gene regulation in the dorsal root ganglion after nerve injury. *BMC Genomics* 20, 1–18. doi:10.1186/s12864-019-5512-9.

Suarez-Roca, H., Klinger, R. Y., Podgoreanu, M. V., Ji, R. R., Sigurdsson, M. I., Waldron, N., et al. (2019). Contribution of Baroreceptor Function to Pain Perception and Perioperative Outcomes. *Anesthesiology* 130, 634–650. doi:10.1097/ALN.0000000000002510.

Sulaiman, W., and Gordon, T. (2013). Neurobiology of peripheral nerve injury, regeneration, and functional recovery: From bench top research to bedside application. *Ochsner J.*

Taenzer, A. H., Clark, C., and Curry, C. S. (2000). Gender affects report of pain and function after arthroscopic anterior cruciate ligament reconstruction. *Anesthesiology* 93, 670–675. doi:10.1097/00000542-200009000-00015.

Talebi, G. A., Saadat, P., Javadian, Y., and Taghipour, M. (2020). Comparison of two manual therapy techniques in patients with carpal tunnel syndrome: A randomized clinical trial. *Casp. J. Intern. Med.* 11, 163–170. doi:10.22088/cjim.11.2.163.

Tapp, M., Wenzinger, E., Tarabishy, S., Ricci, J., and Herrera, F. A. (2019). The Epidemiology of Upper Extremity Nerve Injuries and Associated Cost in the US Emergency Departments. *Ann. Plast. Surg.* 83, 676–680. doi:10.1097/SAP.0000000000002083.

Tawa, N., Rhoda, A., and Diener, I. (2017). Accuracy of clinical neurological examination in diagnosing lumbo-sacral radiculopathy: A systematic literature review. *BMC Musculoskelet. Disord.* doi:10.1186/s12891-016-1383-2.

Taylor, S. S., Noor, N., Urits, I., Paladini, A., Sadhu, M. S., Gibb, C., et al. (2021). Complex Regional Pain Syndrome: A Comprehensive Review. *Pain Ther.* 10, 875–892. doi:10.1007/s40122-021-00279-4.

Temiz, Ç., Yaşar, S., and Kırık, A. (2021). Surgical treatment of peripheral nerve injuries: Better outcomes with intraoperative nap recordings. *Ulus. Travma ve Acil Cerrahi Derg.* 27, 510–515. doi:10.14744/tjtes.2020.95702.

Terkelsen, A. J., Karlsson, P., Lauria, G., Freeman, R., Finnerup, N. B., and Jensen, T. S. (2017). The diagnostic challenge of small fibre neuropathy: clinical presentations, evaluations, and causes. *Lancet Neurol.* 16, 934–944. doi:10.1016/S1474-4422(17)30329-0.

Tölle, T., Fitzcharles, M. A., and Häuser, W. (2021). Is opioid therapy for chronic non-cancer pain associated with a greater risk of all-cause mortality compared to non-opioid analgesics? A systematic review of propensity score matched observational studies. *Eur. J. Pain (United Kingdom)*. doi:10.1002/ejp.1742.

Topp, K. S., and Boyd, B. S. (2006). Structure and biomechanics of peripheral nerves: Nerve responses to physical stresses and implications for physical

therapist practice. *Phys. Ther.* doi:10.1093/ptj/86.1.92.
Torres, J. R., Martos, I. C., Sánchez, I. T., Rubio, A. O., Pelegrina, A. D., and Valenza, M. C. (2015). Results of an Active Neurodynamic Mobilization Program in Patients with Fibromyalgia Syndrome: A Randomized Controlled Trial. *Arch. Phys. Med. Rehabil.* 96, 1771–8. doi:10.1016/j.apmr.2015.06.008.
Tos, P., Crosio, A., Pugliese, P., Adani, R., Toia, F., and Artiaco, S. (2015). Painful scar neuropathy: principles of diagnosis and treatment. *Plast. Aesthetic Res.* 2, 156. doi:10.4103/2347-9264.160878.
Tremlett, H., Bauer, K. C., Appel-Cresswell, S., Finlay, B. B., and Waubant, E. (2017). The gut microbiome in human neurological disease: A review. *Ann. Neurol.* doi:10.1002/ana.24901.
Truini, A. (2017). A Review of Neuropathic Pain: From Diagnostic Tests to Mechanisms. *Pain Ther.* doi:10.1007/s40122-017-0085-2.
Urban, L. M., and Macneil, B. J. (2015). Diagnostic accuracy of the slump test for identifying neuropathic pain in the lower limb. *J. Orthop. Sports Phys. Ther.* doi:10.2519/jospt.2015.5414.
Van Hecke, O., Austin, S. K., Khan, R. A., Smith, B. H., and Torrance, N. (2014). Neuropathic pain in the general population: A systematic review of epidemiological studies. *Pain*. doi:10.1016/j.pain.2013.11.013.
Vanti, C., Bonfiglioli, R., Calabrese, M., Marinelli, F., Guccione, A., Violante, F. S., et al. (2011). Upper limb neurodynamic test 1 and symptoms reproduction in carpal tunnel syndrome. A validity study. *Man. Ther.* 16, 258–263. doi:10.1016/j.math.2010.11.003.
Varallo, G., Scarpina, F., Giusti, E. M., Cattivelli, R., Guerrini Usubini, A., Capodaglio, P., et al. (2021). Does Kinesiophobia Mediate the Relationship between Pain Intensity and Disability in Individuals with Chronic Low-Back Pain and Obesity? *Brain Sci.* 11, 684. doi:10.3390/brainsci11060684.
Verwoerd, A. J. H., Mens, J., el Barzouhi, A., Peul, W. C., Koes, B. W., and Verhagen, A. P. (2016). A diagnostic study in patients with sciatica establishing the importance of localization of worsening of pain during coughing, sneezing and straining to assess nerve root compression on MRI. *Eur. Spine J.* 25, 1389–1392. doi:10.1007/s00586-016-4393-8.
Villafañe, J. H., Silva, G. B., Bishop, M. D., and Fernandez-Carnero, J. (2012). Radial nerve mobilization decreases pain sensitivity and improves motor performance in patients with thumb carpometacarpal osteoarthritis: A randomized controlled trial. *Arch. Phys. Med. Rehabil.* 93, 396–403. doi:10.1016/j.apmr.2011.08.045.
Vos, T., Allen, C., Arora, M., Amare, A. T., Lalloo, R., Melaku, Y. A., et al. (2016). Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* 388, 1545–602. doi:10.1016/s0140-6736(15)60692-4.global.
Wang, J., La, J. H., and Hamill, O. P. (2019a). PIEZO1 is selectively expressed in small diameter mouse DRG neurons distinct from neurons strongly expressing TRPV1. *Front. Mol. Neurosci.* doi:10.3389/fnmol.2019.00178.
Wang, M. L., Rivlin, M., Graham, J. G., and Beredjiklian, P. K. (2019b). Peripheral nerve injury, scarring, and recovery. *Connect. Tissue Res.*

doi:10.1080/03008207.2018.1489381.
Wang, Q., Lin, J., Yang, P., Liang, Y., Lu, D., Wang, K., et al. (2020a). Effect of massage on the TLR4 signalling pathway in rats with neuropathic pain. *Pain Res. Manag.* doi:10.1155/2020/8309745.
Wang, Q. S., Luo, X. Y., Fu, H., Luo, Q., Wang, M. Q., and Zou, D. Y. (2020b). MiR-139 protects against oxygen-glucose deprivation/reoxygenation (OGD/R)-induced nerve injury through targeting c-Jun to inhibit NLRP3 inflammasome activation. *J. Stroke Cerebrovasc. Dis.* doi:10.1016/j.jstrokecerebrovasdis.2020.105037.
Wang, Y., Ma, M., Tang, Q., Zhu, L., Koleini, M., and Zou, D. (2015). The effects of different tensile parameters for the neurodynamic mobilization technique on tricipital muscle wet weight and MuRf-1 expression in rabbits with sciatic nerve injury. *J. Neuroeng. Rehabil.* 12, 1–7. doi:10.1186/s12984-015-0034-4.
Wasan, A. D., Loggia, M. L., Chen, L. Q., Napadow, V., Kong, J., and Gollub, R. L. (2011). Neural correlates of chronic low back pain measured by arterial spin labeling. *Anesthesiology* 115, 364–374. doi:10.1097/ALN.0b013e318220e880.
Williams, S. M., Eleftheriadou, A., Alam, U., Cuthbertson, D. J., and Wilding, J. P. H. (2019). Cardiac Autonomic Neuropathy in Obesity, the Metabolic Syndrome and Prediabetes: A Narrative Review. *Diabetes Ther.* 10, 1995–2021. doi:10.1007/s13300-019-00693-0.
Wolny, T., and Linek, P. (2018). Neurodynamic Techniques Versus "Sham" Therapy in the Treatment of Carpal Tunnel Syndrome: A Randomized Placebo-Controlled Trial. *Arch. Phys. Med. Rehabil.* 99, 843–854. doi:10.1016/j.apmr.2017.12.005.
Wolny, T., and Linek, P. (2019). Is manual therapy based on neurodynamic techniques effective in the treatment of carpal tunnel syndrome? A randomized controlled trial. *Clin. Rehabil.* 33, 408–417. doi:10.1177/0269215518805213.
Wolny, T., Saulicz, E., Linek, P., Myśliwiec, A., and Saulicz, M. (2016). Effect of manual therapy and neurodynamic techniques vs ultrasound and laser on 2PD in patients with CTS: A randomized controlled trial. *J. Hand Ther.* doi:10.1016/j.jht.2016.03.006.
Wolny, T., Saulicz, E., Linek, P., Shacklock, M., and Myśliwiec, A. (2017). Efficacy of Manual Therapy Including Neurodynamic Techniques for the Treatment of Carpal Tunnel Syndrome: A Randomized Controlled Trial. *J. Manipulative Physiol. Ther.* 40, 263–272. doi:10.1016/j.jmpt.2017.02.004.
Wong, A. Y., Karppinen, J., and Samartzis, D. (2017). Low back pain in older adults: risk factors, management options and future directions. *Scoliosis Spinal Disord.* 12, 14. doi:10.1186/s13013-017-0121-3.
Woolf, C. J., and Salter, M. W. (2000). Neuronal plasticity: Increasing the gain in pain. *Science (80-.).* doi:10.1126/science.288.5472.1765.
Xu, N., Wu, M. Z., Deng, X. T., Ma, P. C., Li, Z. H., Liang, L., et al. (2016). Inhibition of YAP/TAZ activity in spinal cord suppresses neuropathic pain. *J. Neurosci.* doi:10.1523/JNEUROSCI.0800-16.2016.
Yamada, Y., Nishiura, Y., Saijilafu, Hara, Y., Ichimura, H., Yoshii, Y., et al. (2009). Repair of peripheral nerve defect by direct gradual lengthening of

the distal nerve stump in rats: Effect on nerve regeneration. *Scand. J. Plast. Reconstr. Surg. Hand Surg.* 43, 305–311. doi:10.1080/02844310903052602.

Yamin, F., Musharraf, H., Rehman, A. U., and Aziz, S. (2016). Efficacy of Sciatic Nerve Mobilization in Lumbar Radiculopathy due to Prolapsed Intervertebral Disc. *Indian J. Physiother. Occup. Ther. - An Int. J.* doi:10.5958/0973-5674.2016.00009.5.

Yang, H., Wu, L., Deng, H., Chen, Y., Zhou, H., Liu, M., et al. Anti-inflammatory protein TSG-6 secreted by bone marrow mesenchymal stem cells attenuates neuropathic pain by inhibiting the TLR2/MyD88/NF-κB signaling pathway in spinal microglia. doi:10.1186/s12974-020-1731-x.

Yeh, T. Y., Luo, I. W., Hsieh, Y. L., Tseng, T. J., Chiang, H., and Hsieh, S. T. (2020). Peripheral Neuropathic Pain: From Experimental Models to Potential Therapeutic Targets in Dorsal Root Ganglion Neurons. *Cells*. doi:10.3390/cells9122725.

Yun, Y.-H., Lee, B.-K., Yi, J.-H., and Seo, D.-K. (2020). Effect of nerve mobilization with intermittent cervical segment traction on pain, range of motion, endurance, and disability of cervical radiculopathy. *Phys. Ther. Rehabil. Sci.* 9, 149–154. doi:10.14474/ptrs.2020.9.3.149.

Zaltsman, Y., Masuko, S., Bensen, J. J., and Kiessling, L. L. (2019). Angiomotin Regulates YAP Localization during Neural Differentiation of Human Pluripotent Stem Cells. *Stem Cell Reports*. doi:10.1016/j.stemcr.2019.03.009.

Zandstra, T. E., Notenboom, R. G. E., Wink, J., Kiès, P., Vliegen, H. W., Egorova, A. D., et al. (2021). Asymmetry and Heterogeneity: Part and Parcel in Cardiac Autonomic Innervation and Function. *Front. Physiol.* 12. doi:10.3389/fphys.2021.665298.

Zeng, W. Z., Marshall, K. L., Min, S., Daou, I., Chapleau, M. W., Abboud, F. M., et al. (2018). PIEZOs mediate neuronal sensing of blood pressure and the baroreceptor reflex. *Science (80-.).* 362, 464–467. doi:10.1126/science.aau6324.

Zhang, M., Wang, Y., Geng, J., Zhou, S., and Xiao, B. (2019). Mechanically Activated Piezo Channels Mediate Touch and Suppress Acute Mechanical Pain Response in Mice. *Cell Rep.* doi:10.1016/j.celrep.2019.01.056.

Zhao, Q., Wu, K., Geng, J., Chi, S., Wang, Y., Zhi, P., et al. (2016). Ion Permeation and Mechanotransduction Mechanisms of Mechanosensitive Piezo Channels. *Neuron*. doi:10.1016/j.neuron.2016.01.046.

Zhu, G.-C. C., Tsai, K.-L. L., Chen, Y.-W. W., and Hung, C.-H. H. (2018). Neural mobilization attenuates mechanical allodynia and decreases proinflammatory cytokine concentrations in rats with painful diabetic neuropathy. *Phys. Ther.* 98, 214–222. doi:10.1093/ptj/pzx124.

Zhuang, Z. Y., Wen, Y. R., Zhang, D. R., Borsello, T., Bonny, C., Strichartz, G. R., et al. (2006). A peptide c-Jun N-terminal kinase (JNK) inhibitor blocks mechanical allodynia after spinal nerve ligation: Respective roles of JNK activation in primary sensory neurons and spinal astrocytes for neuropathic pain development and maintenance. *J. Neurosci.* doi:10.1523/JNEUROSCI.5290-05.2006.