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- To say it in Taylor's words: I cried a lot, but I was so productive! -

Abstract

Chronic pain is a major global health problem with high prevalence and numerous comorbidities. Neuropathic pain, a subtype of chronic pain, affects approximately 7-10% of the population worldwide. Yet, effective treatment options remain elusive, as the mechanisms underlying neuropathic pain are poorly understood.

Using proteomic approaches, this thesis studied neuropathic pain in mice, induced by the spared nerve injury (SNI) model, to comprehensively deepen the molecular understanding of this maladaptive disease.

The first study of this thesis investigated the impact of sex and age on neuropathic pain, two important variables, whose impact are still not fully understood. This is partly due to the historical bias of male-focused studies and the usage of wide age ranges in preclinical studies. Proteomic analysis of dorsal root ganglia (DRG), which harbor peripheral sensory neurons and diverse non-neuronal cell types involved in pain, revealed sex- and age-dependent proteome alterations post nerve injury. Cross-referencing this data to human DRG data, showed evolutionary conserved proteins, allowing us to explore their potential as drug targets, with the aim to benefit male and female pain patients of different age groups.

The second study in this thesis investigated changes in the gut microbiome, a topic with increasing recognition in the field of pain. Using metaproteomics, which has the advantage of elucidating taxonomic and functional activity of the microbiome, as well as its impact on the host, revealed changes in the bacterial composition and metabolic activities linked to neuropathic pain. Furthermore, proteome changes indicate the interaction between microbes and the host's immune system during neuropathic pain.

In summary, this research uncovers comprehensive insights into the molecular changes associated with nerve injury, highlighting potential new drug targets and the gut's involvement in neuropathic pain modulation. These findings pave the way for developing more effective treatments for neuropathic pain.

Zusammenfassung

Chronische Schmerzen sind ein globales Gesundheitsproblem, mit einer hohen Prävalenz und zahlreichen Begleiterscheinungen. Eine Unterform chronischer Schmerzen sind neuropathische Schmerzen, die weltweit etwa 7-10% der Bevölkerung betreffen. Derzeitige Behandlungsmöglichkeiten sind jedoch größtenteils ineffizient und der Bedarf an neuen Therapieoptionen hoch. Um diese zu ermöglichen, müssen molekulare Mechanismen, die neuropathischen Schmerzen zugrunde liegen, verstanden werden.

Im Rahmen dieser Arbeit wurden neuropathische Schmerzen in einem Mausmodell, ausgelöst durch Nervenverletzung, untersucht. Dabei wurden Proteom-basierte Ansätze verwendet, um das molekulare Verständnis dieser Erkrankung zu vertiefen.

Die erste Studie dieser Arbeit untersuchte den Einfluss von Geschlecht und Alter auf neuropathische Schmerzen, da deren Auswirkungen noch immer nicht vollständig verstanden werden. Dies liegt zum Teil daran, dass präklinische Studien mehrheitlich am männlichen Geschlecht, sowie in einer breiten Altersspanne, durchgeführt worden sind. Proteomanalysen von Dorsalwurzelganglien, die periphere sensorische Neuronen und verschiedene nicht-neuronale Zelltypen beherbergen, ergab geschlechts- und altersabhängige Veränderungen des Proteoms nach Nervenverletzung. Durch den Vergleich mit menschlichen Daten, konnten evolutionär konservierte Proteine entdeckt werden, die potentielle Angriffspunkte für Medikamente sein könnten, hilfreich für männliche und weibliche Schmerzpatienten verschiedener Altersgruppen.

Die zweite Studie im Rahmen dieser Arbeit untersuchte Veränderungen im Mikrobiom des Darms, ein Thema, dass in der Schmerzforschung zunehmend an Bedeutung gewinnt. Mittels Metaproteomik können Mikroorganismen identifiziert und dessen Funktion, sowie Auswirkungen auf den Wirt, aufgeklärt werden. Dabei konnten Veränderungen in der bakteriellen Zusammensetzung und den Stoffwechselaktivitäten im Zusammenhang mit neuropathischen Schmerzen festgestellt werden. Darüber hinaus weisen Veränderungen des Proteoms auf die Interaktion zwischen Mikroben und dem Immunsystem des Wirts während neuropathischer Schmerzen hin.

Durch diese Studien konnten umfangreiche Einblicke in die molekularen Veränderungen neuropathischer Schmerzen gewonnen werden. Es wurden potentielle neue Ansätze für Medikamente, sowie die Beteiligung des Darms an der Modulation neuropathischer Schmerzen aufgezeigt. Diese Erkenntnisse ebnen den Weg für die Entwicklung wirksamer Behandlungen neuropathischer Schmerzen.

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Chapter 1 - Introduction

1.1 Pain definition

Worldwide, the most common symptom for which patients seek medical care, is pain [1-3]. It is a universal, yet very personal experience, defined by the International Association for the Study of Pain (IASP) as “an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage” [4].

Nociception describes the process where nociceptors, i.e., peripheral nerve fibers, detect potentially harmful stimuli such as temperature, pressure, or chemical signals [5]. These nociceptors are excited when the stimulus intensifies to the noxious range [6], which can be objectively measured as neuronal activation [7, 8]. Although nociception may induce pain sensation, these two terms should not be used synonymously [9]. Pain is a multidimensional, subjective experience and not a linear representation of nociceptor activation [8, 9]. It is modulated by the biological and social environment, as well as personal experiences, and is therefore, not objectively measurable [4].

Pain can be broadly divided into two subtypes: acute and chronic pain. Acute pain is an important evolutionary adaptive response, necessary to detect and limit the exposure to damaging or noxious stimuli [6, 10]. The importance of this mechanism is shown in people with a rare genetic disease called congenital insensitivity to pain. These patients are unable to sense nociceptive stimuli or tissue damage, leading to a higher incidence of injuries and therefore also increased mortality rates in early life [11, 12]. In contrast to this protective function of acute pain, stands chronic pain. This maladaptive type of pain persists beyond the perceived threat or healing process [6, 10], and has only recently been recognized as a disease itself [13, 14]. According to the IASP, chronic pain, which has a global prevalence of approximately 20% [15], is defined as pain that lasts or recurs for more than three months [16]. While the origin can often be acute pain, e.g., from an injury, it becomes pathological when alterations of the pain pathway exceed the utility of the protective warning system or the healing process and the physiological pain sensitivity is exacerbated [6, 10, 17].

Another way of categorizing pain is by its underlying mechanisms. Pain can be either nociceptive or pathological. Nociceptive pain arises from tissue damage or

inflammation, whereby nociceptors are activated in response to harmful stimuli [10]. Certain diseases, such as osteoarthritis, can generate persistent noxious stimuli, leading to a chronification of nociceptive pain [18]. Inflammatory pain, a subtype of nociceptive pain, results from immune system activation. It is characterized by increased sensory sensitivity following actual tissue damage. This prevents physical contact and movement of the injured area and thereby, supports the healing process, reducing the risk of further damage, and serving a protective role. Nevertheless, in the case of chronic inflammation, such as rheumatoid arthritis, a reduction of this ongoing inflammation is needed [10, 19]. In contrast, pathological pain serves no (currently known) protective role. It arises from abnormal functioning of the somatosensory system, either with underlying damage of the nervous system, as is the case in neuropathic pain, or without an identifiable trigger, referred to as dysfunctional pain [10]. Hereby, a specific form is nociplastic pain. This type of pain is characterized by altered nociception, without clear indicators of actual or threatened tissue damage, nerve injury or a disease of the somatosensory system [20, 21].

1.2 Pain sensation

The brain's interaction with the external and internal environment relies on sensory input, provided by sensory receptors. Somatic sensation can be broadly categorized based on function into four main types: touch sensation, mediated by mechanoreceptors; temperature sensation, mediated by thermal receptors; proprioception, which is the sensation of movement and limb position mediated by specialized mechanoreceptors; and nociception, mediated by nociceptors [22]. Nociceptors are peripheral free nerve endings from primary sensory neurons that detect potentially harmful stimuli, such as mechanical forces, harmful temperatures or chemical substances [5, 22, 23]. In order to perform this function, specialized ion channels are necessary, to detect and transduce noxious stimuli. In particular, transient receptor potential (TRP) channels appear to play a key role in the sensation of pain. For example, noxious heat and capsaicin activate TRPV1 (vanilloid 1), while cold stimuli and menthol are detected by TRPM8 (melastatin 8), and TRPA1 (ankyrin 1) responds to reactive chemical irritants, such as allyl isothiocyanate (AITC), which is the active compound in wasabi and mustard oil, cinnamaldehyde from cinnamon, or allicin from garlic [24, 25] .

The soma of primary sensory neurons are located in dorsal root ganglia (DRG) or, for cephalic sensory innervation (i.e., head and face), in the trigeminal ganglia (TG) [26, 27]. These neurons have a pseudo-unipolar structure, with one axon branching into peripheral tissues and organs, and a central axon terminating in the superficial layers (laminae I and II) of the dorsal horn of the spinal cord [6], or, for TG, in the trigeminal nucleus caudalis, a region of the dorsal brainstem [28, 29]. As shown in Figure 1, when nociceptors detect potentially harmful stimuli the information is converted into electrical signals [6, 30] and transmitted from the periphery, via these primary neurons, to the spinal cord, where they synapse with second-order neurons in the dorsal horn [6, 31]. Through ascending (spinothalamic and spinoreticulothalamic) pathways, the signal is transmitted to the thalamus, where it is projected via third-order neurons to the somatosensory cortex. In the brain, the perception of pain arises through the integration of sensory, emotional and cognitive components [6, 31-33]. Furthermore, descending pathways, from higher brain centers to the spinal level, can modulate the nociceptive processing in both inhibitory and facilitatory ways, changing the transmission of nociceptive signaling to the brain. For example, neurons originating in the brainstem can release inhibitory neurotransmitters, such as norepinephrine, 5-hydroxytryptamine (i.e., serotonin), and endogenous opioids, which then activate inhibitory interneurons in the dorsal horn, leading to a reduction of nociceptive signals and thereby decrease of pain perception [18, 34]. However, in certain behavioral, emotional and pathological conditions, such as peripheral injury, this system can shift towards facilitation, for example via the activation of glial cells in the brainstem, and thereby enhance pain signaling [35-37].

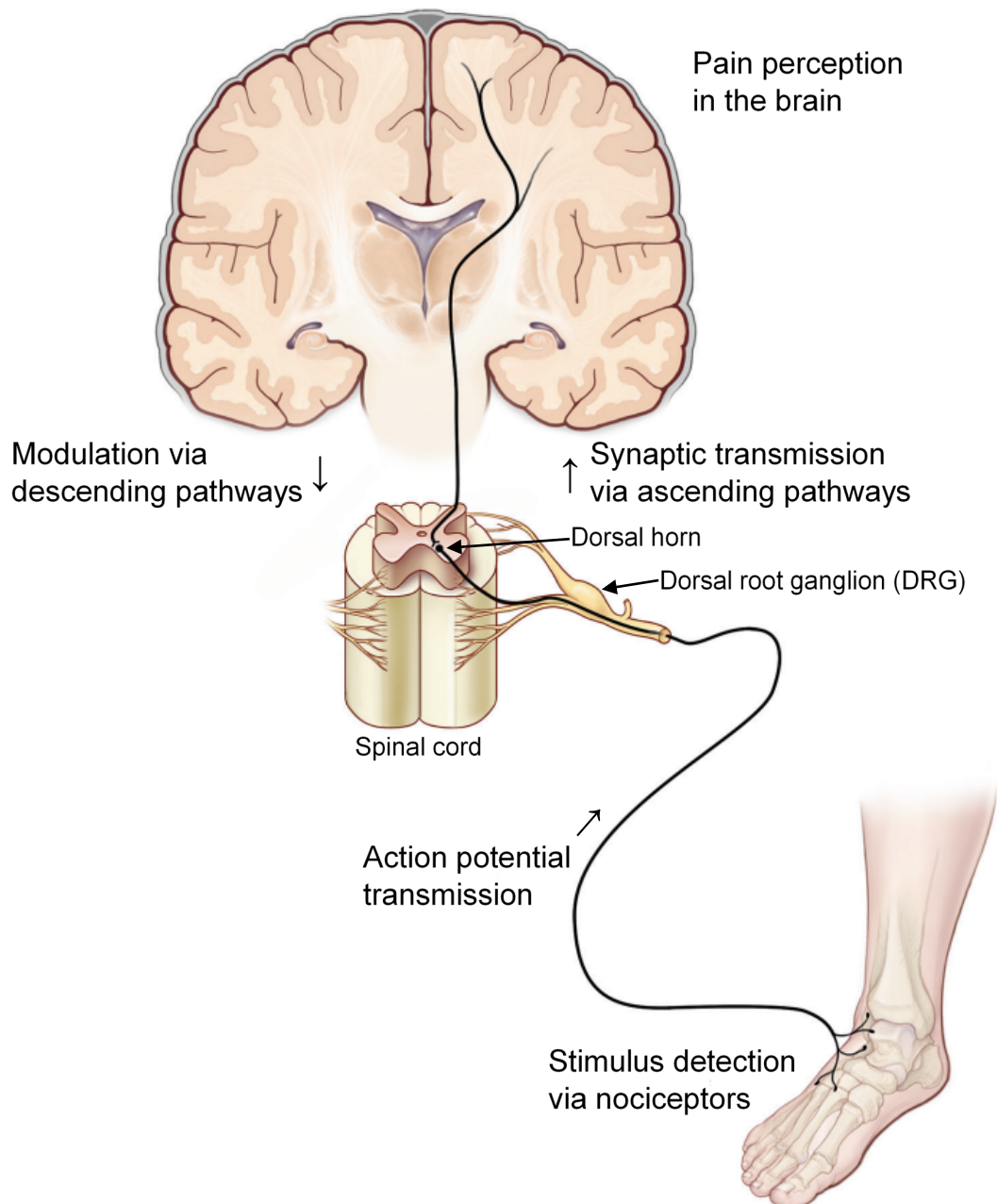


Figure 1. Schematic view of the nociceptive pathway adapted from Fitzcharles *et al.*, 2021

Nociceptors are peripheral free nerve endings of primary sensory neurons, responsible for detecting potentially harmful stimuli. The soma of these neurons lies in the dorsal root ganglia (DRG). When a noxious stimulus is detected, an action potential is generated and transmitted via primary sensory neurons to second-order neurons in the dorsal horn of the spinal cord. From there, nociceptive signals ascend to third-order neurons of the brain, where pain perception is processed. Modulation of this nociceptive information occurs via descending pathways, originating from higher brain centers and projecting to the spinal level. Figure adapted from Fitzcharles *et al.*, 2021 [21].

Sensory neurons show a remarkably high heterogeneity, and are therefore classified into different subtypes, based on their transcriptional patterns, functional annotations, and molecular markers. In murine DRGs, 10 major populations of sensory neurons have been identified [38], whereas in human DRGs, transcriptomic analysis revealed 12 distinct subtypes [39].

Mechanoreceptors are traditionally categorized into A β , A δ and C fibers, based on their conduction velocity, axon diameter, and myelination (Figure 2). A β fibers typically belong to the class of low-threshold mechanoreceptors (LTMRs). These myelinated afferents, terminate into spinal lamina III-V, have a large diameter and a rapid conduction velocity, responding to innocuous mechanical stimulation, e.g., light touch [6, 40]. However, under neuropathic pain conditions, they can start to produce pain [41, 42]. Additionally, A β fibers, functioning primarily as nociceptors, have been identified [39, 43]. In contrast, A δ and C fibers predominantly belong to the class of high-threshold mechanoreceptors (HTMRs), and are primarily associated with nociceptive signaling, terminating into spinal laminae I-II. A δ fibers are myelinated and have a medium diameter. They conduct at a rapid velocity, responsible for “primary pain”, mainly of a mechanical or thermal nature [6, 27, 40]. In contrast, C fibers are unmyelinated and small in diameter, resulting in a low conduction velocity, transmitting “secondary pain”. These C fibers make up the majority of nociceptors [6, 27]. Both classes of nociceptors can further be subdivided. A δ can be characterized based on their functional specification into type I and type II. While type I nociceptors respond to mechanical and chemical stimuli, they have a relatively high threshold for heat (>50°C). Only under persistent heat stimulation, a response at lower temperatures is given. Furthermore, after tissue injury, these nociceptors will sensitize, with a drop in heat and mechanical threshold. In contrast, type II nociceptors are responsible for sensing heat stimuli, but have a high threshold for mechanical stimuli [6]. In mice, C fibers have been traditionally subdivided based on their neuropeptide expression, into peptidergic (PEP) and non-peptidergic (NP) nociceptors. PEP express the neurotrophic receptor tropomyosin receptor kinase A (TrkA). They depend on nerve growth factor (NGF), and express substance P and calcitonin gene-related peptide (CGRP) [44, 45], encoded by the gene calcitonin-related polypeptide alpha (CALCA) [39]. These nociceptors innervate the epidermis’ basal layer and internal organs, terminating in lamina I of the dorsal spinal cord and are mainly responsible for detecting heat [44, 46]. NP express the tyrosine kinase c-Ret. These nociceptors depend on glial cell line-derived

neurotrophic factor (GDNF) [45]. They perceive mechanical stimuli from superficial layers of the epidermis, terminating in lamina II of the dorsal spinal horn [46, 47]. Furthermore, they express cell surface glycol conjugates, recognized by isolectin B4 (IB4) [45]. A subgroup of these cells, which are IB4-negative, corresponding to C-LTMRs, express the vesicular glutamate transporter VGlut3 and tyrosine hydroxylase (TH) [47, 48]. However, transcriptome analysis showed that NP nociceptors can express the neuropeptides substance P and CGRP, making the distinction into PEP and NP less accurate [38]. Furthermore, this distinction into PEP and NP does not hold for human nociceptors, where nociceptors also share expression of these neuropeptides [49-52].

NOCICEPTIVE NEURONS			
A-beta-fibers	Type I A-delta-fibers	Type II A-delta-fibers	C-fibers
			
Light touch Pressure Vibration	Gross touch Fast pain High heat (>53°C) Capsaicin-insensitive	Gross touch Fast pain Low heat (>43°C) Capsaicin-sensitive	Slow pain Low pH Heat Capsaicin-sensitive
>22 μm	12-22 μm	12-22 μm	< 12 μm

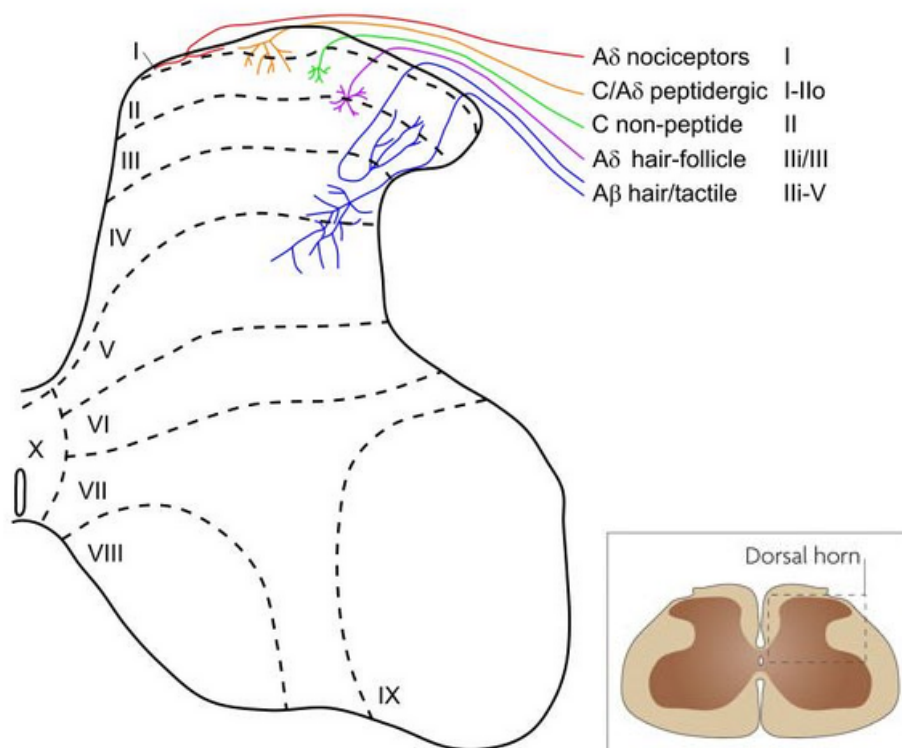


Figure 2. Schematic representation of mechanoreceptors and location in the spinal dorsal horn adapted from Tender *et al.*, 2008 and Todd *et al.*, 2010

Mechanoreceptors can be categorized based on their conduction velocity, axon diameter, and myelination. The upper panel shows the differentiation of mechanoreceptors into A β fibers, A δ (type I and type II) fibers, and C fibers. Myelinated A β fibers have a large diameter and are responsible for sensing innocuous mechanical stimuli, such as light touch, pressure or vibration. A δ and C fibers are associated with nociceptive signaling. While myelinated A δ fibers are of medium diameter and responsible for primary, fast pain, unmyelinated C fibers are of smaller diameter and responsible for secondary, slow pain. The lower panel shows termination of those fibers within the dorsal horn of the spinal cord. A δ nociceptors (red) terminate into laminae I, while A δ hair-follicle afferents (not nociceptive; pink) end in lamina II-III. C fibers (peptidergic; orange and nonpeptidergic; green) terminate in laminae I-II. A β fibers (blue) end in laminae III-V, with some extension into lamina II. Figures adapted from Tender *et al.*, 2008 (upper panel) and Todd *et al.*, 2010 (lower panel) [53, 54].

Despite this, rodents are an important and necessary model organism to study pain [52]. On the RNA level, the landscape of DRG in rodents and humans has been studied extensively. Several studies comparing mice/rats with humans have shown already the strong conservation of DRG-enriched genes between those species, making mice/rats a reliable model for studying human DRGs [55, 56]. Since preclinical models can suffer from their translatability to the clinics [57], studies investigating molecular similarities and differences between human and rodents is of utmost importance [52]. Thereby, evolutionary conserved regulatory programs, which potentially serve as new therapeutic options, can be identified [55].

1.3 Neuropathic pain

1.3.1 Definition and prevalence

Neuropathic pain is defined as “pain caused by a lesion or disease of the somatosensory system” [58]. This type of pain is a form of chronic pain, either persisting continuously or manifesting with recurring painful episodes [59], and is always maladaptive [60].

Neuropathic pain can be divided into peripheral and central neuropathic pain, with many subtypes [59]. Peripheral neuropathic pain is caused by lesions of the peripheral nervous system (PNS), leading to pathophysiological changes of the PNS and/or the central nervous system (CNS). The cause of these lesions can be, for example, mechanical trauma, metabolic diseases, infection, neurotoxic chemicals, or

tumor invasion [61, 62]. Subclasses of chronic peripheral neuropathic pain are classified by the IASP as trigeminal neuralgia, chronic neuropathic pain after peripheral nerve injury, painful polyneuropathy, postherpetic neuralgia, painful radiculopathy, as well as, a subclass of other specified, and unspecified chronic peripheral neuropathic pain types. The chronic central neuropathic pain subtypes include chronic central neuropathic pain associated with spinal cord injury, chronic central neuropathic pain associated with brain injury, chronic central post-stroke pain, chronic central neuropathic pain caused by multiple sclerosis and again, a subclass of other specified, and unspecified chronic central neuropathic pain types [59]. The degree to which pain is associated with a specific form of neuropathic pain varies greatly and can be the single or predominant manifestation of the disease, e.g., in postherpetic neuralgia, or it can occur only in a subset of patients affected by the same disease, such as in chemotherapy-induced peripheral neuropathies. Furthermore, pain symptoms often differ, even among those patients affected by the same disease [63].

Worldwide, the prevalence for neuropathic pain is estimated to be between 7% and 10% [64-66], although defining precise numbers is difficult due to a presumably high number of undiagnosed cases [67]. In general, the incidence of neuropathic pain is higher in women than men, and increases with age [65, 68-70], which is addressed in more detail in section 1.4 and 1.5.

The studies presented in chapter 2 and 3 address neuropathic pain after peripheral nerve injury. This type of pain is the result of a peripheral nerve lesion.

1.3.2 Symptoms

Neuropathic pain patients can experience a decrease or increase of sensory symptoms [59, 71, 72]. Hypoalgesia describes the decreased sensitivity to a nociceptive stimulus [73]. This deficit or complete loss of somatosensory function [59, 71] can occur due to the lesions of the nervous system, peripherally or centrally, in the innervated or affected area [74]. Painful symptoms can be categorized into evoked (stimulus induced) and non-evoked pain (spontaneous pain, i.e., without a stimulus). As shown in Figure 3, evoked pain can be either an exaggerated pain response to a noxious stimulus, referred to as hyperalgesia, or a pain response to a normally innocuous stimulus, termed allodynia [59]. The trigger of evoked pain may be of a mechanical or thermal nature, whereby in the case of thermal stimuli, patients of neuropathic pain seem to be more sensitive to cold [71, 74]. It is suggested that

retention or loss of afferent fibers can determine if evoked pain occurs [74]. However, the majority of neuropathic pain patients suffer from spontaneous pain symptoms, which manifest themselves as burning, shooting, tingling, or pricking [75, 76]. This type of pain is difficult to investigate with current animal pain models, which mainly assess stimulus-evoked pain [77-81].

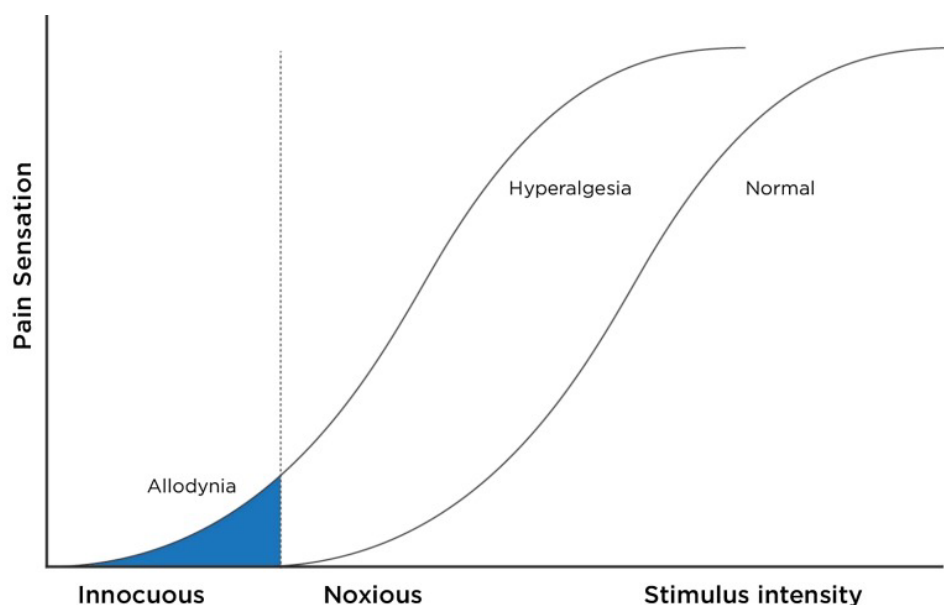


Figure 3. Schematic view of altered pain sensation after injury from Louw *et al.*, 2017

Pain sensation changes after injury. The curve on the right shows the relationship of stimulus intensity and magnitude of pain sensation under healthy conditions. Pain sensation is evoked when the stimulus intensifies to the noxious range, which is indicated as vertical dotted line. After injury, there is a shift, as indicated by the curve on the left. Under these conditions, the pain sensation to noxious stimuli is amplified, which is referred to as hyperalgesia, and innocuous stimuli can already evoke pain, referred to as allodynia (blue). Figure from Louw *et al.*, 2017 [82].

Importantly, neuropathic pain patients suffer not only from the symptoms of the disease itself, but also from numerous comorbidities, such as sleep deprivation, anxiety, depression, inability to work, disturbed social relationships, and in general, a reduced quality of life [83-86]. This leads to a vicious circle, as stress, dissatisfaction, depression, etc. can predetermine the development of chronic pain after acute episodes [87, 88]. Furthermore, the context in which painful stimuli are experienced can impact its mode of perception [60], and cognitive and emotional factors influence the patient's reaction to (chronic) pain [89].

1.3.3 Neuropathic pain mechanisms

The nervous system, both peripherally and centrally, reacts to neural damage with a combination of compensatory and decompensatory mechanisms. An imbalance of these processes is a major contributor to the development of neuropathic pain after peripheral nerve injury [18].

A cascade of alterations in the injured nerve and surrounding tissue are induced, one of which is peripheral sensitization (Figure 4). This phenomenon refers to a reduction in the activation threshold and an increase in the sensitivity of peripheral nociceptors. This typically occurs in inflamed or injured tissues, contributing to the heightened pain perception, which is often observed in neuropathic pain patients. After nerve injury, nociceptors and non-neuronal cells release a variety of chemical mediators, including cytokines (e.g., interleukin (IL)-6, and tumor necrosis factor (TNF)- α), neuropeptides (e.g., CGRP and substance P), growth factors (e.g., NGF), prostaglandins, serotonin and other signaling molecules [30, 90-92]. The release of these inflammatory mediators activates various receptors and ion channels, which reduces the threshold for transduction and increases membrane excitability [18].

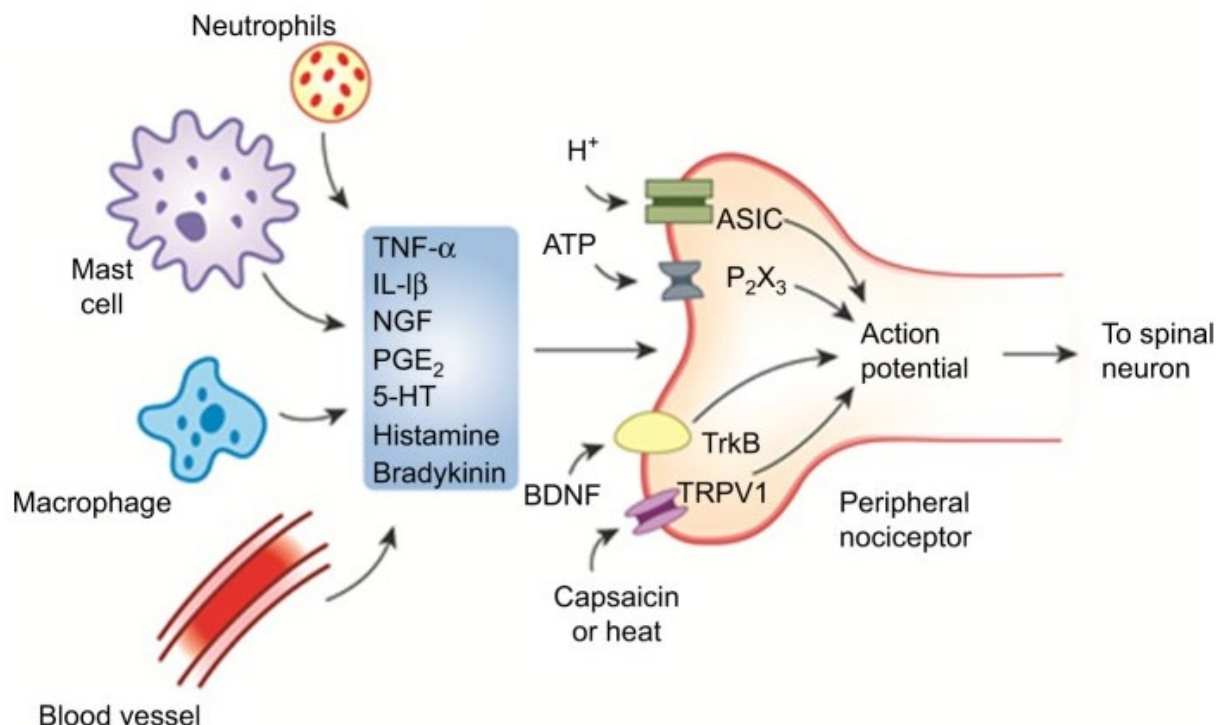


Figure 4. Schematic representation of peripheral sensitization adapted from Dureja *et al.*, 2017

Peripheral sensitization occurs after injury and describes the increased sensitivity of peripheral nociceptors, which is mediated by the release of inflammatory and other chemical mediators, including

TNF- α , IL-1 β , NGF, PGE₂, serotonin, histamine and bradykinin. Thereby, ion channels and receptors are altered, leading to decreased threshold in nociceptor activation and increased generation of action potential, whereby enhanced signals are transmitted towards the spinal cord, to second order neurons. Abbreviations: tumor necrosis factor-alpha (TNF- α), interleukin-1beta (IL-1 β), nerve growth factor (NGF), prostaglandin E₂ (PGE₂), 5-hydroxytryptamine (5-HT, i.e., serotonin), hydrogen (H⁺), adenosine triphosphate (ATP), brain-derived neurotrophic factor (BDNF), acid-sensing ion channel (ASIC), purinergic receptor P2X 3 (P₂X₃), tyrosine receptor kinase B (TrkB), transient receptor potential vanilloid receptor (TRPV1). Figure adapted from Dureja *et al.*, 2017 [93].

Beyond peripheral changes, peripheral nerve injury can cause persistent changes in the CNS, i.e., brain and spinal cord (Figure 5). Central sensitization describes the increased reactivity of nociceptors and circuits in the CNS, due to enhanced excitability and synaptic efficacy of nociceptive pathways, as well as a decrease of inhibitory synaptic inputs at the level of the spinal cord and supra-spinal cord. Key mechanisms are proposed to include activation of N-methyl-D-aspartate (NMDA) receptors, recruiting of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and expression changes of the potassium-chloride transporter member 5 (KCC2) [94, 95]. These modifications might be induced by glutamate, CGRP, and substance P, released by primary neurons, or brain derived neurotrophic factor (BDNF), TNF- α , and IL-1 β , released by immune cells [96, 97]. Nonetheless, additional studies on central sensitization are required, as recent evidence also suggests sex-dependent mechanisms in both clinical and preclinical studies [98].

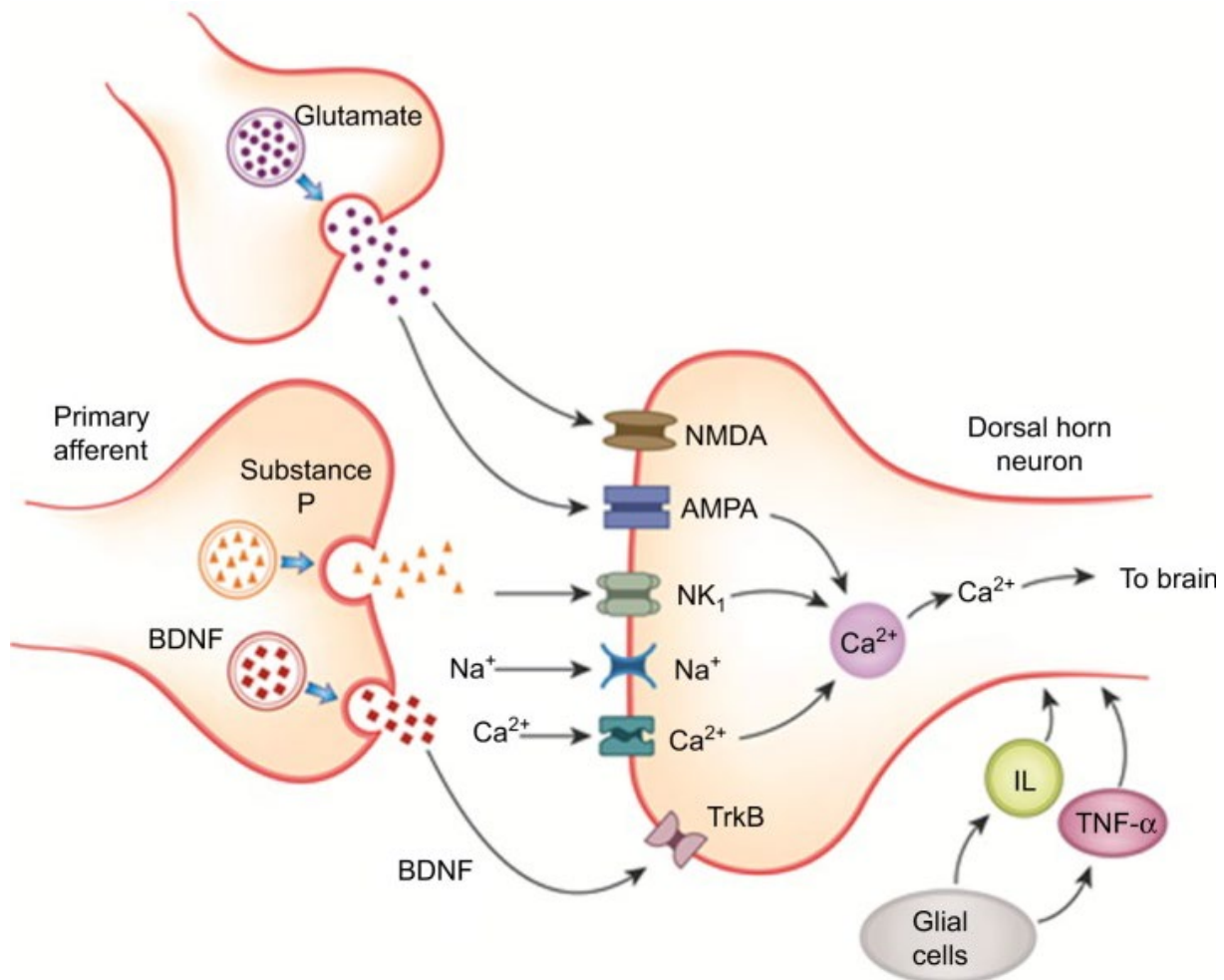


Figure 5. Schematic representation of central sensitization adapted from Dureja *et al.*, 2017

Central sensitization represents a process within the central nervous system (CNS), which develops in response to peripheral injury and persistent inflammation, leading to an increase in the excitability of nociceptive pathways. Thereby, neurotransmitters, such as glutamate, substance P and BDNF are released, leading to prolonged depolarization of postsynaptic neurons. The activation of receptors, including NMDA, AMPA and NK₁ lead to increased Ca²⁺ influx, further intensifying synaptic transmission within the dorsal horn of the spinal cord. Additionally, glial cells release inflammatory mediators, such as TNF-α and ILs, contributing to neuronal hyperexcitability and persistent pain. Abbreviations: brain-derived neurotrophic factor (BDNF), N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), neurokinin-1 (NK₁), sodium (Na⁺), calcium (Ca²⁺), tyrosine receptor kinase B (TrkB), interleukin (IL), tumor necrosis factor-alpha (TNF-α). Figure adapted from Dureja *et al.*, 2017 [93].

An additional contributor to pain processing and central sensitization is ectopic activity, a spontaneous activity generated from injured sensory neurons and their intact neighbors at locations other than the normal impulse generation site [99, 100]. This aberrant activity is transmitted to the spinal cord, causing alterations of central sensory

processing, i.e., central sensitization [99]. Due to changes in ion channel expression (e.g., Nav1.7, Nav1.8 and Nav1.9), it can lead to aberrant action potential generation and ectopic activity [101]. This altered activity is often observed in injured A-fibers. Under naïve conditions, C-fibers are responsible for the induction of central sensitization. However, under neuropathic pain conditions, A-fibers can become stimulated by activated immune cells in the DRG, leading to central sensitization by LTMRs [41, 42, 102]. Persistent ectopic activity can also lead to degeneration of spinal neurons [103], supporting the use of neuroprotective treatments as a strategies to treat neuropathic pain [18].

Furthermore, peripheral sprouting can occur in response to peripheral nerve injury. Due to an actively growing state of sensory neurons, damaged axons are reconnected with their targets [104]. However, this can cause changes in the spinal cord, with sprouting of the central axon terminals [105]. Studies suggest that A β fibers, which typically respond to innocuous touch, can grow into lamina I and II upon nerve damage [106], regions where A δ fibers and C-fibers normally terminate [5, 107]. This may allow innocuous stimuli to activate nociceptive circuits, potentially contributing to allodynia [105, 108-111].

Another key factor in modulating pain involves descending pathways, which can act in an inhibitory and facilitatory manner. Under certain conditions, such as nerve injury, descending pathways can shift from inhibition to facilitation, and thereby contributing to glial cell activation and central sensitization, leading to increased pain sensitivity [35-37, 112, 113].

Moreover, neuropathic pain is not only based on sensory neurons and their associated pathways. The nervous system is thought to communicate in a bidirectional way with the immune system, needed to maintain homeostasis, and adequate response after injury [114, 115]. Peripheral sensory neurons release neuropeptides, such as substance P and CGRP, directly activating nociceptors and increasing the infiltration of immune cells to injury sites [116-118].

Immune cells and glial cells, which are also located in DRG, are important players in the development of pain [119-121]. After peripheral nerve injury, inflammatory mediators of the PNS and CNS are activated. Within DRG, an increase of T-cells and macrophages can be observed, persisting even months after the initial injury. This ongoing inflammation might lead to chronic neuron stimulation and

therefore prolongation of pain. Another suspected cause of pain chronification is the dysregulation of glial cells, which normally support and protect primary neurons. However, after injury, they can lead to persistent inflammation by releasing inflammatory mediators and neurotrophic factors. The latter promotes glial cell proliferation, leading to the compression of neuronal cell bodies and further contributing to persistent inflammation [122].

Microglia, macrophage-like cells in the CNS, have been immensely investigated over the last years for their role in pain modulation and neuroinflammation. This cell type is known for its crosstalk with neurons, representing an example of neuroimmune communication [123]. After nerve injury, microglia are activated and release mediators, such as BDNF, TNF- α , IL-1 β , and IL-18, leading to altered neuronal activity and promoting neuropathic pain [96]. However, a profound sex-difference was observed in mechanical hypersensitivity in mice, where the underlying mechanisms are mediated by microglial signaling in males, whereas in females adaptive immune cells, i.e., T-cells, play a dominant role [124]. Nonetheless, different routes of microglia-dependent promotion of pain hypersensitivity have been identified in recent years. For example, the release of cytokines to amplify excitatory synaptic transmission [125] and the suppression of inhibitory nerve signaling via pro-inflammatory cytokines [125, 126]. Additional mechanisms include lowering the inhibitory threshold [95, 127], removal of inhibitory synapses through engulfment [128, 129], and impact of perineuronal nets of the extracellular matrix [130]. It is highly likely that some of these pathways are sex-specific, while others are shared across both sexes [68].

Beyond neuronal and immune-related mechanisms, genetic factors seem to play a role in determining susceptibility to neuropathic pain, either enhancing or protecting an individual from developing neuropathic pain [18].

Interestingly, it is suggested that evoked and spontaneous pain have different underlying mechanisms. Allodynia and hypersensitivity are assumed to result from peripheral sensitization [18]. In contrast, spontaneous pain is considered to be caused by ectopic activity in nociceptors [131]. Central sensitization may contribute to both, stimulus-evoked pain (e.g., allodynia and hyperalgesia) and spontaneous pain [105, 132]. In the case of spontaneous pain, a connection between afferent fiber types and pain symptoms is hypothesized. Tingling and pricking sensations are likely mediated by myelinated A δ fiber and potentially A β fibers [133], which transmit primary pain and

light touch, respectively [6, 40]. Burning sensations seem to be associated with unmyelinated C fibers [133] which play a key role in persistent pain [6, 40]. These potential differences highlight the complexity of pain mechanisms and the importance of targeting specific pathways for effective pain management.

Although the causative factor of neuropathic pain is damage of the somatosensory system, the associated symptoms usually persist beyond the resolution of the original cause [18]. In 30-60% of patients with damage of the sciatic nerve, the trauma results in persistent neuropathic pain [134, 135]. The reasons behind this chronification and why it only develops in some patients is still not clear [74]. Some studies suggest that the likelihood of chronification increases with the severity of acute postoperative pain. However, whether inadequate management of acute postoperative pain increases the risk for chronic pain, or people who suffer more from intense acute pain also have a higher susceptibility to develop chronic pain is currently still unknown. Furthermore, as mentioned previously, pain is a personal experience, and different factors, including emotional and cognitive aspects, influence pain sensation and pair up with the multifaceted molecular changes, leading to the complex nature of neuropathic pain [89].

1.3.4 Diagnosis

The diagnosis of neuropathic pain is complex, requiring the identification of the pains' initial cause and linking it to the corresponding pain profile [59]. Moreover, the presence of sensory disturbance symptoms increases the diagnosis certainty but does not always lead to a definite conclusion as patients' symptoms can vary greatly [59, 71].

Currently, the diagnosis of neuropathic pain is guided and based on different screening tools, using common verbal pain descriptors, i.e., the Leeds assessment of neuropathic symptoms and signs (LANSS) [136], *douleur neuropathique en 4 questions* (DN4) [137], PainDetect [138], neuropathic pain questionnaire (NPQ) [139], and ID-Pain [140]. Evidence for the cause of pain is examined using neurophysiological tests and imaging techniques, confirming the involvement of the somatosensory system [141]. Adjunctive diagnostic tests are often used as well, such as quantitative sensory testing (QST) in order to evaluate the patient's somatosensory phenotype [142-144]. Thereby, the response to mechanical, thermal and vibration stimuli are measured, quantifying somatosensory deficits, hyperalgesia subtypes and

allodynia [71]. Additionally, a grading system was invented, which should assist in classifying neuropathic pain [141, 145]. This grading system is based on four criteria, namely the patient's history, distribution of pain, sensory symptoms, and a diagnostic test, to confirm the lesion or disease of the somatosensory system. Following this grading system, patients are divided into the categories; unlikely, possible, probable and definite, to define neuropathic pain. However, this grading system has limitations. Firstly, it has no proper validation in terms of inter-rater reliability and test-retest. Secondly, many neuropathic pain patients do not consult a neurologist and lastly, this grading does not include patients suffering from mixed pain syndromes, such as postsurgical pain or cancer pain [71].

1.3.5 Treatment options

Treatment options for neuropathic pain can be split into pharmacological and non-pharmacological approaches, i.e., interventional, physical, and psychological therapy [146-148]. Unfortunately, current pharmacological treatment options are ineffective to relieve neuropathic pain symptoms, only work for a minority of patients, and/or have many adverse effects [60, 146, 149]. A reason for this could be the fact that molecular mechanisms underlying neuropathic pain are still not comprehensively understood [18] and precise targeting is still challenging [60]. The conventional approach for the treatment of neuropathic pain is based on the underlying disease [150]. However, neuropathic pain manifests as a maladaptive plasticity in the nervous system, whereby a cascade of changes, triggered by the initial nerve injury, lead to neuropathic pain. Treating the primary pathology is crucial, however, understanding the molecular mechanisms responsible for these maladaptive alterations is important and offers specific therapeutic opportunities [18].

The IASP's Neuropathic Pain Special Interest Group (NeuPSIG) provides pharmacological treatment options for neuropathic pain. For example, as first-line drugs, gabapentinoids (i.e., gabapentin and pregabalin), tricyclic antidepressants (TCA; e.g., amitriptyline), and serotonin-noradrenaline reuptake inhibitors (SNRI; i.e., duloxetine and venlafaxine) are recommended [59, 147, 148]. When these first-line treatments do not provide satisfactory results [147], subsequent treatment options, including weak opioids (e.g., tramadol and tapentadol) and strong opioids (e.g., morphine and oxycodone), are recommended [147, 148]. However, as opioids are

known to have severe side effects and addictive potential, their use has been restricted sharply [151].

Furthermore, over the last years, many non-pharmacological approaches have been tested in clinical trials. Interventional therapy, such as neurostimulation of the DRG, has shown promising results to alleviate neuropathic pain [152, 153, 154]. Physical therapy may be used additionally, when pharmacological treatments do not lead to satisfactory results. Psychological treatments are intended to improve pain intensity, level of suffering, and general mood. While this form of treatment is a good addition, it is usually not sufficient to alleviate severe pain [148].

1.3.6 Spared nerve injury (SNI) model

To study the pathophysiology of pain, rodents, especially mice and rats are commonly used [78]. A variety of rodent models have been developed to investigate neuropathic pain. These models are all based on damaging the nervous system (e.g., by trauma, chemicals or inflammation), to induce a pain response [155-157].

A widely employed model for inducing neuropathic pain through nerve trauma is the spared nerve injury (SNI) model. As shown in Figure 6, this model is based on the ligation of two terminal branches of the sciatic nerve, the common peroneal and tibial nerve, while sparing the sural nerve. Thereby, mechanical and cold allodynia are evoked on the ipsilateral side of the skin area of the sural nerve, and to a lesser extent also saphenous nerve [158]. An altered behavioral response can already be observed as early as 24h post nerve injury, and persists over time [158-160]. However, a sex-specific resolution of these symptoms was found only in female mice six to nine months after nerve injury [160]. Regardless, SNI is a well-accepted model to unravel the symptomatic and underlying mechanisms of neuropathic pain. The behavioral symptoms in mice mimic the evoked pain symptoms in neuropathic pain patients [158] and, compared to other models, such as the chronic constriction injury (CCI), where the injured nerve can regenerate, the long-lasting pain behavior produced by SNI via transection and partial removal of the nerve branches better reflects the clinical presentation of neuropathic pain patients [74, 161].

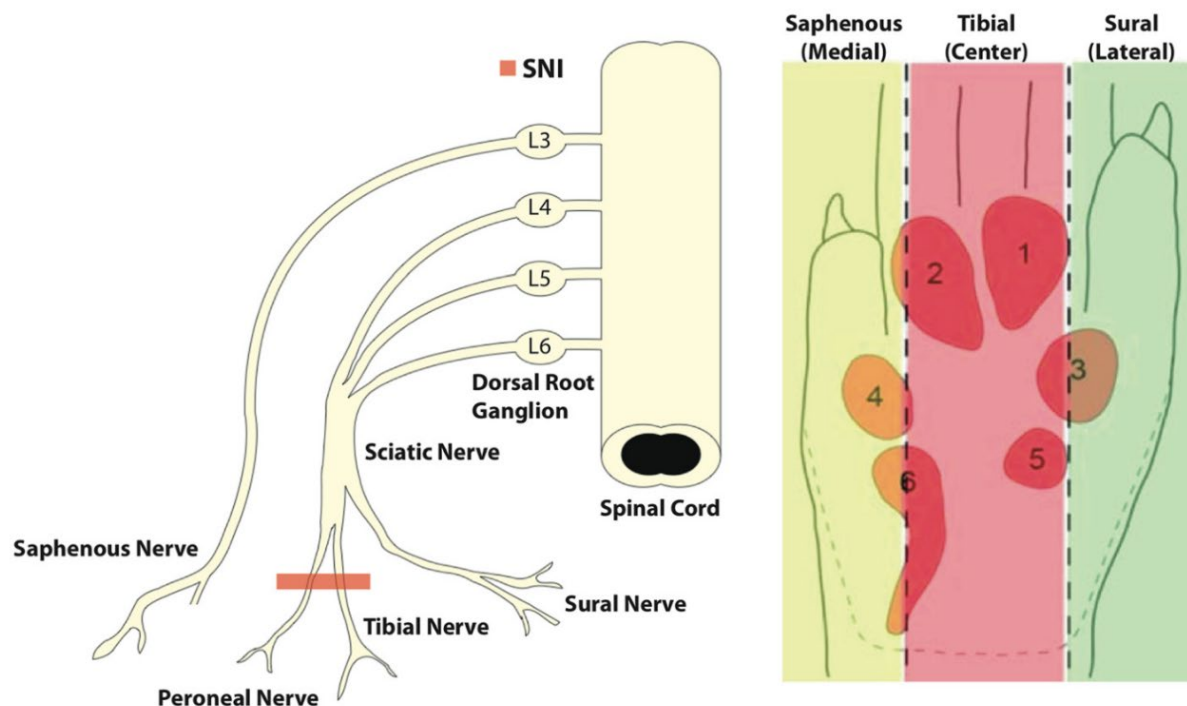


Figure 6. Schematic view of the spared nerve injury (SNI) model from Duraku *et al.*, 2012

In the spared nerve injury (SNI) model, the common peroneal and tibial nerve, two branches of the sciatic nerve, are ligated and transected. The third branch, the sural nerve is spared, causing mechanical and cold allodynia on the ipsilateral side of the skin area of the sural nerve (green), and to a lesser extent also saphenous nerve (yellow). Figure from Duraku *et al.*, 2012 [162].

1.3.7 Behavioral assessments of neuropathic pain in animal models

As it is not possible to measure pain directly in rodents, many behavioral tests to quantify pain-like behavior have been developed. Those assessment tools can be divided into instruments measuring stimulus-evoked nociception and spontaneous (non-stimulus) nociception [73]. The vast majority of these tests focus on stimulus-evoked measurements, quantifying allodynia and hyperalgesia [73, 158]. Since differentiation of those two pain responses is very difficult in animals, these terms are often used incorrectly or synonymously in the literature. In general, stimulus-evoked tests are based on the withdrawal response from a nociceptive stimulus [73], for example, von Frey (manual and electronic) [163] and Randall-Selitto [164] used for mechanical stimuli and the Hargreaves test used for thermal assessment [165]. A major limitation of these stimulus-based assessment tools is their subjectivity. The results rely on the experimenter's judgement to determine whether a withdrawal response was triggered by the stimulus itself or by unrelated factors, such as grooming or ambulation. Additionally, evaluation of the pain response is binary, either the

response was present or absent, whereas behaviors range across a spectrum of intensities [73].

Recently, the clinical translatability of these stimulus-evoked behavioral tests has been questioned, as neuropathic pain patients suffer primarily from spontaneous pain [73, 79, 166-173]. Therefore, many tools investigating spontaneous pain-like symptoms in rodents have been tested, such as burrowing, weight bearing, conditioned place preference and gait analysis [73], but further development is still needed [80, 81].

An important topic in animal experimentation is the protection of the animal's welfare. For this purpose, international standards and guidelines have been developed [174]. These guidelines ensure compliance with the 3Rs principle, i.e., replacement, reduction, refinement. Since animal replacement is particularly challenging in behavioral pain experiments, the focus is set on reduction and refinement [73]. The goal is to design behavioral experiments that minimize animal suffering and distress, while also improving data homogeneity, reducing subjective measurements, and increasing statistical power for more reliable and reproducible results [175].

1.4 Sexual dimorphism

1.4.1 Overview

Various factors are known to influence the perception of pain, with sex being one major factor. Sex and gender are terms which are sometimes used incorrectly, but should be examined separately. Gender is the individual's own identity, influenced and shaped by social and/or cultural values [176, 177], while the term sex refers to biological attributes, based on chromosomes, reproductive anatomy, hormones and gene expression [178]. Although some human studies include gender and investigate its impact on pain, most human studies, and to current knowledge, all preclinical animal studies, are based on the biological sex [177], including this thesis.

Despite the highly subjective nature of pain perception, there is clear evidence of sexual dimorphism in pain sensitivity. Females represent the majority of chronic pain patients [179, 180] and many studies report a greater pain susceptibility and lower pain tolerance in women compared to men [68, 179, 181-186]. However, it is still not completely clear to which extent the pain experience and underlying mechanism are

different or if the sexual dimorphism is biased due to the fact that women are more likely to use health care [187] and report their pain symptoms in comparison to men [182, 188, 189].

Nonetheless, when speaking of sexual dimorphism under pain conditions, it should be considered that already without any experimental conditions, i.e., under naïve conditions, such sex-specific differences exist on both the human and animal level [26, 190, 191]. Still, our knowledge remains incomplete, given the historical bias in research, with male subjects being disproportionately studied in animals (Figure 7), and to some extent, also human studies [185, 192, 193]. Additionally, many studies fail to state the sex of the experimental subjects, or do not analyze the variable sex in the results [194]. This imbalance is limiting the ability of making valid assumptions about sex-specific mechanisms of pain and their treatment outcomes. Clinical trials for pharmacological treatment options may have failed due to reliance on preclinical studies conducted in male subjects only, and therefore might result in treatments which are not equally effective in females [68]. A well-known example of analgesic drug response, with a sexual dimorphism, is K-opioid. In male animals, K-opioid analgesia is dependent on NMDA receptors [195-199], while in female humans and rodents it seems to depend on melanocortin 1 receptor [200-202]. Given this fact, an increasing number of studies are now investigating sex differences at both the behavioral and molecular level.

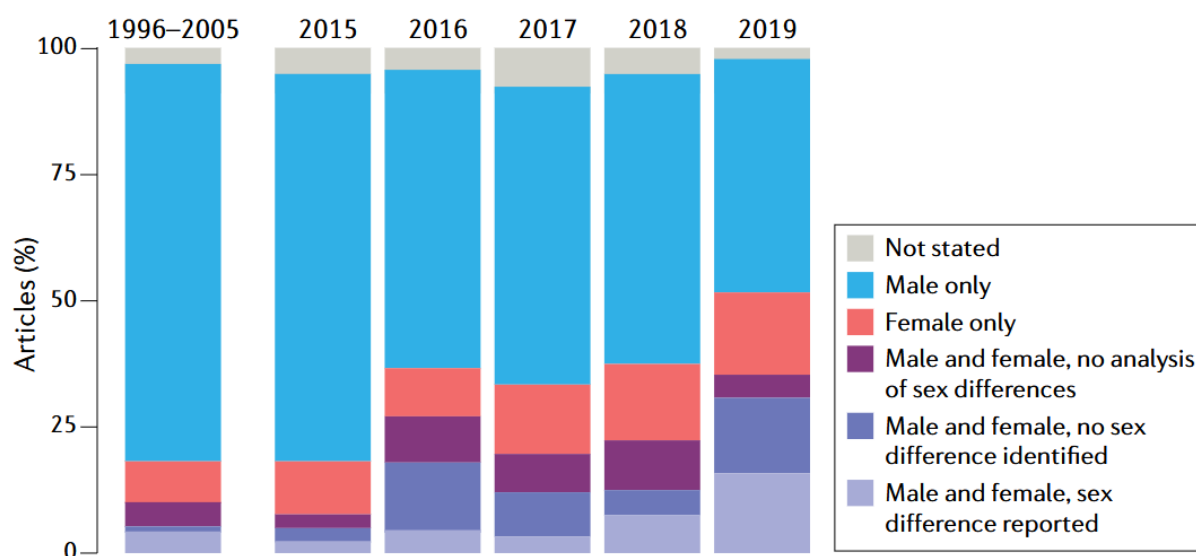


Figure 7. Usage of male and female animal subjects in studies published in the journal *Pain* adapted from Mogil *et al.*, 2020

The bar plot shows the percentage of studies having declared the sex of animals used in studies published in the journal *Pain* from 1996 – 2005, and from 2015 to 2019. The majority of studies report using males only, with a tendency over time to more female, and mixed-sex studies. Despite the awareness in the field of possible sex differences, the largest proportion of studies were still being performed with male-only cohorts in 2019. Figure adapted from Mogil *et al.*, 2020 [185].

1.4.2 Sex hormones and their role in pain

Many studies have focused on investigating the role of sex hormones in pain, and showed, for example, a link between the level of testosterone and the prevalence of fibromyalgia [203] and cluster headaches [204], as well as associations of estrogen levels with rheumatoid arthritis, temporal mandibular joint disorder [205], and irritable bowel syndrome [206]. Testosterone is suggested to have anti-nociceptive and protective effects [207], while estradiol and progesterone may have both pro- and anti-nociceptive impacts [208]. In a preclinical CCI study, administration of estrogen could alleviate allodynia until complete resolution in female mice [209]. Furthermore, preclinical studies suggest that the activity of TRPV1 is regulated via sex hormones. For example, estradiol seems to increase the expression of TRPV1 and promote pain in females [210-212], while testosterone was shown to inhibit TRPV1 expression [213]. Additionally, nuclear receptors, such as the peroxisome proliferator activated receptor (PPAR), show sex-hormone-dependent expression patterns. While PPAR α expression is increased in males with higher levels of testosterone, PPAR γ expression was shown to be increased with increased levels of estrogen in females. In both cases, it leads to the suppression of inflammation [124, 214].

In general, it seems that rather than absolute hormone levels, the interaction and fluctuation of sex hormones impact pain sensitivity. Importantly, different phases of the menstrual cycle may have an impact on the activation of different brain regions, involved in cognitive and motor function. Studies suggest that pain perception in healthy, pain-free menstruating people is not directly influenced by the menstrual cycle but the body's system being sensitive to it [215].

In children, studies on experimental pain mainly show no sex differences. In the minor cases of reported sexual dimorphism, results mimic studies in adults, with girls having elevated pain sensitivity and decreased tolerance to painful stimuli compared to boys. This was especially pronounced with increasing age (> 12 years) of the

children [216], pointing also towards an impact of sex-hormones in the establishment of pain [177].

1.4.3 The immune system and sex-specific pain mechanisms

Another major player in sex-specific pain mechanisms is the immune system. Adult women are known to have a stronger innate and adaptive immune response compared to adult men. Consequently, women show faster clearance of pathogens after an infection, however, this leads also to a greater susceptibility to inflammatory and autoimmune diseases [217]. On a mechanistic level, as indicated in section 1.3.3, Sorge *et al.*, 2015 [124] showed a marked sex difference in mechanical hypersensitivity in mice, as pain perception in males depends on microglia activity, while females seem to rely on adaptive immune cells, i.e., T-cells. In an inflammatory (Complete Freund's Adjuvant; CFA) and neuropathic pain (SNI) model, it could be shown that blocking microglia reverses mechanical hypersensitivity in male but not female mice [124]. The level of microgliosis was equal in both sexes [124, 218-220], suggesting that this sexual dimorphism in pain modulation may be driven by factors released by microglia, which influence pain in a sex-specific manner. A male-specific, testosterone-dependent effect is hypothesized and was shown in different studies [68, 218, 221-237]. Similarly, a testosterone-dependent mechanism was observed following intrathecal administration of lipopolysaccharide (LPS) in the spinal cord of a neuroinflammatory model [238]. LPS, which binds to the toll-like receptor 4 (TLR4) resulted in mechanical allodynia in male, but not female mice [238]. TLR4 is a pattern recognition receptor (PRR), which can be found on various cells of the CNS and PNS [239-245], and which activates the innate and adaptive immune response [246], including microglia [238]. When TLR4, or other PRRs, such as NLR family pyrin domain containing 3 (NLRP3), become activated, neurons become sensitized towards a pain state, increasing the pain sensitivity in females compared to men [247, 248]. It is suggested that the signaling cascade of TLR4 varies between males and females. However, exact sex-specific mechanisms of TLR4 are still not fully understood [123]. A sexually dimorphic response is also seen in the cytokine production in primary astrocytes. LPS stimulation increased TNF- α expression in male mice, whereas in females IL-10 levels were elevated [249]. However, a human study inducing systemic inflammation via intravenous LPS in healthy volunteers found higher levels of TNF- α , IL-6, and cortisol in female plasma, compared to male. Surprisingly, elevated TNF- α levels could be linked to increased pain sensitivity in men, but decreased pain sensitivity in women [250].

Differences in immune cell infiltration in DRG further support a sexual dimorphism in the immune response following nerve injury. A greater infiltration of B-cells within DRG after spinal nerve ligation could be observed in male mice, while female mice showed an increase of T-cell infiltration [220]. Furthermore, in male mice, an increased senescence of spinal cord microglia could be observed post SNI, which contributed to longitudinal pain hypersensitivity [251]. Another profound sexual dimorphism was found at the RNA level of DRG from neuropathic pain patients. While DRGs from male patients were associated with changes in inflammatory cytokines, such as TNF- α , IL-1 β and oncostatin-M (OSM), female patients seem to rely on type I and type II interferons. This highlights sex-specific mechanistic differences in neuropathic pain [252].

Moreover, the cytokine colony stimulating factor 1 (CSF1), which is involved in the differentiation of macrophages, seems to be expressed in higher levels in females, compared to males, post injury [253]. Furthermore, CSF1 can change the immunosuppressive activity of regulatory T-cells (T_{reg}) in females, via a crosstalk between lymphocytes and microglia [225]. Since the receptor for CSF1 can also be found on peripheral macrophages, it is suggested that they also have a role in CSF1-mediated neuropathic pain injury, in a sex-specific manner [123].

It is currently assumed that the proportion of immune cells within the PNS and CNS varies in male and female animals, which could explain the sex-specific responses after injury [177]. Preclinical studies have shown greater levels of chemokines and CD4⁺ T-cells in females [254]. Studies in humans support this theory [177], showing an increase of CD4⁺ and CD8⁺ T-cell activation in gut mucosal samples of healthy women compared to men [255], as well as elevated levels of B-cells in blood samples [256].

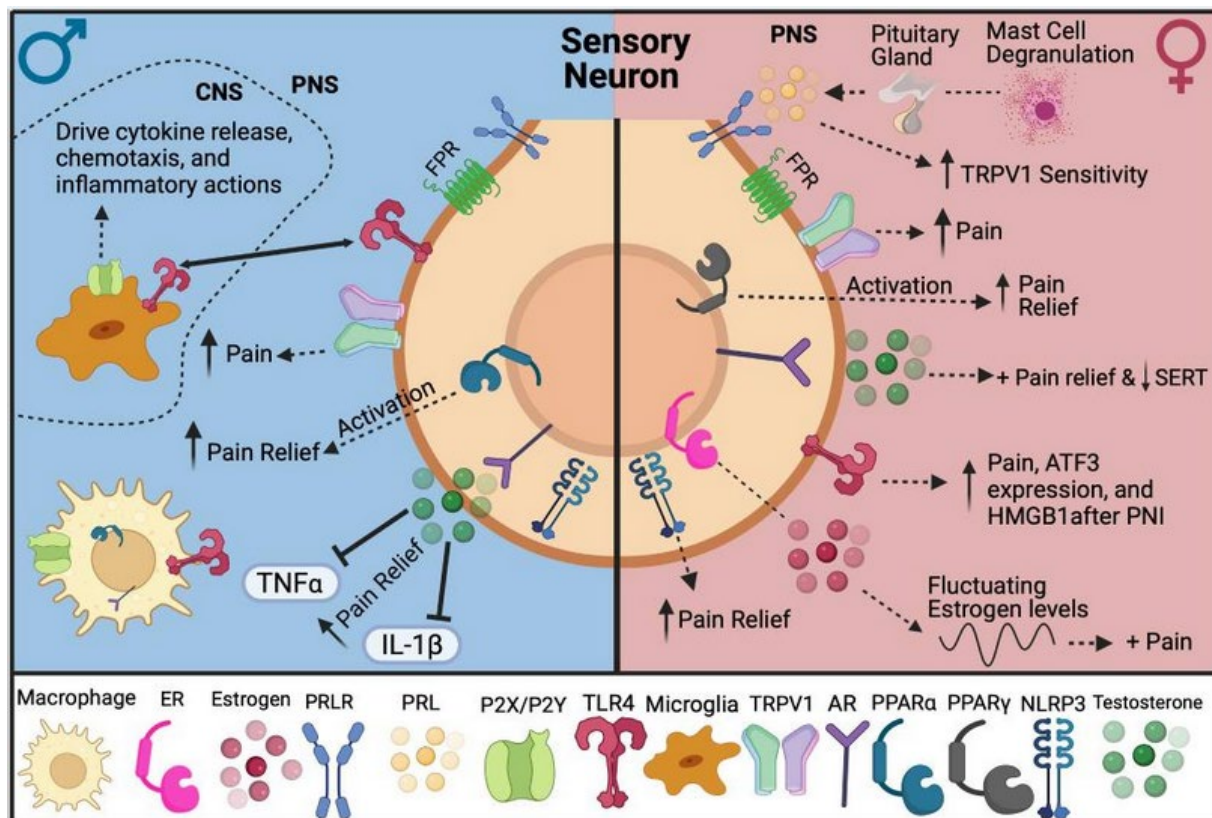


Figure 8. Schematic view of sexual dimorphic pain mechanisms from Alexander *et al.*, 2024

The nervous system and immune system communicate with each other in a bidirectional way. This figure illustrates an example of neuroimmune signaling with a sexual dimorphism in pain. In males (left side) and females (right side), sex hormones and immune factors influence sensory neurons and pain modulation differently. While in males testosterone seems to have a pain-relieving effect by inhibiting the release of pro-inflammatory cytokines (e.g., TNF- α and IL-1 β), estrogen in females is shown to increase pain sensitivity, possibly via increasing the expression of TRPV1. Sex hormones also seem to have an impact on PPARs, with testosterone increasing the expression of PPAR α and estrogen elevating PPAR γ levels, both resulting in suppressed inflammation. Additionally, in immune signaling pathways, including TLR4 and NLRP3, distinct mechanism of pain regulation between males and females is suggested. These examples highlight sex-dependent mechanistic differences in pain modulation. Abbreviations: central nervous system (CNS), peripheral nervous system (PNS), N-formyl peptide receptor (FPR), tumor necrosis factor-alpha (TNF- α), interleukin-1beta (IL-1 β), transient receptor potential vanilloid receptor (TRPV1), serotonin transporter (SERT), activating transcription factor 3 (ATF3), high mobility group box 1 (HMGB1), peripheral nerve injury (PNI), estrogen receptor (ER), prolactin receptor (PRLR), prolactin (PRL), purinergic receptor P2X/P2Y (P2X/P2Y), toll-like receptor 4 (TLR4), androgen receptor (AR), peroxisome proliferator activated receptor alpha/gamma (PPAR α / γ), NOD-like receptor protein 3 (NLRP3). Figure from Alexander *et al.*, 2024 [123].

1.4.4 Sex differences involving other cell types

Sexual specific mechanisms in pain perception can also be found on the level of various other cell types. For example, in male mice, mechanical hypersensitivity, following SNI, was reduced when Merkel cells, mechanosensitive cells, were absent. In female mice, this alleviation could not be observed [257]. In humans, a spatial transcriptomic analysis of DRG revealed a female-specific increased expression of the gene CALCA, encoding CGRP, in pruritogen receptor-enriched nociceptors, which are sensory neurons detecting pruritogenic, i.e., itch-inducing, stimuli [39].

1.4.5 Sexual dimorphism on the behavioral level in animals

When looking at sexual dimorphism on the behavioral level in rodent neuropathic pain models, the results are contradictory. While some studies report increased hypersensitivity in females [258-260], others show higher hypersensitivity in males [261-263], as well as time point dependent sex differences in both directions [264]. Differences in those results can be due to the genetic background of the experimental subject [265], age of the subjects [266], different type of pain model [267], and the subjective evaluation of the experimenter [73]. Furthermore, as already mentioned, current behavioral assessment tools do not reflect the clinical neuropathic spontaneous pain symptoms and new assessment methods need to be developed [77-81, 268].

1.5 Age-dependent differences

1.5.1 Overview

While the variable sex is increasingly studied, few studies focus on investigating the impact of age on pain perception and mechanisms. Across a lifespan, the sensory system changes functionally and anatomically, with a decrease in sensitivity for vision, hearing, smell, taste, and touch [269, 270]. Pain sensitivity also changes due to maturation processes of the pain axis [271-273]. Some systems, such as modulating descending pathways, are not yet active in newborns, leading to a diminished production of serotonin and norepinephrine, compared to adults [274, 275]. Furthermore, in children, the myelination of nociceptive fibers is still incomplete, leading to a slower conduction velocity, although this is compensated by the shortened distance of the pain pathway [276]. Thus, to some extent, differences in the anatomy

can explain differences in pain perception and sensitivity. However, the direction in which pain sensitivity develops with increasing age is still controversial, as shown in animal and human studies [269, 273, 277-293].

1.5.2 Age bias in preclinical research

In preclinical research, the age choice of experimental animals is often not due to scientific reasons, but rather based on practical ones, such as costs and maintenance (Figure 9). Furthermore, age as a variable is often poorly reported, making cross-study comparisons and assumptions difficult. When age is reported, preclinical studies, including those in pain research, use mostly adult rodents within the age range of six to twenty weeks (Figure 9). This illustrates that the term “adult” can cover a wide age range, where many systems are still developing and therefore may have an impact on outcomes of the study. This could be avoided by precise age specification in days, weeks or months [266]. In addition, to this unspecific age-specification, the use of different species, testing methods, and aforementioned sex differences can lead to contradictory results [285].

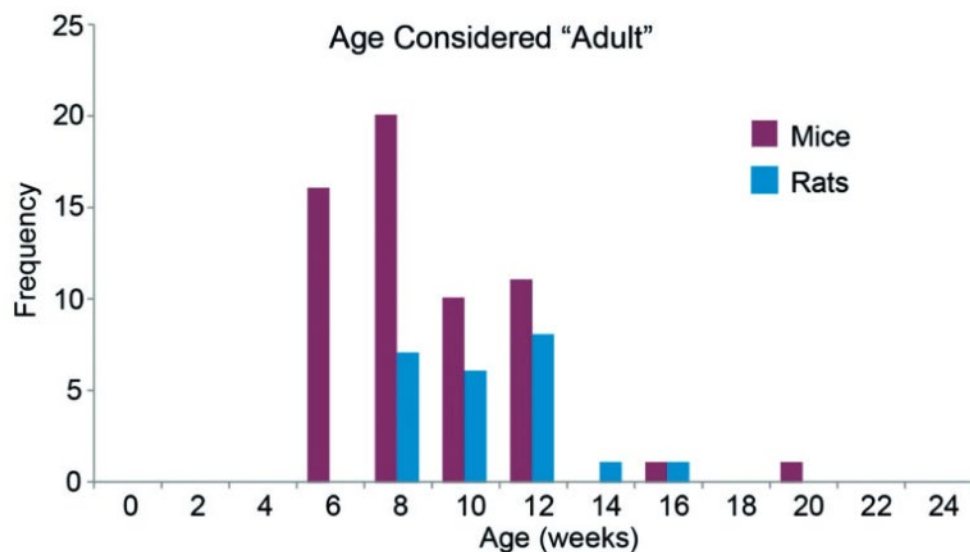
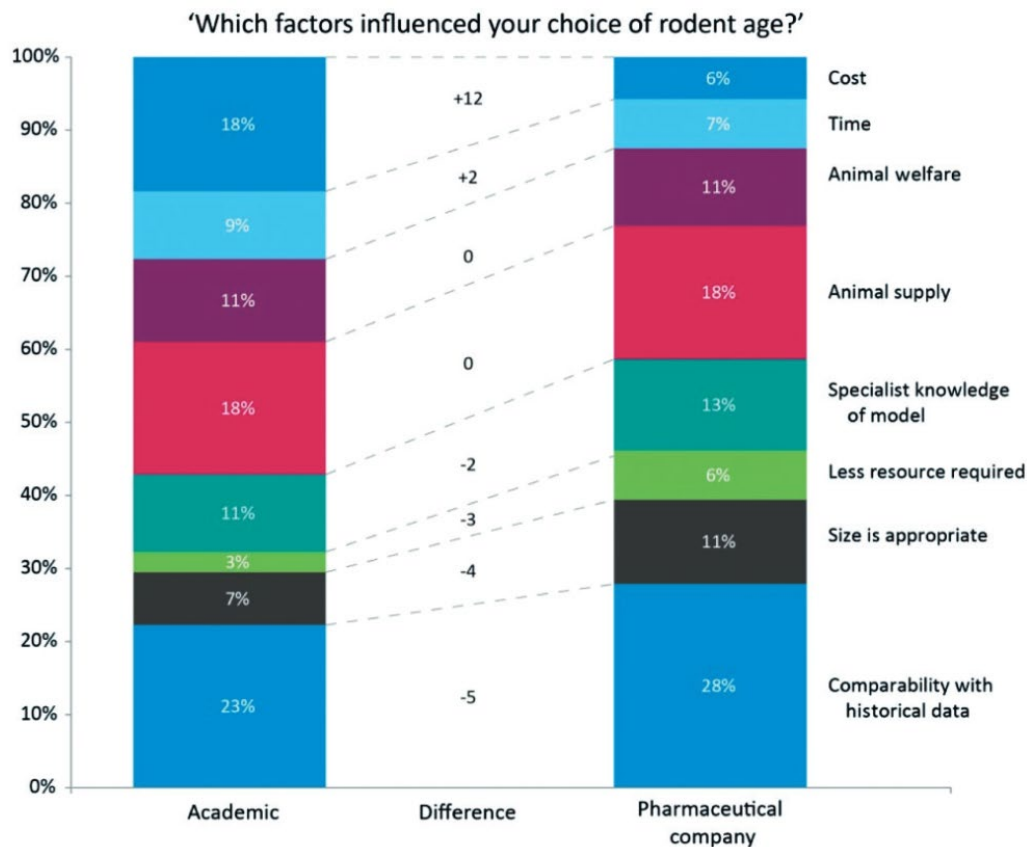


Figure 9. Age bias in preclinical studies adapted from Jackson *et al.*, 2017

In preclinical studies, the age of animal subjects significantly impacts experimental outcomes, yet the selection criteria for age remain inconsistent and poorly defined. The upper panel shows a comparison of age selection in academia-based versus industry-based studies highlights the influence of "practical" factors, such as cost and time in academia. The lower panel indicates the wide age range for which the term "adult" is used, varying from 6 to 20 weeks in mice and 8 to 16 weeks in rats, limiting the reproducibility across studies and underscoring the need for precise indication of age in days, weeks or months. Figures adapted from Jackson *et al.*, 2017 [266].

1.5.3 The impact of age on neuropathic pain development

Preclinical studies on neuropathic pain suggests an enormous impact of age on the development and manifestation of pain after injury [294]. For example, in the first postnatal days, SNI does not result in immediate development of neuropathic pain symptoms [272, 295]. However, longitudinal investigation of animals undergoing nerve injury at juvenile age, show pain hypersensitivity at adolescent age, implying a delayed response to the nerve injury [295, 296]. The mechanisms underlying this delayed response may vary depending on when the injury takes place. In the first postnatal week (until postnatal day 7; i.e., equivalent to preterm infants in humans [294]), animal studies show that the survival of sensory neurons is highly dependent on peripheral skin and muscles [297, 298]. If a nerve injury occurs during that time, sensory neurons in DRG undergo cell death and compensatory sprouting, leading to reorganization of the somatosensory system [294, 299-301]. A reason for the prevention of neuropathic pain, if the injury takes place during the first postnatal weeks (postnatal day 10-21; i.e., equivalent to infants and children in humans [294]), could be the synaptic reorganization of the spinal somatosensory circuits. The neuroimmune response is shifted into an anti-inflammatory sense, releasing IL-4 and IL-10, whereby the neuronal sensitization in the dorsal horn is suppressed. Neuropathic pain is therefore actively suppressed in the early phase, rather than completely absent [294, 296, 302-305]. Due to natural maturation of the central nervous system in aging animals, the neuroimmune response becomes pro-inflammatory, releasing cytokines such as IL-1 and TNF, leading to neuronal sensitization and pain response [306-308]. Furthermore, sexual dimorphic mechanisms, as discussed in section 1.4, should be considered across age groups [309], along with other factors, such as stress. Stress may have a contribution on the likelihood of maladaptive immune response, as chronic stress can lead to increased inflammation [310].

1.5.4 Developmental changes in nociception and pain modulation

In clinics, neuropathic pain following nerve injury is extremely rare in infants, with only a few reports before the age of 5-6 years [311-313]. As the age at which the nerve damage occurs increases, the incidence of neuropathic pain rises [314], with a median age of 13 years at the onset of pediatric neuropathic pain syndromes [313]. However, it needs to be considered that neuropathic pain is likely to be underreported in groups such as pre-verbal infants [315].

Some studies have shown that the nociceptive system is functional as early as gestational week 20, with further maturation occurring throughout growth and development [316]. However, in newborns, the distinction between touch and pain is more difficult, as connections between nociceptive neurons and the spinal cord are yet to be fully organized [317]. A noxious stimulus can trigger an increased immune reaction in newborns, which, though, does not lead to neuroinflammation. However, the immature immune system after birth may be influenced by this early pain, potentially causing its priming and reactivation during later life [318]. Thereby, hyperalgesia and greater risk of suffering from chronic pain may develop [319, 320]. If repetitive, untreated, painful stimuli occur in the preterm period, a link between increased likelihood for depression or post-traumatic stress disorder may be seen [321]. Furthermore, evidence is given that neonatal inflammation may be linked to heightened tactile sensitivity, persisting even beyond the inflammation and might influence pain perception later in life [322]. Indeed, there is growing evidence that the prevalence of chronic pain conditions increases during adolescence, particularly in females [323-325], and persist into adulthood [326]. This period, characterized by puberty and emerging sex differences, might be important in the development of chronic pain, however, little is known on the mechanistic differences in adolescents [327].

1.5.5 Challenges in pediatric pain

Assessment and treatment of pediatric pain is still challenging [328, 329], with symptoms often being untreated or with pharmacological pain treatments being neurotoxic [329, 330]. Certainly, the long-believed inability to perceive pain in infants is not true and pain incidence in children might be comparable to adults. Nevertheless, our understanding of pain during adolescence is underdeveloped [329] and investigations of mechanisms underlying pain perception are critically needed for the improvement of pediatric care and the design of effective treatment strategies [331, 332]. Moreover, it will improve our understanding of mechanisms leading to long-term consequences of early-life pain [328].

1.6 Microbiome

In recent years, studies have confirmed the bidirectional way in which the brain and the gut communicate. This is known as the gut-brain axis. This communication is

achieved by immunological, hormonal, and neural signals [333], which might be influenced by microorganisms in the gut [334, 335]. The human gut microbiome consists of approximately 10^{14} aerobic and anaerobic microorganisms [335-338], such as bacteria, archaea, viruses, and fungi [336, 337, 339, 340], whereby bacteria make up the majority [341]. Within the first years of life, the gut microbiome is established [342, 343]. At this time, it is influenced by genetics [340] and external factors, such as birth delivery (vaginal or caesarean section), diet (whether breastfed or not), infections and antibiotic medication [342, 343]. Throughout life, the composition still changes constantly, due to external and internal factors [344]. In order to maintain health, homeostasis between the host and its gut microbiome is of utmost importance [345, 346]. An imbalance of this system can lead to multiple diseases, such as cardiovascular, neurological and metabolic diseases [337, 347]. Furthermore, growing evidence has been given for a link between stress, anxiety, depression and neurological disease with malfunctions of the microbiome [348-350]. More recently, the role of the gut microbiome in chronic pain has received increasing attention [351]. Several studies on visceral and abdominal pain, and the role of the gut microbiome have already been conducted [351-370]. Initial preclinical animal studies have shown the role of the gut microbiome in inflammatory pain [371] and neuropathic pain [372, 373]. A recent study performed in rats, investigated neuropathic pain and anhedonia, a common symptom of depression. Depression, as already mentioned, is a frequently occurring comorbidity of neuropathic pain. Sequencing the 16S ribosomal RNA, of the gut microbiome of SNI rats, revealed alterations in the gut microbiome composition in rats that showed anhedonia-like behavior, compared to rats without anhedonia and sham rats. Subsequently, they transplanted the fecal microbiota of these rats into antibiotic-treated mice. The recipient mice exhibited pain and depression-like symptoms, which worsened after the transplantation of the fecal microbiota from rats displaying anhedonia. In contrast, transplantation of fecal microbiota from rats without anhedonia led to an improvement of pain and depression-like symptoms in the mice, highlighting the gut microbiome's potential role in modulating pain and depression [372]. Another study investigated changes of the gut microbiome in neuropathic pain mice, induced by CCI. After antibiotic administration, they reported a decrease in neuropathic pain symptoms and a disturbed T-cell immune response [373].

As indicated in Figure 10, it is hypothesized that the gut microbiome influences neuropathic pain through several pathways, including pro-inflammatory mediators,

excitability of nociceptors and activity of glial cells [374]. An imbalance in the gut microbiome may cause alterations of immune and neuronal interactions, linked to neuropathic pain. Additionally, metabolites derived from the gut's microbiome may have an impact on neurotransmitter balance and thereby further modulate pain perception [374].

Mediators of the microbiome can directly and indirectly influence pain sensitization. Direct excitability of nociceptors occurs via activation of pain-related receptors and ion channels, such as γ -aminobutyric acid (GABA) receptors or TRP channels [119, 120, 375]. Moreover, enteroendocrine cells (EECs) may contribute to the communication of the gut-brain axis and thereby modulate pain through diverse signaling molecules, such as serotonin [374]. Indirect regulation occurs through activation of non-neuronal cells. Pathogen-associated molecular patterns (PAMPs), such as LPS, are released by the gut microbiome and bind to PRR, such as TLR4 on immune cells and sensory neurons, causing their activation and sensitization [246, 376, 377], leading to the release of pro-inflammatory cytokines (e.g., IL-1 β , IL-6 and TNF- α), anti-inflammatory cytokines (e.g., IL-4), chemokines (e.g., C-C motif chemokine ligand 2 - CCL2, and C-X-C motif chemokine ligand 1 - CXCL1), and neuropeptides (e.g., opioids) [375, 378, 379]. Another important route in the microbiome's role in neuropathic pain is the regulation of glial cell activation. For example, bacterial derived short-chain fatty acids (SCFAs) can regulate the activity of microglia and thereby pain processing [374]. Furthermore, SCFAs play a key role in modulating the immune response via T_{reg} and may thus contribute to the development of chronic pain [380-384]. Another role, where the microbiome is suggested to have an impact on neuropathic pain is via activating astrocytes. Tryptophan, taken up via diet and metabolized by the gut microbiome, acts on astrocytes via aryl hydrocarbon receptor (AHR), leading to a decrease in inflammation and neurodegeneration [385, 386].

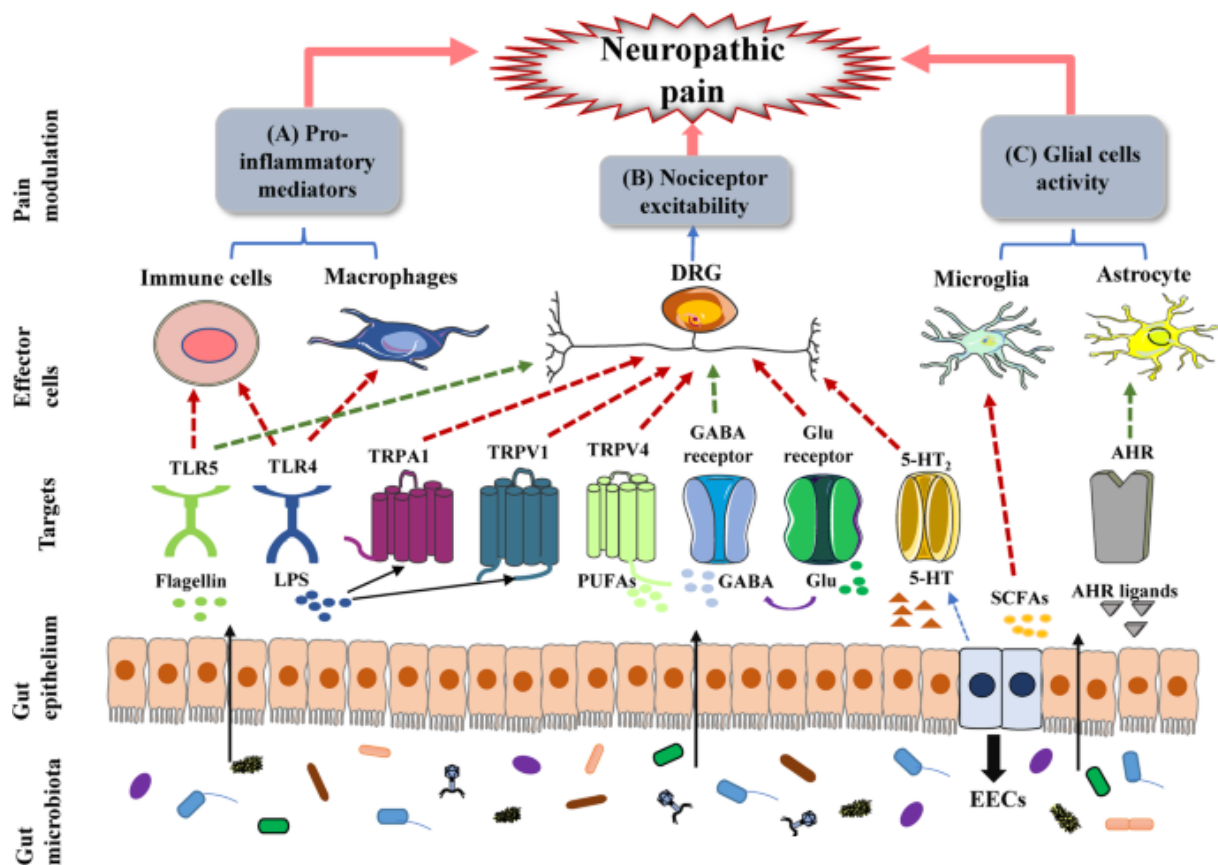


Figure 10. The potential impact of the gut's microbiome in neuropathic pain from Lin *et al.*, 2020

A representation of potential mechanisms, including pro-inflammatory mediators, nociceptor excitability and glial cells activity, that might, in combination, influence neuropathic pain. Mediators released by the gut microbiome act on diverse receptors and channels, causing the alteration of the depicted cell types. Dotted arrows show pathways of pain exacerbation (red) or alleviation (green). Abbreviations: dorsal root ganglia (DRG), toll-like receptor (TLR), lipopolysaccharide (LPS), transient receptor potential cation channel subfamily A, member 1 (TRPA1), transient receptor potential cation channel, subfamily V, member 1 (TRPV1), transient receptor potential cation channel, subfamily V, member 4 (TRPV4), polyunsaturated fatty acids (PUFAs), γ -aminobutyric acid (GABA), glutamate (Glu), serotonin receptor 2 (5-HT₂), serotonin (5-HT), short-chain fatty acids (SCFAs), aryl hydrocarbon receptor (AHR), enteroendocrine cells (EECs). Figure adapted from Lin *et al.*, 2020 [374].

Probiotics, which are living bacteria, can influence the host's gut microbiome in terms of health [388-394], including chronic pain [395-397]. In multiple preclinical studies of visceral pain, hypersensitivity could be alleviated using probiotics [398-400]. For example, using the probiotic VSL#3 could decrease visceral hypersensitivity in rats. It is hypothesized that this effect is mediated via the mast cell-protease-activated receptor 2 (PAR2)-TRPV1 pathway, leading to the release of mediators affecting the enteric nerves and smooth muscles [398]. Furthermore, in rheumatoid arthritis,

evidence shows that probiotics can help to relieve pain [401]. In other pain conditions, such as abdominal pain, clinical studies with probiotics are still contradictory [395-397, 402, 403]. Nonetheless, targeting the gut microbiome holds promise as a novel therapeutic tool for the treatment of pain [341]. However, further studies are needed in investigating the functional roles of gut microbiota in different pain models.

1.7 Proteomics

1.7.1 Technical details

As described in the sections above, neuropathic pain is a complex disease with many changes at the molecular level. Current treatment options for neuropathic pain intervene on the single protein level. However, to achieve better efficiency, a “mechanism-based” approach might be useful [404-409]. To implement this, a comprehensive profiling of the pain axis is required first.

Omic studies measure a particular type of molecule from various sample types regarding its character and quantity. The concept lies in treating a dataset as a unit [410] and capturing molecules in a particular state and time [411]. Multiple -omic studies exist, i.e., genomics (study of DNA molecules), epigenomics (study of the chemical states of DNA, including its binding proteins), transcriptomics (study of RNA), proteomics (study of proteins) and metabolomics (study of metabolites) [410].

Organisms interact and react to their environment, directed by their genomic DNA, which is then transcribed into messenger RNA (mRNA), and translated into proteins [412]. Although significant efforts have been made to characterize RNA, which remains the most comprehensive technique for molecular tissue analysis, the transcriptome corresponds only to a limited extent to the proteome, especially under pathological conditions. The reasons for this restricted overlap are cellular buffering mechanisms [413, 414], such as translational- and post-translational modifications, alternative splicing and protein degradation/stability [415, 416]. Thereby protein abundance undergoes modifications which are beyond the corresponding RNA-level [413, 414]. Hence, the state and expression level of proteins can better reflect diverse physiological states at the cellular level [413, 414, 417], making proteomics, i.e., the comprehensive study of proteins [418], a relevant technique to characterize the state of a biological system [413, 417]. While various techniques exist for studying the

proteome, such as immunoassays and 2D-PAGE, mass spectrometry (MS) has become one of the most widely used tools. MS enables the rapid identification of peptides by analyzing their fragmentation patterns (mass spectra) and comparing them with available sequence databases [419].

The studies in chapter 2 and 3 utilize tandem mass spectrometry (MS/MS), combining it with either data-dependent acquisition (DDA; chapter 3) or data-independent acquisition (DIA; chapter 2 and 3), along with Parallel Accumulation-Serial Fragmentation (PASEF) for enhanced peptide identification and quantification.

As in shown in Figure 11, peptides, obtained from proteome digestion of cells or tissues, are first separated by liquid chromatography (LC) before entering the mass spectrometer. Within the mass spectrometer, trapped ion mobility spectrometry (TIMS) is used to accumulate and separate ions. The mass spectrometer used in this thesis has two TIMS cartridges responsible for the accumulation of ions over a defined time period (TIMS1) and the ion elution in a mobility-resolved manner (TIMS2). TIMS enhances separation of ions based on their ion mobilities, leading to the improvement of peak capacity and resolution. Once ions are separated based on their mobilities, the quadrupole selectively isolates precursor ions and their mass-to-charge (m/z) ratios are measured (MS1) [421, 422]. Different acquisition methods, i.e., methods on MS have been developed over past decades to accommodate various applications [421]. A commonly used acquisition scheme is data-dependent acquisition (DDA) [147, 423-426]. In this semi-stochastic technique, the most intense precursor ions detected at the MS1 level are prioritized for fragmentation. This acquisition approach ensures high specificity, however, peptides with lower intensity may not be detected, resulting in reduced reproducibility and sensitivity [423, 427]. In contrast, data-independent acquisition (DIA) selects and fragments nearly all precursor ions, regardless of their MS1 intensity (within the detection limits of the mass spectrometer and instrumental settings) [428-430]. This approach improves the detection of low abundant peptides, while maintaining high reproducibility in quantification [427-430], making it particularly advantageous for systematic studies of pathology-related proteomic changes [430-434]. After the selection of precursor ions, fragmentation occurs in the collision cell, followed by analysis in the time-of-flight (TOF) mass analyzer, representing the MS2 level [422].

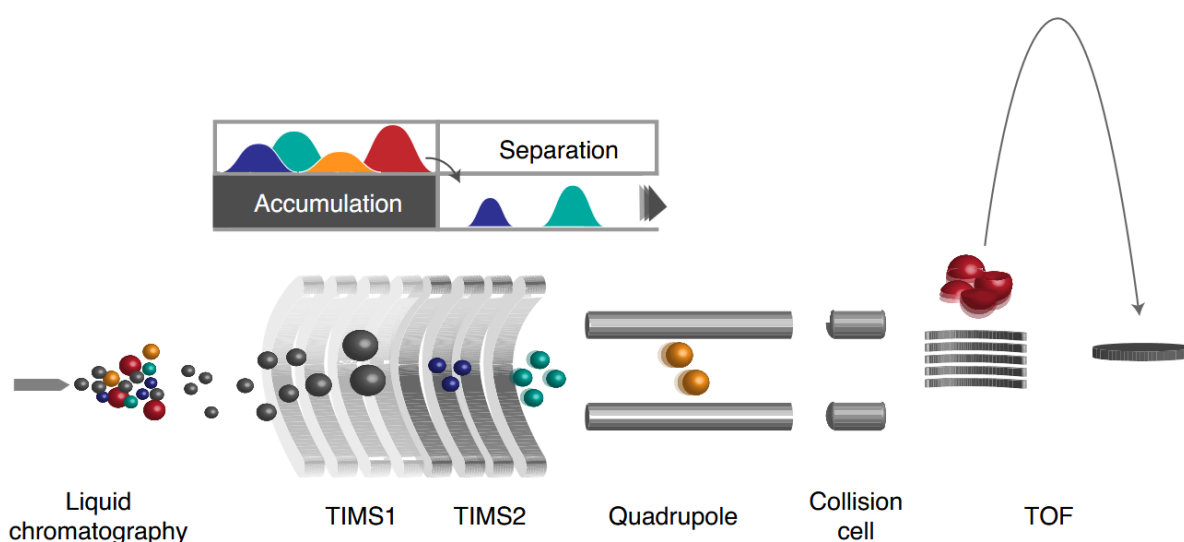


Figure 11. Schematic view of mass spectrometry technique used in the studies presented in this thesis adapted from Meier *et al.*, 2020

Peptides are separated in the liquid chromatograph. In trapped ion mobility spectrometry (TIMS) ions are accumulated (TIMS1) and separated (TIMS2). Precursor ions are selected by the quadrupole, followed by fragmentation in the collision cell and analysis by time-of-flight (TOF). Figure adapted from Meier *et al.*, 2020 [422].

PASEF enhances both DDA and DIA by utilizing ion mobility separation in TIMS. Instead of analyzing ions sequentially, PASEF efficiently cycles ions with similar mobility, significantly increasing the number of MS/MS (MS2) scans without compromising sensitivity. This optimized workflow enhances sequencing speed, signal intensity, and overall proteome coverage, making it a powerful approach for high-throughput and accurate protein identification [422].

1.7.2. Proteomics in neuropathic pain

Few chronic pain studies on the proteome level have been conducted [436-440], yet a system-level understanding of protein network organization and dynamics is still incomplete. However, due to recent significant progress and continuing efforts of ongoing research, knowledge is steadily advancing. For example, proteome investigation of inflammatory (CFA) and neuropathic pain (SNI) models, in murine DRGs, showed significant alterations in signaling pathways, such as mitochondrial function and protein synthesis. Moreover, single protein candidates of these networks showed their different involvements in the two pain models. *In vivo*, inhibition of mitochondrial complex I and protein disulfide isomerase attenuated chronic inflammatory pain, highlighting their potential as therapeutics [441]. A subsequent

study, which investigated the proteome of DRG, sciatic nerve, and spinal cord, identified previously unknown tissue-specific proteins during neuropathic pain (SNI) and alterations in pathways related to e.g., mitochondrial dysfunction [442]. A study of DRG and sciatic nerve, in mice with type 2 diabetes, showed alterations and impairment of metabolic pathways, and proposed the resulting low adenosine triphosphate (ATP) bioavailability as a main mechanism of neurodegeneration in diabetes [443]. In humans with diabetic peripheral neuropathy, remodeling of the extracellular matrix was suggested to be related [444]. Investigation of postoperative pain (plantar incision) in a mouse model demonstrated differential expression of proteins from DRG being involved in immune and inflammatory pathways, and proposed annexin A1 (*Anxa1*) as a novel pain candidate [445]. Another incision study on humans and mice showed the different modulation of protein-protein interactions when comparing the two species. Furthermore, in humans, a link could be made between protein signature and hyperalgesia. While altered proteins in low responders, i.e., small incision-related hyperalgesic area, were linked with anti-inflammatory pathways, high responders, i.e., large incision-related hyperalgesic area, showed protein signatures associated with persistent inflammation and a distinct proteolytic environment. Since high responders have an increased likelihood to develop chronic post-surgical pain, these findings may be potentially relevant for the prevention of chronification [446]. Despite their important insights, a common limitation for all of these studies is the exclusive use of male subjects (except for Doty *et al.* [444]) and in terms of mice, adult age. This highlights the critical need for the integration of variables such as sex and age for a more comprehensive understanding.

1.7.3 MESSAGE FINDING FOR SPATIO-TEMPORAL ORDERING (MEFISTO)

In -omics studies, datasets have become increasingly complex due to several data modalities and sample groups [447, 448]. Managing these sophisticated relationships is therefore difficult. As a result, innovative strategies for analyzing such data have become increasingly important. MESSAGE FINDING FOR SPATIO-TEMPORAL ORDERING (MEFISTO), an extension of the multiomics factor analysis (MOFA), is a novel computational method used for such datasets. MEFISTO is particularly suited for datasets with spatio-temporal dependencies, as it identifies pattern differences, distinguishes smooth changes from irregular variations, and manages imperfect alignment and missing data. By reducing spatio-temporal dimensionality, MEFISTO enhances interpretability and accuracy in uncovering underlying patterns [449]. The

study in chapter 2 uses MEFISTO and compared its result with the classical approach of differential expression analysis.

1.7.4 Metaproteomics

Metaproteomics is the large-scale study of all proteins expressed by microorganisms within an ecosystem at a given moment [450]. In comparison to metagenomics, which characterizes microorganisms and indicate the functional potential of the microbiota, metaproteomics has the advantage of being able to explore directly the microorganisms' functions and is therefore more suitable for studies investigating functional alterations as well as the host response [451-453]. Thereby complex host-microbe interactions, e.g., under pain conditions, can be demonstrated simultaneously. Microbial samples, such as feces, are of high complexity [454], with many low abundant bacteria species. Therefore, the classically used proteomics acquisition method DDA, has reached its profiling limit [453]. As mentioned before, DIA mass spectrometry can overcome these limitations [441], and its performance in metaproteomics has been evaluated recently [455-458]. Still, sampling efficiency and complex peptide spectra remain challenging [422], increasing the need for improved proteomic acquisition methods.

Chapter 3 of this thesis explored this challenge by employing different acquisition methods (DDA versus DIA) in combination with PASEF to increase the depth and resolution of metaproteomics, and further applied to analyze the mouse gut microbiome in a neuropathic pain model.

1.7.5 Limitations

Although MS has significantly advanced our understanding of proteome alterations in pain conditions, a major limitation remains the generation of bulk data. Proteins are analyzed from heterogeneous tissue samples, such as DRG, which harbor multiple cell types. Consequently, while global abundance changes are detected, the resolution to attribute these changes to specific cell types is lacking. A promising solution to overcome this limitation is single-cell mass spectrometry, which aims to achieve proteomic profiling at the level of individual cells. However, this technology is still in its early stages and faces challenges in sample preparation, protein yields, and sensitivity limitations [459].

In proteomics, a key challenge is that some of the identified peptides can correspond to multiple proteins [451], which is known as protein inference issues due to the low sequence coverage of identified proteins. In metaproteomics, the magnitude of complexity increases, as peptides and protein identifications are not from a single organism but many species [451, 460], often with close homology [461]. Unlike proteomics, in metaproteomics we do not know in advance which type of species are present in a sample. This uncertainty makes the choice of database particularly challenging, and will affect the sensitivity of peptide identification [451, 462, 463]. Databases need to be broad enough to capture the diversity of a sample, however, this may strongly influence sensitivity and false discovery rate (FDR) estimation [464, 465].

Chapter 2

Deep proteomics and network pharmacology reveal sex- and age-shared neuropathic pain signatures in mouse dorsal root ganglia

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Major Aims

The overall goal was to deepen the understanding of neuropathic pain on the molecular level. For this, we focused on a comprehensive experimental design, by investigating nerve injury-related proteome changes in male and female mice, of two age groups. Sex and age are two biological variables, known to play a major role in pain perception and its underlying mechanism, still, a comprehensive understanding of sex-dependent pain mechanisms, in different age groups, is lacking. For age, we decided to investigate adolescent and adult mice, the latter being mainly used in preclinical studies. In comparison, adolescent age is rarely investigated, although we know that pain sensitivity changes during a lifespan, with young patients being particularly vulnerable. To analyze proteome changes, we isolated the dorsal root ganglia (DRG), a tissue known for their role in pain transmission, at the initial phase of neuropathic pain establishment and analyzed them with mass spectrometry. The goal was to identify a sex- and age-shared protein signature of neuropathic pain, which potentially represent impactful drug targets for pain therapy, benefiting men and women of different age groups.

Contributions

For this study, I contributed to many aspects of the experimental design as well as being responsible for a large portion of the experimental, observational, and analytic work, and drafting the manuscript itself. Experimentally, I was primarily responsible for performing surgeries on the subjects and control subjects (SNI and Sham). This includes preparation of the subjects for surgery, the surgeries themselves and post-operative care. Furthermore, I conducted most of the assessments of both, evoked

and non-evoked behavior, and performed the subsequent analysis of these data. Regarding tissue isolation, I coordinated and contributed to the tissue isolation of different tissues from all subjects, including naïve mice, with assistance from colleagues. I was responsible for preparing these samples for further analysis, including protein extraction from DRG and SP3-assisted protein digestion, in order for samples to be analyzed via mass spectrometry. The mass spectrometry run and DIA-NN analysis was conducted by my colleagues. Also, the subsequent MEFISTO analysis was performed by a collaboration partner. In the analytical part, I was responsible for analyzing the differentially expressed proteins, obtained from mass spectrometry, including their biological analysis and interpretation. I also took the lead in presenting these results, drafting the manuscript and, generating figures for publication, as well as responding to reviewer comments.



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Deep proteomics and network pharmacology reveal sex- and age-shared neuropathic pain signatures in mouse dorsal root ganglia

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ABSTRACT

Our understanding of how sex and age influence chronic pain at the molecular level is still limited with wide-reaching consequences for adolescent patients. Here, we leveraged deep proteome profiling of mouse dorsal root ganglia (DRG) from adolescent (4-week-old) and adult (12-week-old) male and female mice to investigate the establishment of neuropathic pain in the spared nerve injury (SNI)-model in parallel. We quantified over 12,000 proteins, including notable ion channels involved in pain, highlighting the sensitivity of our approach. Differential expression revealed sex- and age-dependent proteome changes upon nerve injury. In contrast to most previous studies, our comprehensive dataset enabled us to determine differentially expressed proteins (DEPs), which were shared between male and female mice of both age groups. Among these, the vast majority (94 %) were also expressed and, in part, altered in human DRG of neuropathic pain patients, indicating evolutionary conservation. Proteome signatures represented numerous targets of FDA-approved drugs comprising both (i) known pain therapeutics (e.g. Pregabalin and opioids) and, importantly, (ii) compounds with high potential for future re-purposing, e.g. Ptpcr-modulators and Epoetins. Protein network and multidimensional analysis uncovered distinct hubs of sex- and age-shared biological pathways impacted by neuropathic pain, such as neuronal activity and synaptic function, DNA-damage, and neuroimmune interactions. Taken together, our results capture the complexity of nerve injury-associated DRG alterations in mice at the network level, moving beyond single-candidate studies. Consequently, we provide an innovative resource of the molecular landscape of neuropathic pain, enabling novel opportunities for translational pain research and network-based drug discovery.

Abbreviations: 5HT3A, 5-hydroxytryptamine receptor 3A; ACN, Acetonitrile; Atf3, Activating transcription factor 3; C1qb, Complement C1q B chain; C5ar1, Complement C5a receptor 1; Cacna2d1, Calcium voltage-gated channel auxiliary subunit alpha2delta 1; Camk2A, Calcium/calmodulin dependent protein kinase II alpha; Ctss, Cathepsin S; DEPs, Differentially expressed proteins; DIA, Data-independent acquisition; DRG, Dorsal root ganglia; DTT, Dithiothreitol; Epoetin alpha/beta, Erythropoietin alpha/beta; FA, Formic acid; FDA, Food and Drug Administration; Fgf12, Fibroblast growth factor 12; GO, Gene Ontology; Hcn channels, Hyperpolarization-activated cyclic nucleotide-gated channels; IAA, Iodoacetamide; Irs2, Insulin receptor substrate 2; Itgam, Integrin subunit alpha M; Kcnab2, Potassium voltage-gated channel subfamily A regulatory beta subunit 2; Kcnb2, Potassium voltage-gated channel subfamily B member 2; Kcns1, Potassium voltage-gated channel modifier subfamily S member 1; LC-MS, Liquid chromatography-mass spectrometry; LED, Light emitting diodes; Lrrk2, Leucine-rich repeat kinase 2; Map4k4, Mitogen-activated protein kinase 4; MEFISTO, MESAging Finding for Spatio-Temporal Ordering; MOFA, Multiomics factor analysis; MS, Mass spectrometry; MRNA, Messenger ribonucleic acid; Nedd4l, NEDD4 like E3 ubiquitin protein ligase; Nefn, Neurofilament heavy chain; NEP, Non-evoked pain; Ntrk1, Neurotrophic receptor tyrosine kinase 1; Parp3, Poly-ADP-ribose-polymerase family member 3; PASEF, Parallel accumulation-serial fragmentation; PBS, Phosphate-buffered saline; Pla2g7, Platelet activating factor acetyl hydrolase; PPI, Protein-protein interaction; Ptpcr, Protein tyrosine phosphatase receptor type C; Scn channels, Sodium voltage-gated channels; SDS, Sodium dodecyl sulfate; SNI, Spared nerve injury; SP3, Single-pot, solid-phase-enhanced sample preparation; STable, Supplementary table; Tgm1, Transglutaminase 1; Trpm8, Transient receptor potential cation channel subfamily M member 8; Trpv1, Transient receptor potential vanilloid channel 1; Vav1, Guanine nucleotide exchange factor 1.

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1. Introduction

Chronic pain is a major public health burden, with a prevalence of 20 % in the global population [1–5]. With its high prevalence, associated comorbidities, and the lack of adequate treatment options, the demand for detailed knowledge of the molecular underpinnings of chronic pain is increasing [5–7]. Although (chronic) pain has been studied extensively over the last decades, its complexity is far from being well understood. Among the many factors that affect its intensity and treatment success are sex and age. For example, some pain conditions are more prevalent in women compared to men [8–11]. These sex differences are subject to major (pre)clinical research efforts [12–15] with the goal to design tailored treatment options. In contrast, the impact of age (i.e. age as in lifespan) on chronic pain is less commonly investigated. In fact, ontogeny, i.e. the organism's lifespan throughout development and aging, plays a significant role for shaping nociception and pain [16]. This has significant implications for the treatment of pediatric and adolescent patients [17] facing limited efficacy and substantial risk of adverse effects [5,18]. These young patient groups are particularly vulnerable, as clinical evidence suggests that pain in early-life correlates with negative outcomes in adulthood, such as pain chronification, depression or anxiety [19–21]. Work on rodent pain models is consistent with these findings showing altered pain sensitivity in pups and juveniles compared to adults [16,22–25]. However, the molecular processes governing pediatric and adolescent pain and how its molecular patterns differ from adult pain are largely unknown.

Proteins serve as cellular building blocks. Their abundance and activity control cellular function and, ultimately, phenotypes, and behaviors [26–29]. Thus, proteomics based on quantitative mass spectrometry (MS) is an important tool for examining dynamic conditions and diseases, such as chronic pain.

Here, we harnessed deep proteome profiling of mouse dorsal root ganglia (DRG) derived from the spared nerve injury (SNI) model of chronic neuropathic pain. We focused on DRG, as they harbor peripheral sensory neurons alongside diverse non-neuronal cell types, all of which are crucially implicated in pain [30–33]. To gain insights into early-life pain, we investigated adolescent (4-week-old) male and female mice in comparison to adult (12-week-old) mice. By applying two different analysis strategies, we determined sex- and age-dependent differences and similarities upon SNI. We specifically focused on sex- and age-shared proteome signatures, matched these to human DRG data, and revealed protein networks that may represent impactful drug targets. By incorporating sex, age, and human biology we offer an innovative resource of enormous translational value, potentially accelerating discoveries in understanding and targeting pain chronification upon nerve injury.

2. Materials and methods

2.1. Experimental mice

Wildtype C57BL/6J mice were purchased from Charles River Laboratories (Massachusetts, USA) and further bred in-house. All mice were group-housed according to sex, age, and experimental condition (SNI, Sham, Naïve) with 2–4 mice per cage, in open housing cages, in a 12 h light/dark cycle with water and food *ad libitum*. Experiments were performed on male and female mice in two age groups (adolescent, i.e. 4 weeks at nerve injury, and adult, i.e. 12 weeks at nerve injury) and divided into three different cohorts, according to their day of sacrifice (day (D) 7, D10, and D14 post nerve injury). We included naïve non-treated mice as additional controls specifically for proteome data analysis. Of note, our animal licence limited the possibility to include the acute early phase after SNI (prior D7), which is the reason why sampling and analysis started at D7. Each cohort consisted of 5–6 animals (for behavior analysis data from different cohorts were combined resulting in high replicate numbers, i.e. up to 35). Mouse handling (weighing,

operation, behavioral experiments and sacrifice) was performed by female experimenters only, as suggested by Sorge *et al.* [34]. Animal experiments were approved by the IACUC at the University of Vienna and the Austrian Ministry for Education, Science and Research (BMBWF; license number 2021–0.138.925), following international ARRIVE guidelines [35] and the EU recommendations (Directive 2010/63/EU) for studies on animals.

2.2. Spared nerve injury (SNI) model of neuropathic pain

Neuropathic pain was induced by the spared nerve injury (SNI) model [36], as described previously [37]. In brief, after isoflurane-anesthesia, and carprofen injection (Rimadyl, Zoetis, New Jersey, USA; 0.05 mL/10 g body weight diluted 1:25 with 0.9 % NaCl), the eyes were covered with eye ointment (Bepanthen Augen- und Nasensalbe, Bayer Austria Ges.m.b.H.) to prevent dehydration, and the surgery site was disinfected with Octenisept (Schülke & Mayr GmbH, Norderstedt, Germany). The trifurcation of the left sciatic nerve was exposed by incising the *biceps femoris* muscle and skin above. The common peroneal and tibial nerves were ligated, with a non-resorbable, braided, 7–0 silk (Vömel, Kronberg, Germany) and transected below the bifurcation. To impede regrowth, a 2 mm nerve segment was excised. The skin was closed using a surgical clip (Fine Science Tools, Heidelberg, Germany), applied by AutoClip Applier (Mikron Precision Inc, California, USA). BraunolÄ povidone-iodine (BraunolÄ, B. Braun Melsungen AG, Melsungen, Germany) was used to disinfect the surgery site. Mice received carprofen injection (0.05 mL/10 g body weight) on D1 post-injury. Surgical clips were removed on D5 post-injury using the AutoClip Remover (Mikron Precision Inc, California, USA). As a control group, Sham-operated mice were used, where the same operation was performed as in SNI mice (including carprofen injection before injury and on D1 post-injury, eye ointment and disinfection) but without ligation and transection of the sciatic nerve branches. In all mice included in this study, bleeding during surgery and postsurgical recovery was similar. Additionally, for proteomic analysis, naïve mice (untreated mice) from D7 were used. Weighing of operated mice was done every day until D7 post-injury, and every second day until D14 post-injury. Mice from each cage were randomly assigned to the three experimental conditions (SNI, Sham and Naïve).

2.3. Mouse behavior

All behavioral assays were performed on SNI and Sham-operated mice, between 8:00 am, and 3 pm, by female experimenters only. Experimenters were officially blinded to sex and experimental condition. However, visible features such as body size, sex-specific physical characteristics, hair loss in the surgical area, sutures and a pain-induced gait due to nerve injury do not allow the experimenter to be fully blinded with regard to the age, sex, and pain model of the mice during observation.

2.3.1. Paw print area (non-evoked pain)

To assess non-evoked pain (NEP)-related behavior a video-based technique was used [38]. In brief, mice were placed on a 1 cm thick Plexiglas plate, surrounded by green light emitting diodes (LEDs), covered by transparent plastic boxes (9.5×9.5 cm) and a red LED plate. Mice were filmed ventrally from a distance of 30 cm, using a portable camera (Hero 8 Black, GoPro, California, USA) with the settings 1080p, 60 frames, linear, for 10 min, in a dark room, without experimenters being present. For the analysis of the paw print area, 10 randomly selected images from the video were investigated for the mean of the contact area using ImageJ Fiji [39]. Images were only included in the analysis when both hind paws of a mouse were placed visibly on the Plexiglas plate, no excessive urination was visible, and when the mouse was not rearing. If fewer than 10 images from an individual could be obtained, this individual was excluded from the analysis for that time

point. Print area data are stated as the ratio between the ipsilateral (injury side; left) and the contralateral (non-injury side; right) hind paw, to eliminate the confounding factor of body weight. Each group consisted of 8–35 and 11–32 mice for adolescent and adult, respectively. Analysis of the print area was independently performed by two experimenters to confirm analysis consistency.

2.3.2. Mechanical stimulation (evoked pain)

After assessment of non-evoked pain, mechanical sensitivity was tested using a Dynamic Plantar Aesthesiometer (37450–001, Dynamic Plantar Aesthesiometer Touch Stimulator: 37400–002, Ugo BasileÀ, Gemonio, Italy) as described previously [37]. In brief, mice were accustomed for 4 h to the testing grid, consisting of 5 × 5 mm square holes, in the week before the baseline testing (Pre). Behavioral assessments were conducted after a 2 h acclimatization period on the testing grid on D7, D10, and D14. The paw withdrawal threshold (PWT; in seconds) was measured as mechanical force (Force Intensity: 10.0 g, Ramp Time: 40 s) applied to the lateral side of the plantar hind paw. Five measurements of each hind paw were assessed, with a recovery period of at least 2 min between each measurement. Mice were excluded from analysis for those time points, at which < 3 values/hind paw could be obtained. PWT are stated as the ratio between the ipsilateral (injury side; left) and the contralateral (non-injury side; right) hind paw. Each group consisted of 9–12 and 10–12 mice for adolescent and adult, respectively.

2.3.3. Statistical analysis

Behavioral data was analyzed using GraphPad Prism 8.0 (California, USA). All data are represented as median ± interquartile range. Replicates are biological replicates. As statistical test mixed-effects analysis with Holm-Sidak multiple comparison correction was used. Adjusted *p*-value: * ≤ 0.0005, ** ≤ 0.0001.

2.4. Isolation of lumbar 3–5 dorsal root ganglia (L3–5 DRG)

Mice were randomly assigned into three different cohorts, depending on the day of sacrifice (D7, D10, and D14 post-injury). After euthanization with CO₂, the lumbar 3–5 dorsal root ganglia (L3–5 DRG) were collected by removing the back skin from the mice, detaching the spinal column from the rest of the body and dorsally cutting open the spinal column under a microscope, exposing the DRG (surgical tools from Fine Science Tools (FST), Heidelberg, Germany and Dumont, Montignez, Swiss). L3–5 DRG of the ipsilateral side (injury-side; left) were then collected, rinsed with ice-cold phosphate-buffered saline (PBS) before flash-freezing in liquid nitrogen and stored at -80°C until further use.

2.5. Protein extraction

The protein extraction procedure closely follows the previously described protocol in Xian *et al.* [40], with a few modifications. In brief, L3–5 DRG were transferred to 250 µL lysis buffer, consisting of 100 mM Tris-HCl, 5 % glycerol, 10 mM dithiothreitol (DTT), 2 % sodium dodecyl sulfate (SDS), 1x cOmplete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). Sonication was performed using a Bioruptor Pico (Diagenode, Seraing, Belgium; 30 s on and 30 s off, 4°C, low frequency) for 10 cycles. For the incubation step at 70°C for 10 min, 1500 rpm agitation was used. The supernatant was transferred to a new tube after centrifugation for 5 min at 10000 x g to remove the remaining tissue debris. Lipids were removed using 5x sample volume of ice-cold acetone and incubated for 4 h at -20°C. To remove the acetone and collect the protein sediment, the samples were centrifuged for 30 min at 14000 x g. After a washing step with ice-cold ethanol (80 % v/v), samples were centrifuged for 30 min at 14000 x g. Protein pellets were air-dried for 30 min at room temperature. Reconstitution of the protein pellet was performed in 50 µL lysis buffer and incubated at 60°C for 10 min with 1500 rpm agitation. Before protein concentration was measured with NanoPhotometer N60 (Implen GmbH, München, Germany) at 280 nm, a

1 min centrifugation at 14000 x g was performed. Of each sample, 50 µg of protein was diluted to a volume of 90 µL with lysis buffer and sonicated (Bioruptor Pico, 30 s on and 30 s off, 4°C, low frequency). Reduction of the samples with 5 mM DTT was performed for 30 min at 60°C, with 1000 rpm agitation. After alkylation with 20 mM iodoacetamide (IAA) for 30 min at room temperature in the dark, samples were quenched with 5 mM DTT for 15 min at room temperature.

2.6. SP3-assisted protein digestion

Single-pot, solid-phase-enhanced sample preparation (SP3) was performed as described in Xian *et al.* [40] adapted from Hughes *et al.* [41]. In brief, 10 µL of pre-mixed Sera-Mag SpeedBead beads (Cytiva, Marlborough, Massachusetts, USA) were used for 50 µg protein. Binding of proteins to the beads was initiated using one volume of absolute ethanol. After incubation at 24°C for 5 min with 1000 rpm agitation on a Thermomixer (Eppendorf, Hamburg, Germany) and a 2 min incubation on a magnetic rack (DynaMag™-2 Magnet, Invitrogen by ThermoFisherScientific, Waltham, USA), the supernatant was removed and the beads with attached proteins were rinsed three times with 400 µL of ethanol (80 % v/v). Reconstitution of the beads was done in 50 µL digestion buffer (50 mM ammonium bicarbonate, pH 8). Protein digestion was done with 2 µg of trypsin/Lys-C for 18 hr at 37°C with 950 rpm agitation. Following digestion, 100 % acetonitrile (ACN) was added to a final concentration of 95 %. Samples were incubated for 8 min at room temperature, followed by 2 min incubation on a magnetic rack. After removing the supernatant, the beads were rinsed with ACN to a final concentration of 100 %. After another incubation for 2 min on a magnetic rack and removal of ACN, the samples were air dried for 1 min. Peptide elution was done using 20 µL LC-MS grade water. After measuring the peptide concentration using NanoPhotometer N60 (Implen GmbH) at 205 nm, peptide samples were acidified with formic acid (FA) to a final concentration of 0.1 % and stored at -20°C until LC-MS/MS analysis. An additional preparation step was performed for some samples to completely remove ACN before mass spectrometry analysis. For this, samples were thawed, and centrifuged for 1 min at 17000 x g. Samples were then transferred into a 96 well plate (Eppendorf, Hamburg, Germany) and air-dried for 45 min at 30°C, followed by 20 min at 45°C using Concentrator plus (Eppendorf). Dried peptides were resuspended in 20 µL LC-MS grade water, vortexed for 10 min, and sonicated using Elmasonic S (Elma Schmidbauer GmbH, Singen, Germany) for 5 min. Samples were frozen at -20°C until the next day when they were centrifuged for 1 min at 1000 x g. After the peptide concentration was measured, samples were acidified with FA to a concentration of 0.1 %, centrifuged for 1 min at 1000 x g and stored at -20°C for further use.

2.7. LC-MS/MS setup

Mass spectrometry was similarly done as described by Xian *et al.* [40]. Nanoflow reversed-phase liquid chromatography (Nano-RPLC) was executed using a NanoElute system from Bruker Daltonik (Bremen, Germany). 250 ng of digested peptides were injected and separated using a 25 cm × 75 µm column, packed with 1.6 µm C18 particles (Ion-Opticks, Fitzroy, Australia) with a 60 min gradient. For mobile phase A, 100 % water (LC-MS grade), 0.1 % FA, was used. Mobile phase B consisted of 100 % ACN, 0.1 % FA. For the whole separation, apart from the last 7 min, where the flow rate was set to 400 nL/min, the flow rate was set to 250 nL/min. A linear increase from 4 % to 20 % was used for mobile phase B, for the first 35 min gradient, followed by a linear increase to 35 % within 17 min. Within 0.5 min a strong increase to 85 % was achieved. To elute all hydrophobic peptides, the flow rate was kept at 400 nL/min for 7 min, with 85 % of mobile solvent B. A hybrid TIMS quadrupole TOF mass spectrometer (timsTOF pro, Bruker Daltonik) was coupled to the NanoElute LC, via a CaptiveSpray ion source. Sample analysis was acquired in data-independent acquisition (DIA) mode,

combined with parallel accumulation serial fragmentation (PASEF). For the acquisition, the TIMS analyser was set to 100 % duty cycle with 100 ms of accumulation and ramp time. The ion mobility range was set to 0.65–1.4 (1/k0). Precursors with m/z between 300 and 1150 were defined in 14 scans containing 32 isolation steps with an isolation window of 25 Th in each step, led to a total cycle time of 1.59 sec. Linear collision energy was from 59 eV at 1/k0 = 1.6–20 eV at 1/k0 = 0.6.

2.8. DIA-PASEF data processing

DIA-NN (version 1.8.2 beta 22) was used to process DIA-PASEF data in library-free mode based on the *M. musculus* proteome database as described in Xian et al. [40]. This study was analyzed together with several additional datasets from mouse DRG, which are not included in this study. To predict theoretical peptide spectra, retention time, and ion mobility, a deep learning-based method within DIA-NN was used. For *in silico* digestion, Trypsin/P was used allowing a maximum of 1 missed cleavage. Peptides with a maximum variable modification of 2 were allowed, whereby the variable modifications were specified as methionine oxidation, N-terminal acetylation, and N-terminal methionine excision. Carbamidomethylation on cysteine was set as a fixed modification. Peptide length was restricted from 7 to 30 amino acids. The m/z range was aligned with the DIA-PASEF acquisition method and set to 350–1150 for precursors and 100–1700 for fragment ions. The mass accuracy for MS1 and MS2 was set to automatic determination. Protein inference was set to “Genes”. The option “Heuristic protein inference” was unchecked. For cross-run analysis, match-between-run (MBR) was checked. For quantification, RT-dependent cross-run normalization and “Composite (high precision)” options were used. Additional processing of the DIA-NN search output was done using the R [42] package DIA-NN (<https://github.com/vdemichev/diann-rpackage>), extracting the quantitative intensity for all identified peptides and protein groups (q -value ≤ 0.01 was applied to precursor and protein group level).

2.9. Differential expression analysis

12,778 protein groups were quantified. For differential expression analysis (SNI – Sham) protein groups were selected if they were quantified in $\geq 75\%$ of replicates within each experimental condition (male/female, adolescent/adult and different time points). Quantitative data were imported into the R package ProTIGY (<https://github.com/broadinstitute/protigy>), and $\log_2$ -transformed. Two-sample moderated t -test was used to test for statistical significance of individual contrasts between SNI-Sham (F = female, M = male, 4w = adolescent, 12w = adult, D7/10/14). Proteins with adjusted (Benjamini and Hochberg, for multiple testing) p -value ≤ 0.05 (hereafter referred to as the q -value) were considered as differentially expressed proteins (DEPs) in each contrast. For all comparisons of DEPs, the first mapped protein of each protein group was considered.

2.10. Cell type analysis

To determine the likely origin of sex-shared DEPs, single-cell RNA-seq datasets (<https://painseq.webflow.io/>; accessed Dec 2024; [43]) was used. To enable cell type mapping, gene names were used instead of protein names. Gene expression was interrogated using the tools ‘DRG neurons’ and ‘DRG non-neurons’. Due to limited input parameters offered by the web-interface, 50 genes were mapped per iteration. Gene expression was scaled within the 50 input genes, grouped per ‘Atlas annotation’ (for immune cells grouping was done by ‘cell_type’). For DEPs shared by male and female mice (sex-shared), ‘mouse’ was used as species (for immune cells no species was selectable). For sex-shared DEPs matching human transcriptome and proteome data [12,44–47], ‘human’ was used as species.

2.11. Protein-protein interaction network

The protein-protein interaction (PPI) [48] network was built on DEPs shared by male and female mice (sex-shared), combined from both age groups (adolescent and adult). The PPI network was investigated and visualized using Cytoscape (v3.10.1) [49] and the stringApp (STRING: protein query) [50]. *Mus musculus* was used as species. To assemble the PPI network, the full STRING network option and a confidence (score) cut-off of 0.7 (high confidence) was used. Node size is based on the node degree. Edge thickness is related to score. With the aim to identify genes for which interactions with FDA-approved drugs are known and which can be potentially adapted for pain therapy, the Drug – Gene Interaction Database (DGIdb v4.2.0, <https://old.dgiddb.org/>) was used [51,52]. A Drug-Gene Interaction search was performed for unique matches, with the preset filter for approved drugs. Nodes are categorized into targets for FDA-approved drugs indicated for pain (thick dark grey outline), targets for FDA-approved drugs (dashed grey outline) or non-drug targets (thin light grey outline). Protein names highlighted in bold are sex-shared DEPs matching a human neuropathic pain study [12]. Single nodes, not being connected within a network, were removed, except for nodes identified in neuropathic pain patients [12] or as drug targets.

2.12. Pathway analysis

For sex-shared DEPs (adolescent and adult sex-shared DEPs combined) a functional Gene Ontology (GO)-analysis was done using Cytoscape (v3.10.1) [49] and the ClueGO app (v2.5.10) [53]. Species was set to *Mus musculus*. GO-Biological Processes (GO-BP), KEGG and Reactome pathways were used as Ontologies/Pathways (all updated May 2024). Network specificity was set to medium. GO Term fusion was used and only pathways were considered with a $p \leq 0.05$. The GO Term/Pathway Network Connectivity (Kappa Score) was set to 0.5 (medium). The other pathway options were left as default. Enrichment/Depletion (Two-sided hypergeometric test) and Benjamini-Hochberg were used. Grouping options were set as default.

2.13. Factor analysis with MEFISTO

We applied multiomics factor analysis (MOFA) (Bioconductor package MOFA2) [54] to find the major axes of biological variation in our proteome dataset. Specifically, to exploit the unique structure of our data, in which samples are no longer independent of each other but exhibit a temporal relationship, we used MESAge FInding for Spatio-Temporal Ordering (MEFISTO) [55], which is an extension of the MOFA framework. We used the muon package (<https://github.com/scverse/muon>), which is a Python framework for multiomics data and allows usage of MEFISTO. Similar to most modern machine learning methods, the MEFISTO algorithm benefits from a high number of features (i.e. quantified proteins) in the input dataset. This is why we included data from naïve mice in this analysis. In addition, not all of the input features are necessarily informative, meaning some might not carry any information about similarity or differences between our experimental groups. Even though MEFISTO is principally able to impute missing data, we did not use this option. Therefore, we removed potential noise and missing data in our proteome datasets in a two-stage process. First, we removed any protein with more than 25 % missing values in any time x condition group by counting the number of missing values and dividing this count by the total number of samples in this group. Subsequently, we used two-way ANOVA as implemented in the statsmodels v0.13.5 (<https://www.statsmodels.org/stable/index.html>) on the $\log(x + 1)$ transformed data to assess the influence of the factors ‘pain condition’ and ‘time’ on the mean abundance of each protein and filtered out those that did not show any significant factor influence. In brief, for each protein we fitted an ordinary least squares regression model using pain condition and time as categorical variables, subsequently testing for a non-zero slope of either variable or their interaction

using the *F*-test. We then used the Benjamini-Hochberg procedure to correct the resulting *p*-values for multiple testing per variable and only retained those proteins with an adjusted *p*-value < 0.1 for either variable or their interaction. In total, we retained 9435 proteins after filtering for missing values, which was further reduced to 6653 proteins after applying the ANOVA filter. We then used the $\log(x + 1)$ transformed and ANOVA-filtered data to fit a 50-factor MEFISTO model. We used the 50-factor MEFISTO model because the variance described by our main metadata features was only subtle, compared to the overall variance when using the default 10-factor model. In brief, we reformatted the transformed and filtered data into an AnnData object using `anndata` v0.8.0 and subsequently used the `MOFApy2` (v0.6.7) interface of `muon` v0.1.2 to fit MEFISTO with `n_factors = 50`, `groups_label = 'condition'`, `center_groups = False`, `n_iterations = 2000`, `smooth_covariate = 'time-factor'`, `smooth_kwargs = {'n_grid': 50, 'start_opt': 50, 'opt_freq': 50}`, `seed = 2023`, `convergence_mode = 'fast'`. Code used for MEFISTO analysis was deposited at Zenodo under the following DOI: 10.5281/zenodo.12772115. The computational results presented were obtained using the CLIP cluster (<https://www.clip.science/>).

3. Results

3.1. Deep proteome profiling in mouse dorsal root ganglia upon peripheral nerve injury

We investigated the proteome of lumbar dorsal root ganglia (lumbar (L) level 3–5, L3–5 DRG) in male and female mice of two age groups (adolescent mice, 4 weeks at nerve injury, and adult mice, 12 weeks at nerve injury) after unilateral spared nerve injury (SNI) [36] (Fig. 1). Sham injury served as control condition. The time points of DRG harvest were chosen to broadly cover the initial phase (days (D) 7–14) of neuropathic pain establishment (Fig. 1). For deep proteome profiling, we performed mass spectrometry, in particular data-independent acquisition with parallel accumulation serial fragmentation (DIA-PASEF) [56] (please see methods for details). In our previous work we have demonstrated the extraordinary performance of DIA-PASEF in combination with DIA-NN for deep profiling of mouse tissues such as paw skin and sciatic nerve [40].

To confirm the induction of neuropathic pain, we assessed pain-related behavior outcomes with commonly used paradigms: on the one hand we determined the mechanical hypersensitivity (evoked pain; Fig. 2A-B) and, on the other hand, we measured the size of the paw print area as a proxy for non-evoked pain (NEP) [38,57] (Fig. 2C-D). As expected, mechanical hypersensitivity and non-evoked pain behaviors were significantly different to baseline and Sham at all time points after SNI (Fig. 2). In accordance with previous reports for male and female mice [58–60], these pain-related behaviors were similar across all sex and age groups and consistent among analyzed time points [58,60,61].

Employing mass spectrometry in DIA-PASEF mode, we quantified over 12,000 protein groups across all experimental conditions (please see methods for quality criteria) (Fig. 3A, STable 1). Compared to previously published DRG proteomes (including our own studies) [38, 62–64] this dataset constitutes the most comprehensive catalogue of the protein-set-up of mouse DRG reported thus far. Besides the high consistency of profiling and low variance (SFig. 1), another indication for the reproducible and sensitive performance of our workflow is the robust quantification of assorted ion channels known to be involved in nociception and pain (Fig. 3B-C). For example, transient receptor potential vanilloid channel 1 (Trpv1), voltage-gated calcium channel subunits (e.g. Cacna2d1), as well as voltage-gated sodium channels (e.g., Scn8a, Scn9a, Scn10a, Scn11a), and voltage-gated potassium channels (e.g. Kcna2, Kcna2b) [65,66] (Fig. 3B-C, STable 1).

3.2. Identification of sex- and age-shared proteome signatures in mice

Based on our proteome data, we asked whether similarities and differences across sex and age groups can be identified. We applied a significance cut-off (*q*-value ≤ 0.05) and determined differentially expressed proteins (DEPs; SNI versus Sham) after nerve injury. DEPs at each time point are listed in STable 2, and respective volcano plots of all comparisons are shown in SFig. 2. Among the DEPs were several aforementioned ion channels (Fig. 3D, STable2). It is noteworthy that the number of DEPs varied in experimental groups among analyzed time points, compare, for example, adolescent female mice at D7 with D14 (STable 2). Technical variability is unlikely to account for this difference among experimental groups given the high quality of our dataset: high

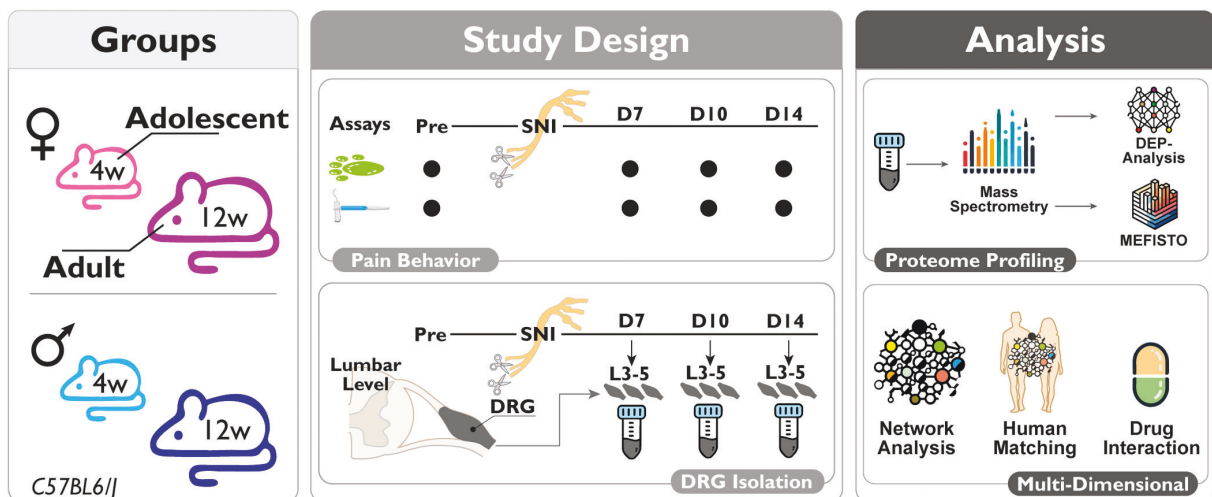


Fig. 1. Experimental design. The experimental groups consisted of adolescent (4w) and adult (12w) male and female mice. We investigated nerve injury-induced proteome dynamics in the spared-nerve injury (SNI) model of neuropathic pain in mice. Sham-treated mice (no nerve injury upon skin incision) served as controls. Pain-associated behaviors were assessed three to four days before nerve injury (Pre) and on D7, D10, and D14 after nerve injury. On days (D) 7, 10 and 14 lumbar 3–5 (L3–5) dorsal root ganglia (DRG) were isolated and submitted to proteome profiling, using LC-MS/MS and data-independent acquisition with parallel accumulation serial fragmentation (DIA-PASEF). Differentially expressed proteins (DEPs) and longitudinal differences (MEFISTO) between SNI vs. Sham were determined, followed by matching to human transcriptome and proteome DRG data, and drug-gene interaction analysis.

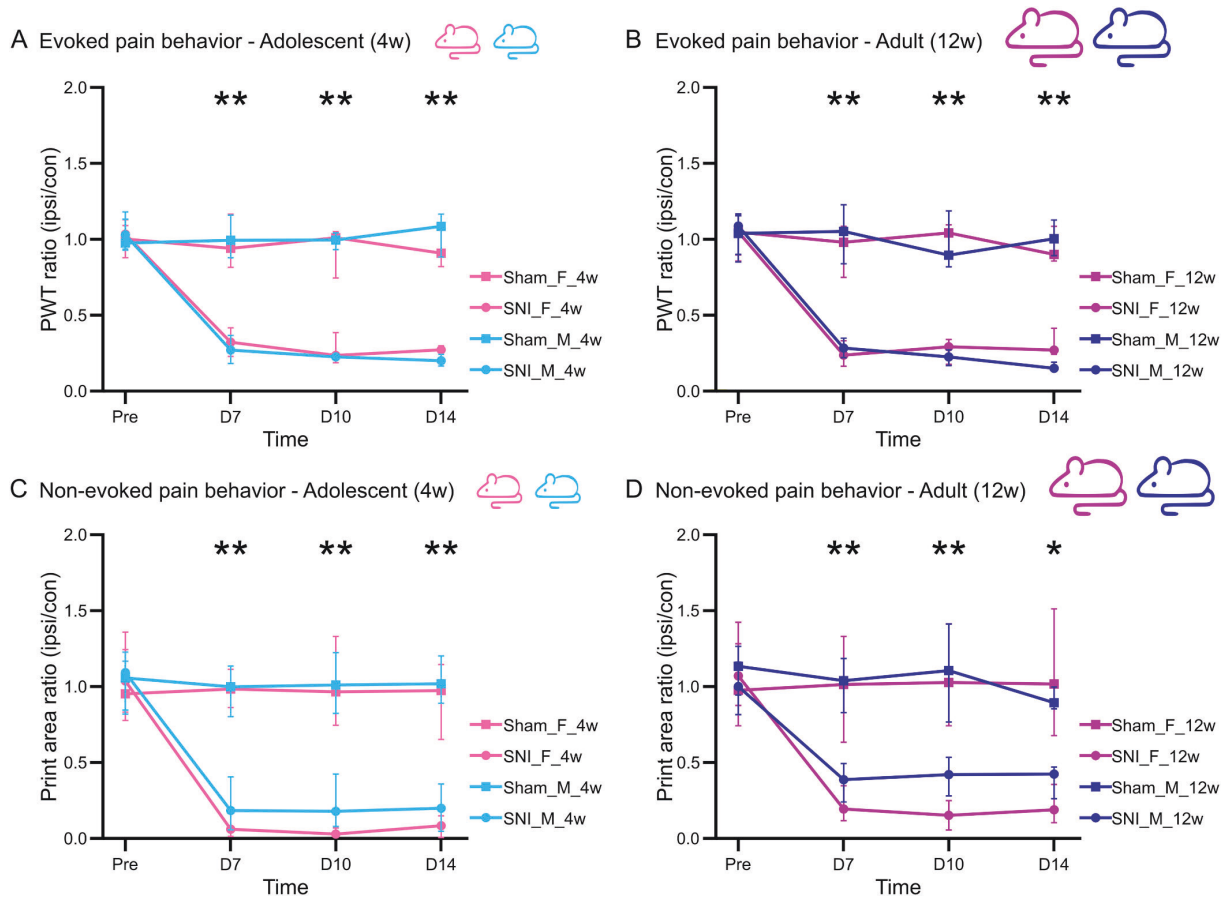


Fig. 2. Non-evoked and evoked pain-related behaviors after peripheral nerve-injury. Evoked (hypersensitivity to innocuous mechanical stimulation) and non-evoked (print area) pain-related behavior was assessed before nerve injury (Pre) and on day (D) 7, 10, and 14 post nerve injury. Mechanical hypersensitivity was assessed by Dynamic Plantar Aesthesiometer (Ugo BasileÅ). Non-evoked pain (NEP) was assessed by determining the size of the hind paw print area. (A-B) SNI resulted in significant mechanical hypersensitivity from D7 to D14 post nerve injury independent of the sex and age of the subjects, as evidenced by a marked reduction in paw withdrawal thresholds (PWT, ratio ipsilateral (injured)/contralateral hind paw) for mechanical stimulation, compared to both, pre-measurements and the Sham group. The line graph indicates the mechanical hypersensitivity in SNI (circle) and Sham (square) (A) adolescent and (B) adult mice of both sexes. (C-D) Upon SNI non-evoked pain (NEP) behavior increased as demonstrated by a significant reduction in the print area of the injured side compared to the unaffected side. The line graph indicates the print area (ratio ipsilateral/contralateral hind paw) as an indication for non-evoked pain in SNI (circle) and Sham (square) (C) adolescent and (D) adult mice of both sexes. Data are represented as median $\pm$ interquartile range. Mixed-effects analysis with Holm-Šidák multiple comparison correction was used. Adjusted p -value: * ≤ 0.0005 , ** ≤ 0.0001 . (A) $N = 9-12$ mice/group and condition. (B) $N = 10-12$ mice/group and condition. (C) $N = 18-35$ mice/group and condition. (D) $N = 11-32$ mice/group and condition.

reproducibility and correlation paired with low variation of protein identifications across all samples (SFig. 1).

Since our aim was to globally investigate the initial establishment of neuropathic pain – a phase, which cannot be assigned to distinct time points – we combined DEPs from all three time points for the remainder of this study. Our data reveal hitherto unrecognized differences in nerve injury-triggered DRG proteome changes between both age and sex groups (Fig. 3D). The abundance of each quantified protein, at each time point, and in all four experimental groups can be found in STable 1 and 2. DEPs, which are preferentially regulated in one sex within an age group are listed in STable 3. Interestingly, many of these have been discovered in previous mouse studies to be associated with SNI, such as C5ar1 [67], Hcn1 [68], Kcns1 [69] or Trpm8 [70], validating our findings.

Besides differential expression, we observed shared expression of distinct DEPs upon SNI, i.e. 143 and 131 DEPs, which overlapped between male and female mice at adolescent and adult ages, respectively (Fig. 3E-G, STable 4). Such a sex-shared set of proteome changes has

largely been undefined in DRG. Also, these sex-shared DEPs exhibited similar abundance changes in male and female mice as suggested by high positive correlation at all investigated time points (SFig. 3). Taken together, we not only present distinct sex- and age-dependent proteome alterations (STables 3), but specifically deciphered sex-shared DEPs exhibiting similar regulation in male and female DRG of either age group after nerve injury (STable 4).

DRG harbour diverse cell types (subpopulations of peripheral sensory neurons and non-neuronal cells like glia and immune cells [71]) and our bulk proteomics approach cannot differentiate among these. This is why we interrogated the most comprehensive single-cell RNA-seq dataset (<https://painseq.webflow.io/>; accessed Dec24; [43]) to determine the likely origin of our sex-shared DEPs (STable 5). Based on this RNA-seq reference, we apparently sampled proteins across different subpopulations of DRG neurons (e.g. Calca-expressing ones and Trpm8-expressing ones) and, prominently, across several immune cell types (e.g. macrophage populations and neutrophils). In addition, we profiled many proteins from DRG neurons expressing the

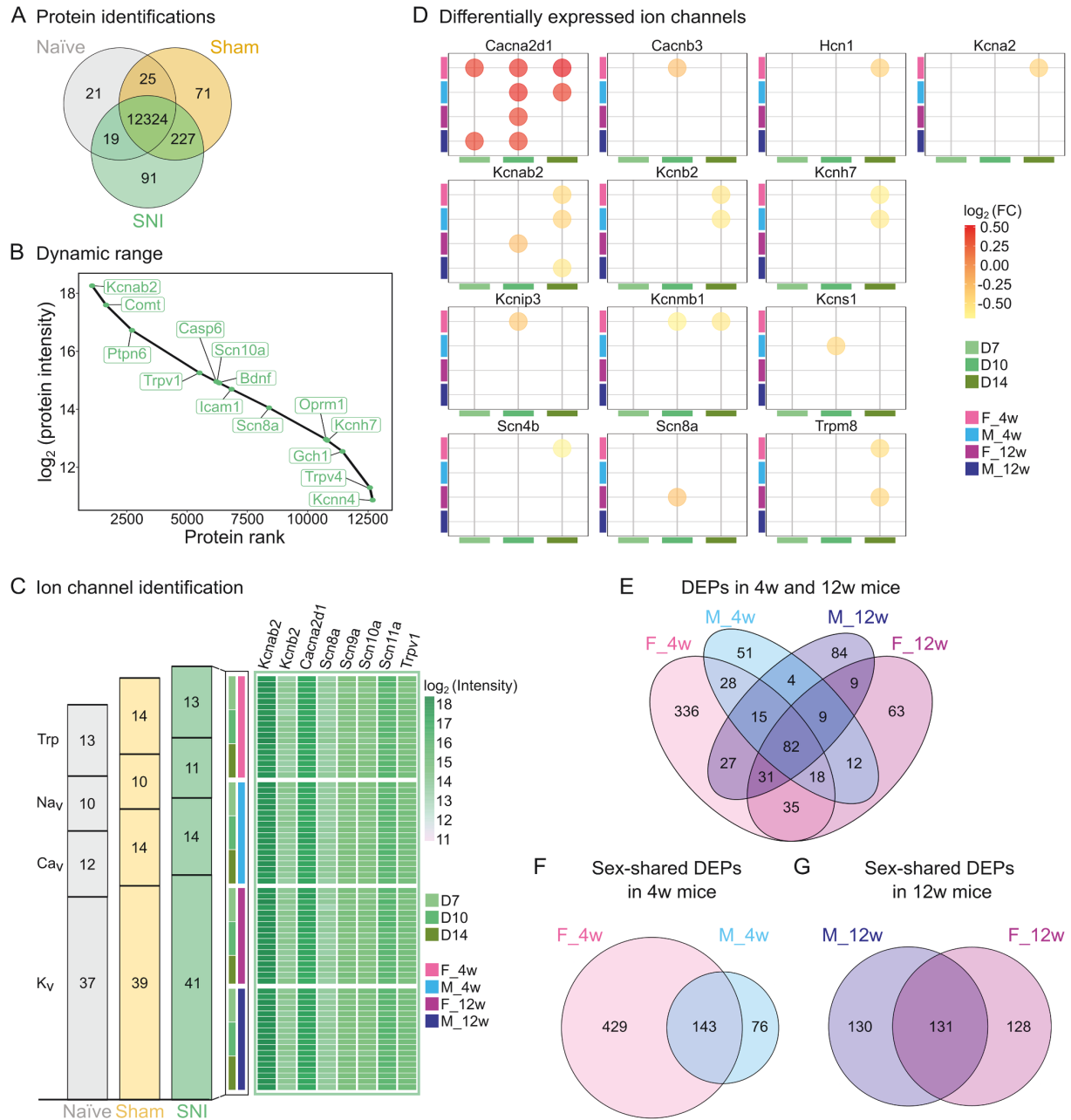


Fig. 3. Deep proteome profiling in mouse DRG upon neuropathic pain. (A) Venn diagram shows unique and shared protein groups (protein IDs) across experimental conditions (Naïve, Sham, SNI). Naïve non-treated mice were included as additional controls for proteome data analysis. In each condition more than 12,000 protein groups were identified (see quantified protein groups in STable1). (B) Representative data of the dynamic range of protein abundance in SNI samples. The y-axis shows the log₂-transformed protein intensity. A selection of pain genes and ion channels known to be involved in nociception and pain are depicted to indicate their relative abundance. (C) Numbers of detected ion channels (classified by Trp, sodium, calcium, and potassium channels) in each experimental condition (Naïve, Sham, SNI). The heatmap on the right shows the abundance of an exemplary selection of ion channels in SNI samples. Color legend reflects log₂-transformed protein intensity of SNI samples. (D) Examples of differentially expressed ion channels when comparing SNI with Sham samples at indicated time points (D7, D10, and D14) and across sex and age groups. Color legend reflects log₂-transformed fold change (FC). (E-G) Venn diagrams show the number of DEPs between SNI vs. Sham (q -value ≤ 0.05) across sex and age groups (irrespective of time point; please see STable 2 for DEPs per time point).

adaptive-response gene *Atf3* (Activating transcription factor 3) validating that we indeed analyzed DRG affected by the nerve injury (STable 5).

3.3. Human-matched proteins for network-level pharmacology of neuropathic pain

Identifying protein signatures that are associated with neuropathic

pain independently of sex represents a highly valuable knowledge base for optimized drug discovery efforts aiming for more broadly relevant “pain targets”. Nonetheless, a pre-requisite for their translatability is the evolutionary conservation in mice and humans [44,47,72–74]. Previous work by Ray and colleagues [44] has shown an evolutionary divergent part in the molecular set-up of mouse and human DRG, which needs to be considered for translational studies. Thus, we intended to validate the presence of identified sex-shared murine protein signatures in human DRG. To this end, we harnessed previous profiling efforts (mostly RNA-based and, sparsely, protein-based) of human DRG [12,44–47] and asked whether DEPs in our dataset have been shown to be expressed in human DRG. 180 of 192 (93.75 %) murine sex-shared DEPs (we combined sex-shared DEPs from adolescent and adult mice) were found to be expressed in human DRG (Fig. 4A, STable 6), i.e. they represent human-matched DEPs. Among these, 75 were shared by both age groups, i.e. they were sex-and age-shared (Fig. 4A; SFig. 4A-B; STable 6).

Furthermore, we compared human-matched DEPs to a recent seminal study on neuropathic pain patients [12] (STable 6). Ray and colleagues [12] investigated transcriptome changes of DRG in a sizable cohort of neuropathic patients undergoing vertebrectomy (for malignant tumors). Fifteen sex-shared murine DEPs matched with transcripts altered in neuropathic pain patients (highlighted in bold in Fig. 4B and STable 6; listed in SFig. 4C-D). Remarkably, the direction of their regulation (up or down) correlated across species in all but two candidates (Btd8 and Lrrk2, see STable 6, SFig. 4D). However, the sexual dimorphism reported in human DRG was not reflected in our sex-shared mouse data. All but one (Tgm1; transglutaminase 1) altered transcript in neuropathic pain patients were either regulated in male or female patients (SFig. 4D). This is in contrast to the sex-shared regulation we observed in mouse DRG. The reasons for this divergence may be manifold ranging from known discrepancies between transcript and protein levels [26,28,29,75,76], differences in the origin of neuropathic pain (nerve injury in mice versus cancer followed by vertebrectomy in humans), and potential species differences, which await detailed study once protein-based data on human DRG will be available.

Nonetheless, the observed overlap of mouse and human neuropathic DEPs strongly suggests their evolutionary conserved implication in neuropathic pain entities.

In a next step, we interrogated protein networks among human-matched DEPs (Fig. 4B; associated biological pathways are listed in STable 7) and we used the Drug Gene Interaction Database (v4.2.0) [51, 77] to ask, whether they may represent targets for approved drugs. Forty-two human-matched DEPs were targeted by FDA-approved drugs (STable 8). Among these were drugs commonly used in pain therapy (targets marked with thick grey outline in Fig. 4B; STable 8): the calcium channel blocker Pregabalin (target: Cacna2d1, Calcium voltage-gated channel auxiliary subunit alpha2delta 1), glucocorticoids (target: Irs2, Insulin receptor substrate 2 and Ntrk1, Neurotrophic receptor tyrosine kinase 1), the opioids Morphine and Fentanyl (target: Itgam: Integrin subunit alpha M), antidepressants such as Mirtazapine and Clozapine (target: 5HT3A, 5-Hydroxytryptamine receptor 3A, also known as HTR3A), and cytokine-targeting inflammatory inhibitors like Etanercept and Infliximab (target: Ptpcr, Protein tyrosine phosphatase receptor type C, also known as CD45).

Other suggested drugs targeting human-matched DEPs (STable 8) have neither been approved for pain nor been routinely implemented in pain therapy. For example, the kinase-inhibitors Imatinib (target: Ntrk1) and Vandetanib (targets: Lrrk2, Leucine-rich repeat kinase 2, and Map4k4, Mitogen-activated protein kinase 4), inhibitors of Parp3 (Poly-ADP-ribose-polymerase family member 3), voltage-gated potassium channel inhibitors, and various pleiotropically acting statins. In addition, Epoetin alpha (target: Vav1, Guanine nucleotide exchange factor 1) and Epoetin beta (targets: Ptpcr and Pla2g7, Platelet activating factor acetyl hydrolase) were suggested.

Overall, neuroimmune interactions have emerged to play a substantial and partly sex-divergent role in pathological pain [78–81].

Accordingly, in our network of human-matched sex-shared DEPs Itgam, Ptpcr and Vav1 represent prominent nodes interconnecting diverse processes associated with inflammatory and immune responses, e.g. T-cell- and B-cell- as well as leukocyte-related pathways (Fig. 4C, STable 7). Not surprisingly, these three nodes are targets of multiple approved drugs including anti-inflammatory corticoids, immune suppressors, and opioids (Fig. 4D).

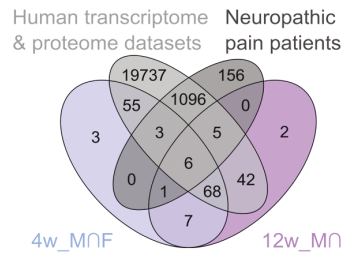
3.4. Multidimensional factor analysis validates sex- and age-shared protein signatures of neuropathic pain

We next assessed nerve injury-induced proteome alterations from a multidimensional point of view with the aim to uncover longitudinal proteome dynamics and exploit the unique structure of our data: Our data were obtained at different times points after nerve injury (D7, D10, D14) and may exhibit a temporal relationship, which is not reflected by classical differential expression analysis presented before (Fig. 3E-G; STable 4). Specifically, we sought to identify major axes of variation in our data to globally interrogate commonalities and differences. To this end, we employed MEFISTO (MEsage Finding for Spatio-Temporal Ordering) [55], an extension of multiomics factor analysis (MOFA) [54]. MEFISTO represents a dimensionality reduction method to account for spatial and, in our case, temporal dependencies between analyzed samples. It has previously been applied for the system-level analysis of multimodal data such as mouse development [55] and identification of COVID-19 severity markers [82]. We fitted a 50-factor MEFISTO model with time (D7, D10, and D14) as the continuous covariate and experimental condition (Naïve, Sham, SNI) as the grouping variable (please see methods for details; SFig. 5A). In this manner, we identified factors that differentiated mice by sex and age but independently from the experimental condition: factor 9 with 237 proteins ($p \leq 0.05$; STable 9) mildly by sex (as judged from the silhouette score and the factor value; SFig. 5B-D) and factor 36 with 333 proteins ($p \leq 0.05$; STable 9) by age (as judged from the silhouette score and the factor value; SFig. 5B-D).

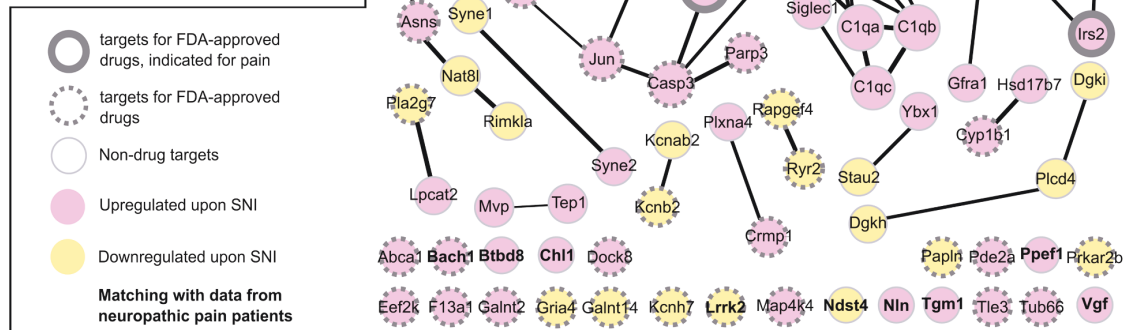
Intriguingly, and translationally highly relevant, MEFISTO also pinpointed two factors that robustly (as judged from the silhouette score SFig. 5B, and the factor value Fig. 5A-B) discriminated SNI-associated proteome dynamics versus those in Sham and Naïve controls (Fig. 5A-B; STable 9): factors 14 and 37 with 431 and 243 proteins, respectively ($p \leq 0.05$). Thus, factors 14 and 37 harbor protein signatures and biological pathways (STable 9) that are shared among adolescent and adult as well as male and female mice. Biological pathways prominently associated with factor 14 and/or 37 were related to (i) protein transport (e.g. kinesin isoforms), (ii) ion transport, ion channel activity and action potential (including voltage-gated ion channels such as Cacna1d, Hcn channels, Scn channels, Trpv1, and other proteins like Lrrk2, Fgf12, Nedd4l), and (iii) synapse-related terms (e.g. several ionotropic glutamate receptors and Camk2A) (Fig. 5C; STable 9). On the other hand, (i) immune and inflammation-related pathways (such as Toll-like receptor signaling, interferon response, leukocyte-, B-cell- and microglial signaling, Jun kinase cascade), (ii) metabolic pathways, and (iii) DNA-damage, apoptosis and defense response pathways were mostly anti-associated with factor 14 and/or 37 (Fig. 5C; STable 9). These findings are in line with the well-established relevance of neuronal function, metabolism, as well as immune/inflammatory signaling for chronic pain [5,83,84]. Importantly, these data go beyond current knowledge and pinpoint candidates and their associated networks involved in these biological processes with unprecedented opportunities for therapeutic targeting. Along these lines, it is noteworthy that identified candidates are shared across experimental groups, i.e. they are largely independent of the sex and age of mice. Consequently, they may hint towards the existence of commonly triggered alterations by nerve injury.

Importantly, roughly 40 % of human-matched sex-shared DEPs (Fig. 4A, STable 6) overlapped with the protein signature in MEFISTO factors 14 and 37 (Fig. 5D, STable 10). This overlap included 30 out of

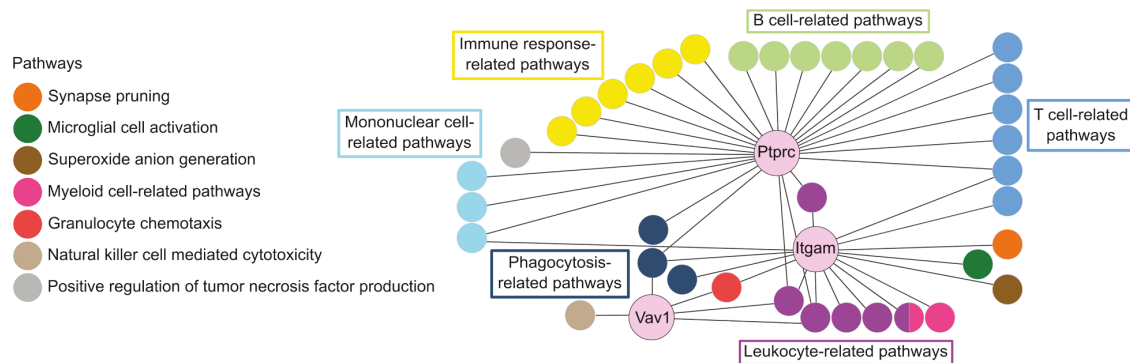
A Human-matched DEPs



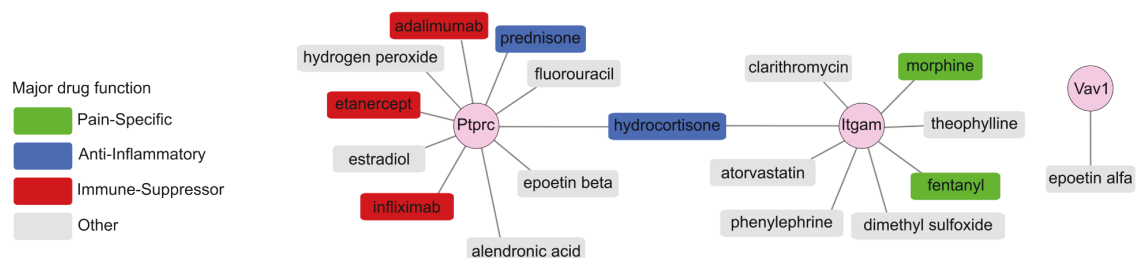
B Network of human-matched DEPs



C Pathways of highly-connected nodes



D Drug-Gene interactions of highly connected nodes



(caption on next page)

Fig. 4. Human-matched protein signatures and systems pharmacology. (A) Venn diagram shows the number of sex-shared DEPs across age matching human transcriptome and proteome DRG data [44–47], as well as data from neuropathic pain patients [12]. 180 proteins are evolutionarily conserved between our sex-shared DEPs (4w and 12w combined) and human DRG data. Among these, 75 DEPs are sex- and age-shared (please see SFig. 4A-B, STable 6). (B) Protein-protein interaction [48] network of sex-shared DEPs (180 adolescent and adult DEPs combined, from 4 A) retrieved from Cytoscape (v3.10.1) [49] and STRING [50]. This interaction map was generated using high confidence (0.7). Nodes are categorized into targets for FDA-approved drugs indicated for pain (thick dark grey outline), targets for FDA-approved drugs (dashed grey outline) or non-drug targets (thin light grey outline) based on the Drug-Gene-Interaction-Database (DGIdb v4.2.0, <https://old.dgiddb.org/>, please see STable 8). Proteins in bold were identified in neuropathic pain patients [12]. The color of the nodes reflects upregulation (red) or downregulation (yellow) of DEPs in mice upon SNI. Node size is relative to the node degree. Edge width is relative to the score. Nodes without any connection are hidden from the network, unless they are identified in neuropathic pain patients [12] or as drug targets. (C) Visualization of pathways associated with highly connected nodes from panel B, i.e. *Itgam*, *Ptpcr* and *Vav1*, using Gene Ontology- Biological Process (GO-BP). Colors represent different pathways (complete pathway analysis is shown in STable 7). (D) Visualization of drugs targeting highly connected nodes from panel B, i.e. *Itgam*, *Ptpcr* and *Vav1*, using the Drug-Gene-Interaction-Database (DGIdb v4.2.0, <https://old.dgiddb.org/>, please see STable 8).

75 sex- and age- shared DEPs (SFig. 4A-B) such as the highly connected hubs *Itgam*, *Ptpcr*, and *Vav1* (Fig. 4C-D). Among these, 11 represent targets for FDA-approved drugs (marked with * in Fig. 5E, STable10). Being identified by two independent analysis approaches (MEFISTO-based factor analysis, and differential expression analysis) these sex- and age-shared proteins (Fig. 5E, STable10) may constitute a high confidence and evolutionary conserved core-set of nerve injury-associated proteins. To interrogate their cell-type specific expression pattern, we again harnessed single-cell RNA-seq data (<https://painsseq.webflow.io>, accessed Dec 2024; [43]) and compared human and mouse DRG. While they appeared quite similar with a prominent immune component, some species-differences became apparent in neurons: several conserved core-set proteins seem to be expressed in human DRG neurons but not in mouse neurons. Among those are *C1qb* (Complement C1q B chain), *Ctss* (Cathepsin S), *Itgam*, *Ptpcr*, and *Vav1* to name a few (SFig. 6).

4. Discussion

Our proteome-based investigation captures the complexity of DRG alterations upon neuropathic pain at the protein network level. In addition, we integrate sex and adolescent (versus adult) age as biological variables. In contrast to most studies, we deliberately focus on biological pathways constituting proteome signatures that are shared across sex- and age-groups. By matching our findings with data from human DRG and drug-gene interactions, we offer a translationally valuable resource for systems pharmacology, drug discovery, and drug repurposing, which may benefit adolescents, women, and men, equally.

Ever increasing evidence has demonstrated the importance of integrating sex and age in pain-related research in both rodent models and humans. Firstly, pain perception and experience can vary considerably between males and females, likely governed by a combination of sex hormones [15,85], differences in neuroimmune interactions [79,86] and hitherto unknown sex-specific biological processes. Secondly, pain sensitivity varies significantly across lifespan [87,88]. All parts of the pain-axis, from peripheral sensory neurons, non-neuronal cell types in the peripheral and central nervous system [89], to diverse brain regions [90] change throughout maturation, development, adulthood, and aging. However, we still lack molecular and systems-level knowledge about these age-dependent changes and similarities in each sex.

Recent RNA-seq approaches have provided a significant leap forward towards systematically categorizing the molecular set-up of DRG and their peripheral sensory neurons using RNA abundance as a proxy for protein abundance [43,44,48,91]. To date, RNA-seq represents the most comprehensive method for molecular characterization of tissues. However, the transcriptome only exhibits a limited correspondence with the proteomic content of a cell especially during pathological conditions. This is largely due to the existence of various cellular buffering mechanisms [28,75,92], e.g. alternatively spliced variants, regulation of translation, post-translational modifications, and protein degradation/stability [26,76], which modify protein abundance beyond corresponding RNA-levels. Consequently, the value of using RNA abundance as a proxy for protein abundance is constrained. For example, *SCN9A* transcripts encoding a pain-relevant voltage-gated sodium channel

(*Nav1.7*) seem to be present in virtually all human DRG neurons, while the protein was less widely distributed and specifically absent from very large diameter proprioceptive neurons [93]. These protein expression data are in line with the clinical phenotype of human patients: loss-of-function mutations in *Nav1.7* render humans insensitive to pain yet with normal proprioception [94]. This discrepancy between transcript and protein levels emphasizes the necessity of conducting investigations at the protein level in order to explore functional significance for phenotypic outcomes. Our study is unique as we analyzed the DRG proteome of male and female mice and, in parallel, two different age groups. The depth of our proteome data is highlighted by the fact that we detected multiple ion channels and receptors (Fig. 3B-D, STables 1 and 2). These are notoriously difficult to be identified in complex tissue samples due to their relatively low abundance (Fig. 3B-D) [95,96]. Consequently, our data extend the current knowledge and provide a highly valuable protein-level view of DRG upon nerve injury.

One limitation of our proteome-based approach is the fact that we generated bulk data from DRG but cannot assign protein abundance (and the modulation thereof) to distinct cell types or neuronal subpopulations. DRG harbor not only peripheral sensory neurons but also non-neuronal cells such as fibroblasts, glia cells and a diverse set of immune cells. The latter being particularly relevant during pathological pain [22,78–81,86]. Furthermore, peripheral sensory neurons can be categorized into distinct subpopulations – a task, at which single-cell RNA-seq has excelled during the last years. In contrast to single cell RNA-seq, single-cell analysis on the proteome level has not become routine yet, in particular not in complex tissue samples. This is why we leveraged the most comprehensive single-cell RNA-seq data set of mouse and human DRG (<https://painsseq.webflow.io>, accessed Dec 2024; [43]) to infer the likely cell type distribution of identified DEPs in our study (STable 5 and SFig. 6). However, such an approach can only serve as a rough estimate and requires experimental confirmation on the protein level once single-cell proteomics has become standard.

In terms of data analysis, we employed two complementary approaches to decipher SNI-associated proteome changes: commonly used analysis of differential expression of single candidate proteins (DEPs, Figs. 3D-G, 4) and multidimensional factor analysis via MEFISTO (Fig. 5). MEFISTO enabled us to capture significant longitudinal patterns in our data. Our analysis identified proteins and associated pathways (represented by factors 14 and 37, Fig. 5) that consistently discriminated SNI from control conditions across all experimental groups. These factors were linked to biological pathways known to be implicated in acute and chronic pain, including neuroimmune and inflammatory signaling as well as neuronal (dys)function, validating our approach. Leveraging the power of both analysis methods, we deliberately put our emphasis on the overlapping similarities of proteome signatures across sex and age (i. e. sex- and age-shared proteins). We then determined a high confidence and evolutionary conserved (in mice and humans) core-set of proteins (Fig. 5E; STable 10), which may reflect fundamental processes triggered by nerve injury and may facilitate optimized drug target discovery. Importantly, cell type-specific expression patterns in both species need to be considered for functional follow-up studies (SFig. 6). For example,

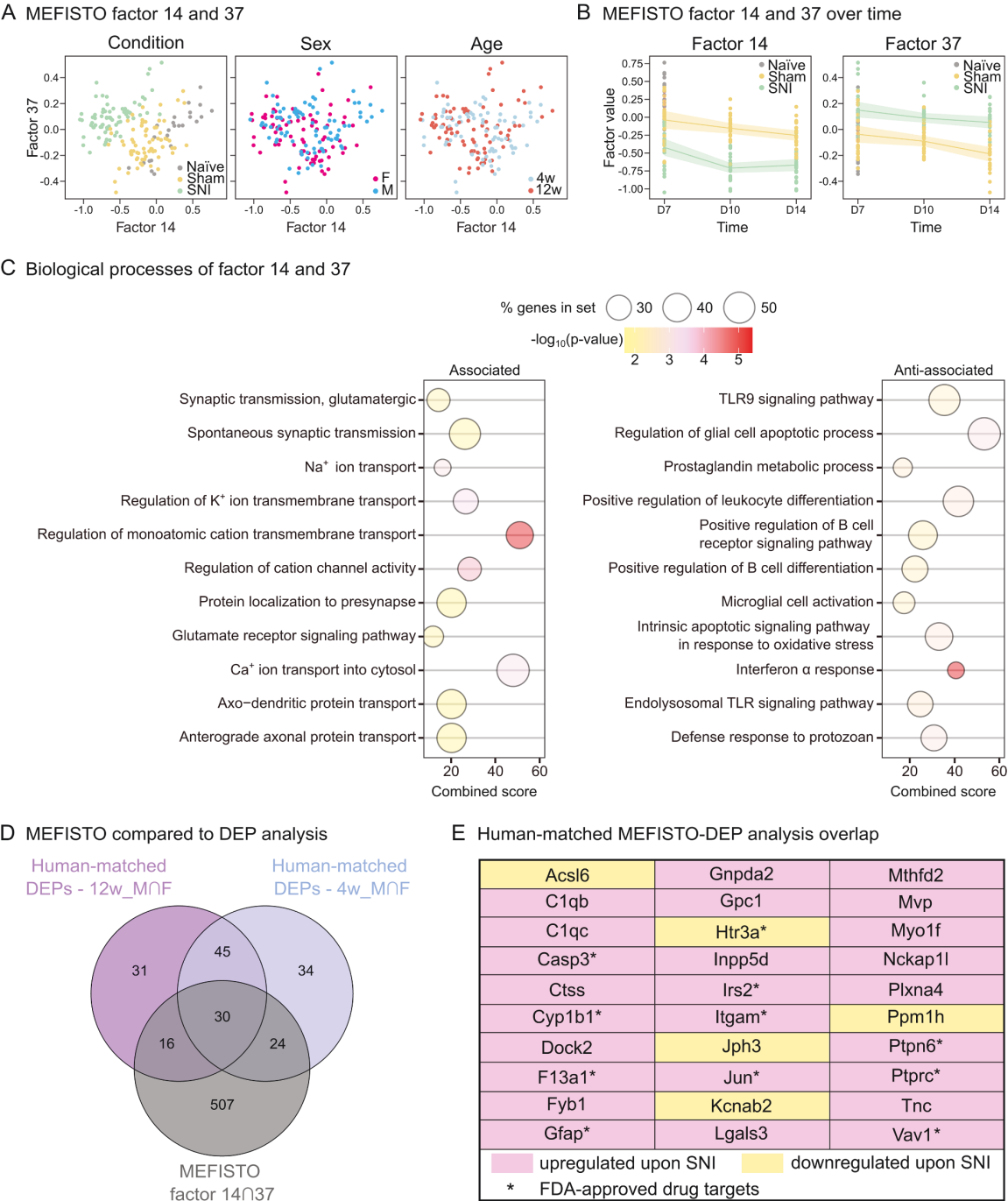


Fig. 5. Global MEFISTO-based factor analysis validates SNI-induced proteome alterations. (A) MEFISTO-based factor analysis revealed factor 14 and 37, which were able to discriminate between condition (SNI vs. Sham and Naive). Plots depict the factor value. (B) Plots show factor values of MEFISTO factor 14 and 37 discriminating SNI vs. Sham and Naive over time (D7, D10, and D14; please see STable 9). (C) Bubble plots show a selection of biological processes (using Gene Ontology-Biological Process) associated (left) and anti-associated (right) with the protein signature represented by MEFISTO factor 14 and 37. Bubble size indicates the percentage of genes in a pathway. Color legend reflects negative $\log_{10}$ -transformed p -values. (D) Venn diagram shows the number of human-matched sex-shared DEPs (Fig. 4) in either age group and of MEFISTO factor 14 and 37 (please see STable 10). Notably, 30 proteins overlap between human-matched sex- and age-shared DEPs (Fig. 4) and MEFISTO factor 14 and 37. (E) Table depicts the 30 human-matched overlapping proteins from Panel D. Color indicates upregulation (red) or downregulation (yellow) upon SNI. (*) indicates FDA-approved drug targets in the Drug-Gene-Interaction-Database (DGIdb v4.2.0, <https://old.dgiddb.org/>, please see STable 8).

the neurofilament Nefh (Neurofilament heavy chain) has been reported to be broadly distributed across human DRG neuron subtypes, while it appears to be restricted to approx. 50 % of mouse DRG neurons [97]. Overall, the here identified core-set of conserved proteins exhibits a similar distribution in mouse and human DRG including their enrichment in immune cells (SFig. 6). Nonetheless, RNA-based comparisons pointed at some species differences in DRG neuron subtypes (SFig. 6). The functional consequences thereof, specifically for neuropathic pain, remain to be elucidated.

Targeting evolutionarily conserved proteins and their associated networks may bridge the gap between mouse and human biology, thereby (i) fostering forward and reverse translational approaches for mechanistic insights, (ii) enhancing the likelihood that preclinical findings can be successfully translated into clinical applications, and (iii) accelerating the development of novel pain treatments. A striking implication of translational research on human-matched results is the fact that it fosters the 3Rs principle (Replacement, Reduction, and Refinement) [98] as unnecessary animal experiments may be avoided and future experiments will be refined.

Our drug-gene-interaction analysis revealed that only around 25 % of human-matched sex-shared DEPs represent targets of FDA-approved drugs (STable 8) highlighting the need for intensified research. Our drug list includes known analgesics and anti-inflammatory agents (e.g. opioids, glucocorticoids) and, importantly, features drugs, which have not yet been approved or routinely used for pain management, such as Ptpcr-modulators or Epoetins. Generally, Ptp-modulators (i.e. modulators of protein tyrosine phosphatases) show promising effects in autoimmune disorders and diverse tumor types [99]. Epoetins are not only known for their therapeutic effects on anemia, but also promote neuronal plasticity and regeneration [100] – a function well-worth investigating in the context of pain and nerve injury. Indeed, Epoetin alpha has shown neuroprotective effects in a rat model of neuropathic pain induced by peripheral nerve injury [101]. Notably, Epoetins are suggested to impact two of the most prominent human-matched hubs revealed in our dataset, i.e. Ptpcr and Vav1 (Fig. 4B-D). Vav1 is a GDP/GTP nucleotide exchange factor (GEF) relevant for microglial activation [102,103]. Furthermore, it is involved in the signal transduction in T cells, thereby affecting T cell development, proliferation and activation [104,105]. Several mRNA expression studies reported Ptpcr to be regulated upon neuropathic pain in humans [12,106] and rodents [107]. Moreover, Ptpcr has been described to impact T- and B-cell activation [108], as well as nervous system development and axon guidance [109]. Both, Ptpcr and Vav1, appear to be expressed in a fraction of human DRG neurons and, broadly, in human immune cells (SFig. 6). Together with Itgam, Ptpcr and Vav1 form a subnetwork connecting diverse immune- and inflammatory pathways (Fig. 4C) rendering this network a promising target (Fig. 4D) for modulating neuroimmune signaling during neuropathic pain.

5. Conclusion

Collectively, our results uncover the proteome landscape associated with neuropathic pain in mice. We reveal differences and similarities of DRG proteomes among adolescent and adult mice of both sexes and emphasize evolutionary conserved similarities bridging the gap between animal models and human painful conditions.

Our comprehensive proteomics resource may be used by the research community to (i) interrogate nerve injury-induced pathophysiology and (ii) mechanistically study neuropathic pain considering similarities and distinctions between adolescent and adult as well as male and female mice. In addition, our dataset will promote future functional validation, bi-directional translational pain research, and network-based drug discovery benefiting adolescents, women, and men equally.

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CRediT authorship contribution statement

Jörg Menche: Resources, Supervision. **Daniel Segelcke:** Writing – review & editing, Methodology, Conceptualization, Visualization, Supervision. **Esther Pogatzki-Zahn:** Resources, Supervision. **Feng Xian:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Software, Data curation. **Daniel Malzl:** Writing – review & editing, Software, Formal analysis, Investigation, Data curation, Visualization. **Sabrina Grundtner:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization, Software, Data curation, Visualization. **Julia Sondermann:** Writing – review & editing, Supervision, Investigation, Conceptualization, Validation. **David Gomez Varela:** Supervision. **Manuela Schmidt:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Methodology, Conceptualization.

Declaration of Competing Interest

In the past, MS received research awards and travel support by the German Pain Society (DGSS) both of which were sponsored by Astellas Pharma GmbH (Germany). MS received one-time consulting honoraria by Grunenthal GmbH (Germany). MS received research awards by the Austrian Pain Society. None of these funding sources influenced the content of this study, and MS declares no conflict of interest. DGV and MS have an ongoing scientific collaboration with Bruker (Center of Excellence for Metaproteomics University of Vienna - Bruker). This collaboration did not influence the content of the manuscript and DGV declares no conflict of interest. During the past 3 yr, EPZ received financial support from Grunenthal for research activities and from Grunenthal (Germany), Medtronic and Merck/MSD for advisory board activities, lecture fees, or both. All money went to the institution EPZ is working for. None of this research support/funds was used for or influenced this manuscript, and EPZ declares no conflict of interest. The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.phrs.2024.107552](https://doi.org/10.1016/j.phrs.2024.107552).

Data availability

Requests for materials should be directed to and will be fulfilled by

the Lead Contact, Manuela Schmidt (manuela.schmidt@univie.ac.at). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [110] partner repository with the dataset identifier PXD055816. Data will be publicly available as of the date of publication. All original code has been deposited at Zenodo and will be publicly available as of the date of publication. DOI: <https://doi.org/10.5281/zenodo.12772115>. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

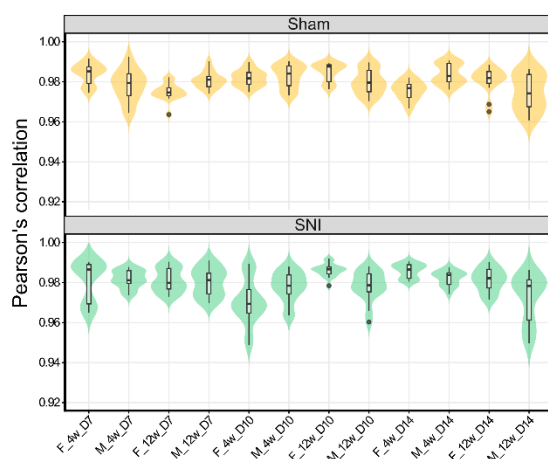
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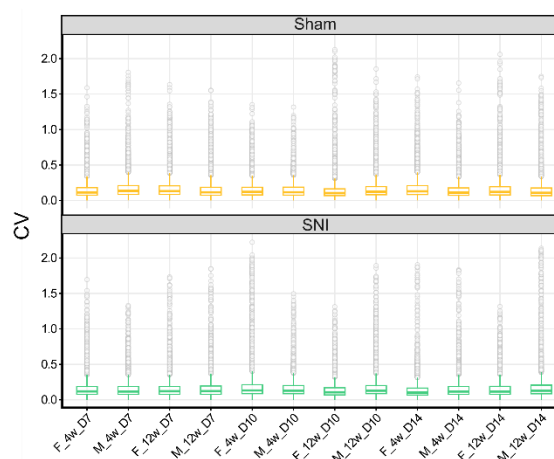
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SFigure 1

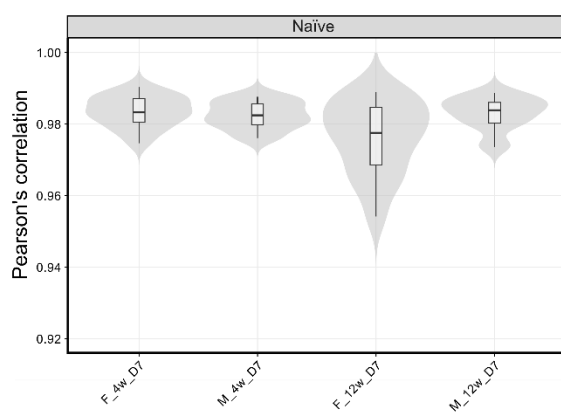
A Intra-group correlation



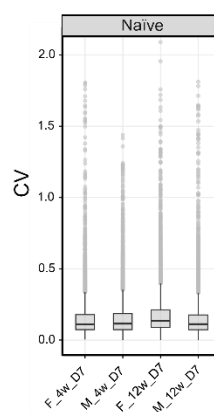
B Coefficient of variation (CV)



C Intra-group correlation - Naïve



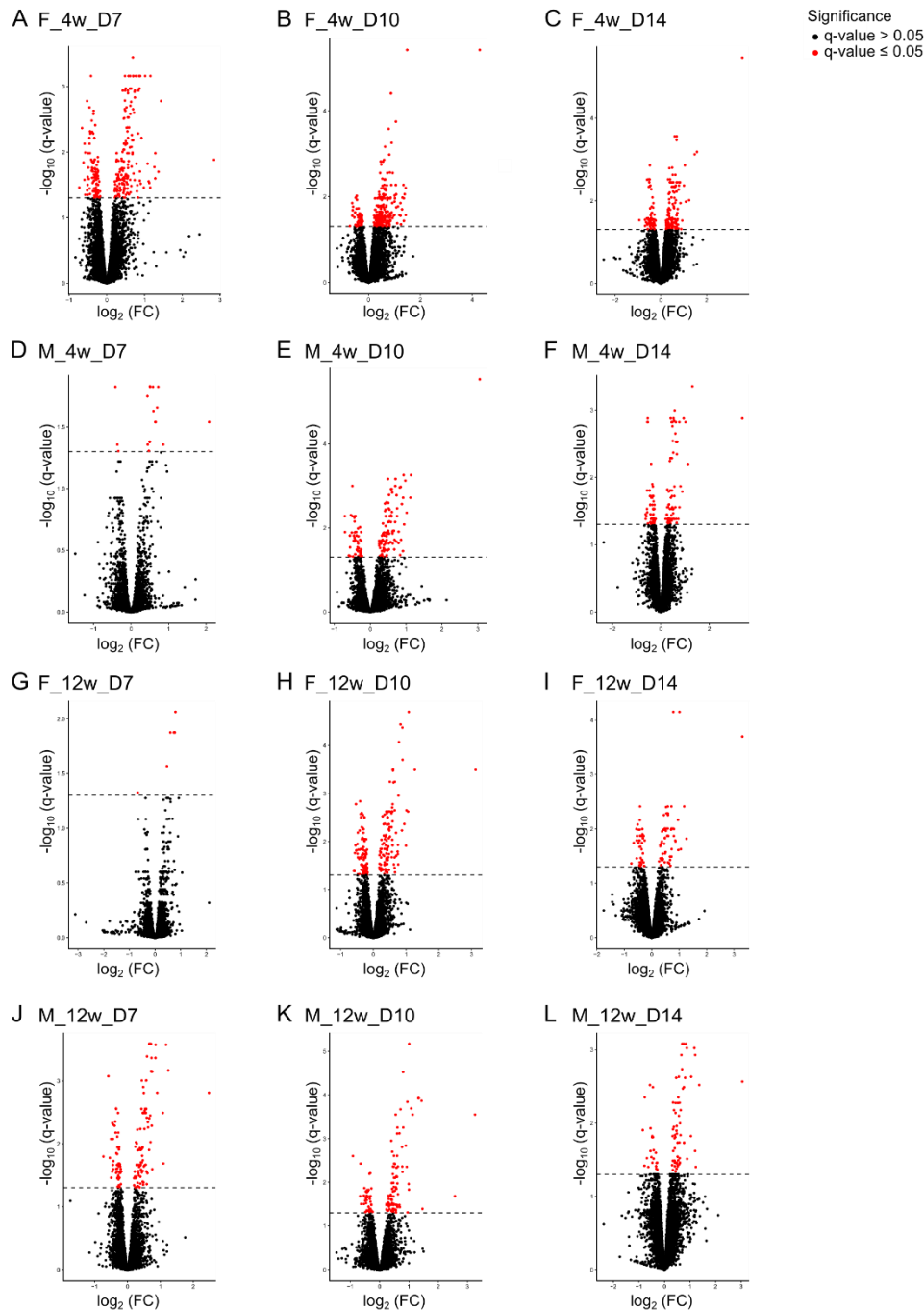
D Coefficient of variation (CV) - Naïve



Supplementary Figure 1. DRG proteome characterization

(A-B) Pearson's correlation (A) and Coefficient of variation (CV) distributions (B) of Sham and SNI DRG proteome data across groups (sex and age) and time points (D7-D14). (C-D) Pearson's correlation (C) and Coefficient of variation (CV) distributions (D) of naïve DRG proteome data across groups (sex and age) and time points (D7-D14).

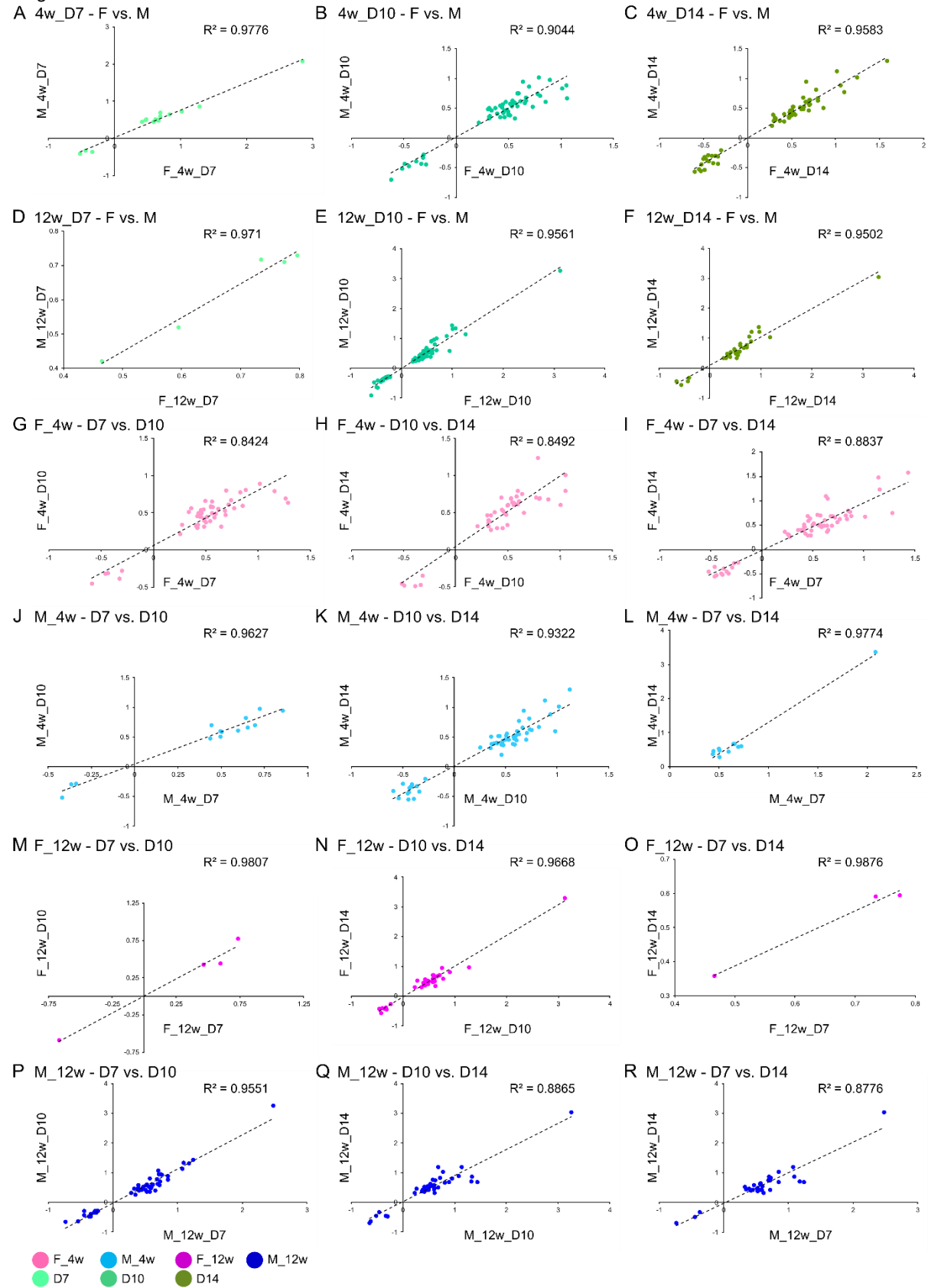
SFigure 2



Supplementary Figure 2. Proteome alterations upon SNI using differential expression analysis

(A-L) Volcano plots depict differentially expressed proteins (DEPs, SNI vs. Sham) across sex and age groups at each time point (D7-D14). The $\log_2$ -transformed fold change SNI/Sham is shown on the x-axis, the negative $\log_{10}$ -transformed q -value is shown on the y-axis. Each point depicts a protein. Proteins changed with q -value ≤ 0.05 are depicted in red. F: female; M: male; 4w: Adolescent; 12w: Adult. List of DEPs in STable 2.

SFigure 3

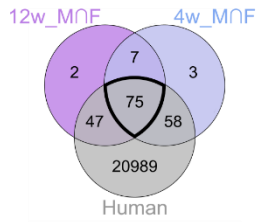


Supplementary Figure 3. Similar abundance changes in DEPs across sex and analysed time points

(A-F) Linear regression of sex-shared DEPs show similar abundance changes in adolescent (4w) and in adult (12w) male and female mice at indicated time points. (G-L) Linear regression of sex-shared DEPs in adolescent (4w) female and male mice show similar abundance changes when comparing indicated time points. (M-R) Linear regression of sex-shared DEPs in adult (12w) female and male mice show similar abundance changes when comparing indicated time points. For each analysis R^2 values are depicted.

SFigure 4

A Human-matched DEPs

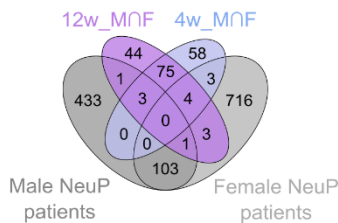


B 75 age- and sex-shared DEPs matched to human data

Abca1	Gfra1	Plxna4
Acsf6	Gnpda2	Ppef1
Apba2	Gpc1	Ppm1h
Arg2	Htr3a	Ptpn6
Btd8	Inpp5d	Ptprc
C1qb	Irs2	Rasgrf2
C1qc	Itgam	Rcc1
Cacna2d1	Jph3	Rimka
Camk1	Jun	Rps6ka1
Casp3	Kcnab2	Sez6l
Chl1	Lgals3	Siglec1
Chst2	Lrrn1	Srxn1
Cnst	Macro2	Stab1
Ctss	Map7d1	Syne1
Cyp1b1	Mrc1	Syne2
Cyth4	Mthfd2	Tep1
Dock2	Mvp	Tes
Dock3	Myo10	Tmem43
F13a1	Myo1f	Tnc
Flrt3	Nckap1l	Tnik
Frrs1l	Oxr1	Uap1
Fry	Pde1a	Ugdh
Fyb1	Pde1c	Vav1
Gap43	Pfkfb4	Vgf
Gfap	Plcd4	Vstm2l

upregulated upon SNI downregulated upon SNI

C Neuropathic pain (NeuP) patient-matched DEPs



D 15 sex-shared DEPs matched to neuropathic pain (NeuP) patients

Bach1 ♀	Gfap ♂★	Ptprc ♀
Btd8 ♂	Lrrk2 ♀	Skap2 ♀
Cd48 ♀	Ndst4 ♂★	Stmn2 ♀
Chl1 ♀	Nln ♀	Tgm1 ♂♀
Fyb1 ♀	Ppef1 ♀	Vgf ♂★

upregulated in NeuP patients downregulated in NeuP patients
♂ regulation for males ♀ regulation for females ★ NA for opposite sex

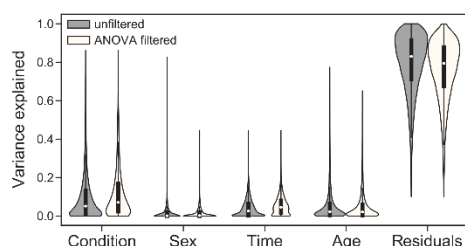
Supplementary Figure 4. Human-matched sex- and age-shared DEPs

(A) Venn diagram shows the number of sex-shared DEPs across age (4w and 12w) matching human transcriptome and proteome DRG data (same datasets as Fig. 4A, human datasets combined for clarity) [12, 44-47]. (B) 75 DEPs matching human transcriptome and proteome DRG data are sex- and age-shared. Colour code: upregulation (red) or downregulation (yellow) of DEPs in mice comparing SNI vs. Sham. List of human-matched sex-shared DEPs in STable 6. (C) Venn diagram shows the number of sex-shared DEPs across age (4w and 12w) matching human neuropathic pain patients [12]. (D) 15 sex-shared DEPs (4w and 12w combined) matching human neuropathic pain patients [12]. Colour code: upregulation (red) or downregulation (yellow) of male (♂) and female (♀) DEPs in neuropathic pain patients.

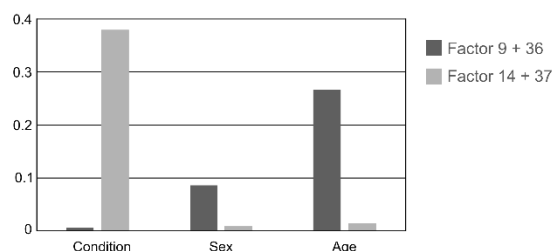
Some data are only available for males; therefore, regulation cannot be indicated for females and is marked with ★ [12]. List of neuropathic pain-matched sex-shared DEPs in STable 6.

SFigure 5

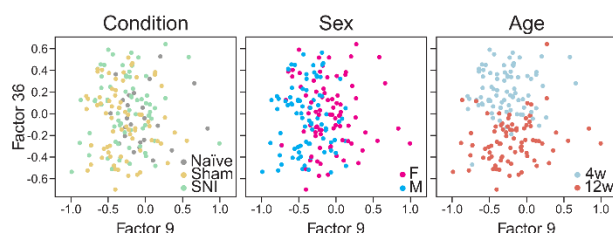
A Variance decomposition



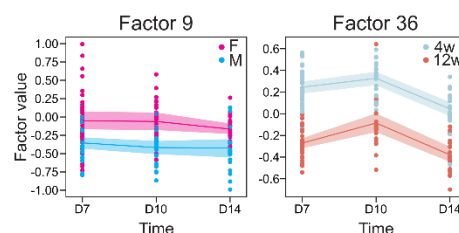
B Silhouette score



C MEFISTO factor 9 and 36



D MEFISTO factor 9 and 36 over time

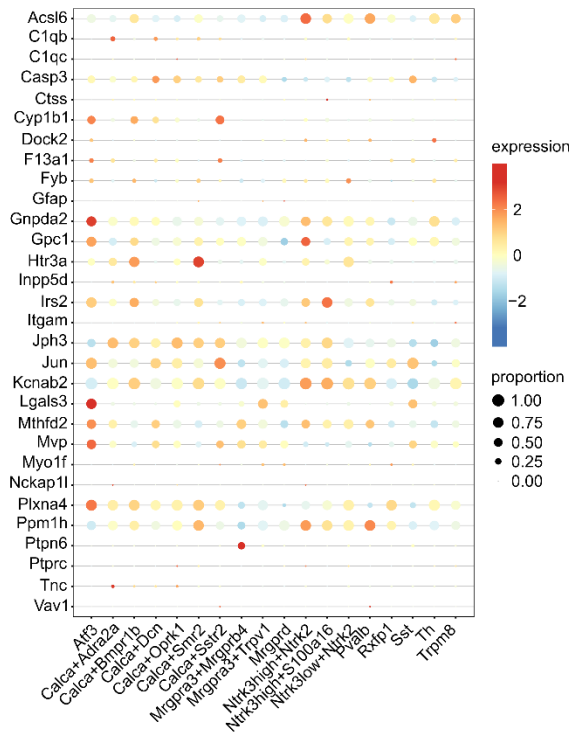


Supplementary Figure 5. MEFISTO-based factor analysis

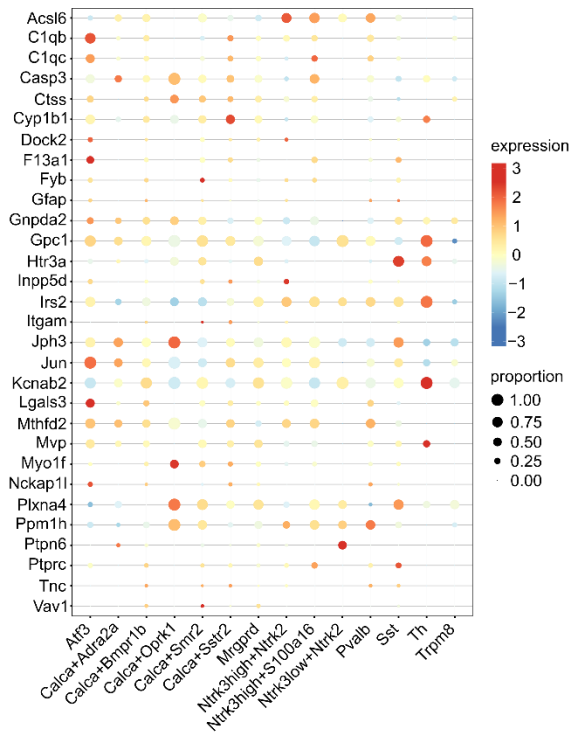
(A) Plot illustrates the variance decomposition from MEFISTO-based factor analysis in dependence on indicated variables (condition, sex, time, and age). On the y-axis the contribution of each variable is displayed. Residuals define a variance, which cannot be explained by any variable known to us. (B) Silhouette score indicates the extent to which a factor can separate a cluster. This score is based on the Euclidean distance. Condition (SNI vs. Sham/Naïve) is discriminated by MEFISTO factor 14 and 37, while sex and age are discriminated by MEFISTO factor 9 and factor 36. (C-D) MEFISTO factor-based analysis revealed factor 9 and 36 to discriminate between sex and age, respectively, yet independently of the condition (C). Plots depict the factor value. (D) Plots show factor values of MEFISTO factor 9 and 36 over time (D7-D14). List of MEFISTO factor 9 and 36 in STable 9.

SFigure6

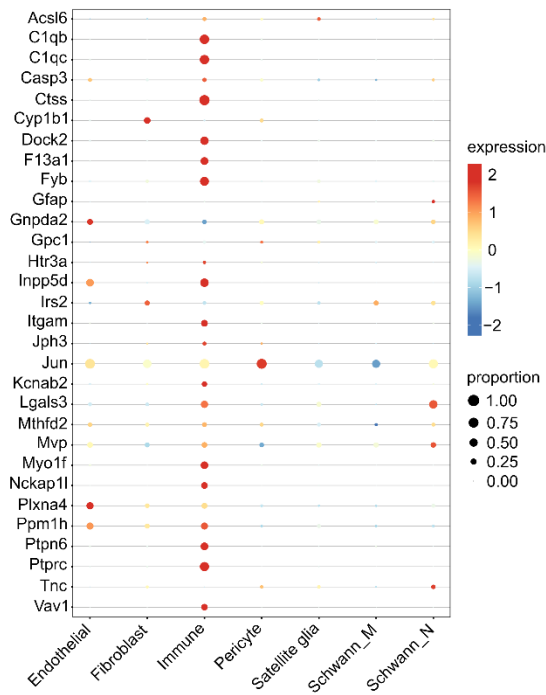
A DRG neurons (mouse-specific cell type mapping)



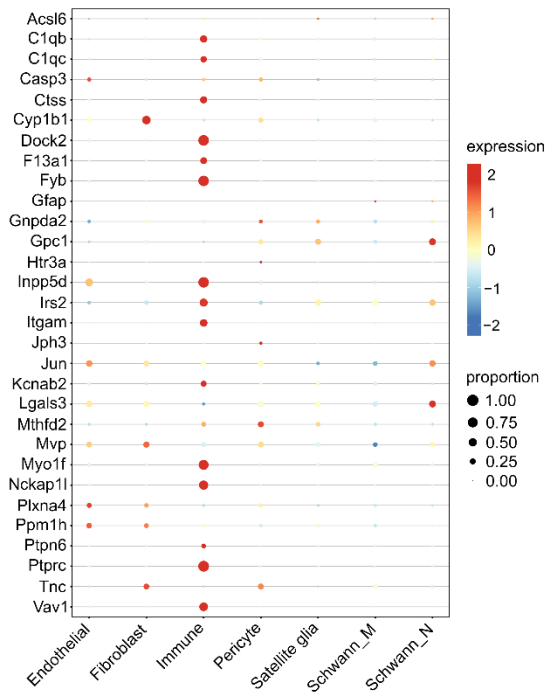
B DRG neurons (human-specific cell type mapping)



C DRG non-neurons (mouse-specific cell type mapping)



D DRG non-neurons (human-specific cell type mapping)



Supplementary Figure 6. Gene expression cell type mapping across species

Bubble plots indicate gene expression of 30 sex-shared, human-matched [12, 44-47] DEPs overlapping with MEFISTO factor 14 and 37 as listed in Fig. 5E (full dataset in STable 10). All

bubble plots were created using <https://painseq.webflow.io> (accessed Dec 2024; [43]), with scaled gene expression in mouse and human datasets. (A) Gene expression in DRG neurons based on mouse data. (B) Gene expression in DRG neurons based on human data. (C) Gene expression in DRG non-neurons based on mouse data. (D) Gene expression in DRG non-neurons based on human data. Gene names were used instead of protein names to enable cell type mapping. Genes were grouped by 'Atlas_annotation'. The gene synonym Fyb was used instead of Fyb1 to enable mapping.

Chapter 3

Increasing taxonomic and functional characterization of host-microbiome interactions by DIA-PASEF metaproteomics

David Gómez-Varela, Feng Xian, Sabrina Grundtner, Julia Regina
Sondermann, Giacomo Carta, Manuela Schmidt

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Major Aims

The microbiome is an ecosystem whose contribution to pain perception has lately gained increasing attention. However, current technologies to investigate the metaproteome, the entirety of all proteins within this ecosystem, of highly complex microbial samples, such as feces, have reached their limit of detection. Therefore, one aim of this study was to find a suitable technique to explore the gut microbiome. The second aim of this study was to apply this technique to a neuropathic pain model, to investigate associated changes in the gut microbiome. The analysis of fecal samples from nerve-injured mice and their control group allowed us to gain functional insights into the microorganisms, and to discover host-gut interactions altered during neuropathic pain. The aim of this descriptive study was to expand the currently limited knowledge of the gut microbiome as a key player in neuropathic pain.

Contributions

In this study, I contributed to the overall experimental design as far as the mice were concerned. Experimentally I was primarily responsible for performing surgeries on the subjects and control subjects (SNI and Sham). This includes preparation of the subjects for surgery, the surgeries themselves and post-operative care. Furthermore, I was mainly responsible for sample collection from experimental mice, including naïve mice. Additionally, I prepared and conducted most of the behavioral experiments and the subsequent analysis of that data, including statistical analysis and visualization of the data. Finally, I also drafted and edited sections of the manuscript relating to the above work in close corporation with the first author.



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Increasing taxonomic and functional characterization of host-microbiome interactions by DIA-PASEF metaproteomics

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Introduction: Metaproteomics is a rapidly advancing field that offers unique insights into the taxonomic composition and the functional activity of microbial communities, and their effects on host physiology. Classically, data-dependent acquisition (DDA) mass spectrometry (MS) has been applied for peptide identification and quantification in metaproteomics. However, DDA-MS exhibits well-known limitations in terms of depth, sensitivity, and reproducibility. Consequently, methodological improvements are required to better characterize the protein landscape of microbiomes and their interactions with the host.

Methods: We present an optimized proteomic workflow that utilizes the information captured by Parallel Accumulation-Serial Fragmentation (PASEF) MS for comprehensive metaproteomic studies in complex fecal samples of mice.

Results and discussion: We show that implementing PASEF using a DDA acquisition scheme (DDA-PASEF) increased peptide quantification up to 5 times and reached higher accuracy and reproducibility compared to previously published classical DDA and data-independent acquisition (DIA) methods. Furthermore, we demonstrate that the combination of DIA, PASEF, and neuronal-network-based data analysis, was superior to DDA-PASEF in all mentioned parameters. Importantly, DIA-PASEF expanded the dynamic range towards low-abundant proteins and it doubled the quantification of proteins with unknown or uncharacterized functions. Compared to previous classical DDA metaproteomic studies, DIA-PASEF resulted in the quantification of up to 4 times more taxonomic units using 16 times less injected peptides and 4 times shorter chromatography gradients. Moreover, 131 additional functional pathways distributed across more and even uniquely identified taxa were profiled as revealed by a peptide-centric taxonomic-functional analysis. We tested our workflow on a validated preclinical mouse model of neuropathic pain to assess longitudinal changes in host-gut microbiome interactions associated with pain - an unexplored topic for metaproteomics. We uncovered the significant enrichment of two bacterial classes upon pain, and, in addition, the upregulation of metabolic activities previously linked to chronic pain as well as various hitherto unknown ones. Furthermore, our data revealed pain-associated dynamics of proteome complexes implicated in the crosstalk between the host immune system and the gut microbiome. In conclusion, the DIA-PASEF metaproteomic workflow presented here provides a stepping stone towards a deeper understanding of microbial ecosystems across the breadth of biomedical and biotechnological fields.

KEYWORDS

metaproteomics, data-independent acquisition, parallel accumulation-serial fragmentation, mouse gut microbiome, chronic neuropathic pain, host-microbiome interactions

Introduction

The characterization of microbial communities has increasingly gained attention due to their crucial role for the health across planetary ecosystems (Clemente et al., 2012; Prescott et al., 2018). However, our understanding of dynamic and multidimensional interactions between members of these ecosystems (hosts, bacteria, pathogens), and of the resulting functional alterations is still limited. Genomic sequencing methods are the most common approaches to studying these interactions by means of characterizing the taxonomic composition and relative abundance of functional genes. Nevertheless, the presence of a protein-coding gene does not always result in its expression under any given condition and even transcript levels can only partially predict protein levels (Liu et al., 2016). Thus, prediction algorithms are used for inferring the functional roles of microorganisms in an ecosystem based on genomic or transcriptomic datasets, yet such algorithms exhibit limited performance (Mills et al., 2019; Sun et al., 2020). Moreover, the inference on how changes in the abundance of specific taxa affect the functional output of a microbial habitat is unclear given the prominent functional redundancy (Fetzer et al., 2015; Tian et al., 2020). In fact, functional changes in the microbiome underlie gut inflammation and xenobiotic treatments without detectable changes in the taxonomic composition of the microbiome (Li et al., 2023). To overcome these boundaries, mass spectrometry-based proteomics applied to the study of hundreds of microbial organisms (termed, metaproteomics (Rodríguez-Valera, 2004; Wilmes and Bond, 2004)) has emerged as an attractive alternative. Metaproteomics has the potential to understand complex host-microbiome interactions as it enables the analysis of entire sets of proteins, providing direct insights into the identity and functionality of microorganisms present in an ecosystem (Stambouliau et al., 2022). However, proteomics acquisition methods classically used in metaproteomics have reached their theoretical profiling limits (Duan et al., 2022).

The predominant method employed for the acquisition of metaproteomic data is data-dependent acquisition (DDA) mass spectrometry (MS). DDA generally fragments only the most intense peptide ions, rendering the majority of remaining peptides unidentifiable (Michalski et al., 2011). Additionally, accurate quantification is challenging, owing to the inconsistent recording of ion intensities along the chromatographic profile. Consequently, reproducibility across repeated analyses is hampered and a sensitivity bias toward high-abundance peptides is introduced. These limitations are exacerbated in

highly complex microbial samples (e.g., feces), which are estimated to be composed of 100 million peptide species (Armengaud, 2023) distributed across a very high dynamic range (Duan et al., 2022). To overcome these constraints, recent studies have evaluated the performance of data-independent acquisition (DIA) mass spectrometry (Aakko et al., 2020; Long et al., 2020; Zhao et al., 2022, 2023). Although DIA offers reproducibility improvements over DDA methods, spectral complexity and sampling efficiency remain challenging (Meier et al., 2020), particularly in highly complex peptidomes. Consequently, improved proteomic acquisition methods with the potential to increase the depth and resolution of metaproteomics are needed.

Previously, we showed how the combination of the Parallel Accumulation-Serial Fragmentation (PASEF) technology (Meier et al., 2015) and data analysis based on deep neuronal networks (Demichiev et al., 2020), significantly increases the number of proteins quantified in mouse tissues (Xian et al., 2022). PASEF incorporates an additional ion mobility separation that allows for the differentiation of peptide signals that would otherwise be co-fragmented (Meier et al., 2015). This results in a more than tenfold increase in MS/MS scan rates without loss of sensitivity. Here, we aimed to investigate whether PASEF can optimize metaproteomics in complex microbial samples, i.e., in mouse feces.

In this study, we show that a DDA-PASEF-based workflow increased peptide identification up to 5 times and significantly improved data consistency and quantification reproducibility compared to previously published classical DDA (Kolmeder et al., 2016) and DIA (Aakko et al., 2020) methods. Moreover, we demonstrated that a DIA-PASEF strategy sets new profiling standards in metaproteomics by enabling the quantification of more proteins than DDA-PASEF while requiring only 1/10 of peptides. In comparison to previous DDA-based metaproteomic studies (Tanca et al., 2017, 2018), DIA-PASEF profiled up to 4 times more taxonomic units using 16 times fewer injected peptides and 4 times shorter chromatography gradients. Furthermore, DIA-PASEF offered comparable taxonomic depth as previous metagenomic studies. When applied to a pre-clinical mouse model of neuropathic pain, DIA-PASEF enabled the quantification of more than 15,000 protein groups and deciphered novel host-microbiome interactions associated with the establishment of chronic pain. Taken together, we expect DIA-PASEF to shed light on previously unexplored regions of the metaproteome and significantly enhance our understanding of microbiological ecosystems.

Materials and methods

Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, Missouri) if not mentioned otherwise. Acetonitrile (ACN) and formic acid (FA) were purchased from Fisher Scientific (Hampton, New Hampshire; both FA and ACN were liquid chromatography-mass

Abbreviations: DIA, Data-independent acquisition; DDA, Data-dependent acquisition; PASEF, Parallel accumulation serial fragmentation; FDR, False discovery rate; LC-MS, Liquid chromatography-mass spectrometry; SNI, Spared nerve injury; MBR, Match between runs; MQ, MaxQuant; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO-MF, Gene Ontology Molecular function terms; SIgA, Secreted immunoglobulin A.

spectrometry (LC–MS) grade). LC–MS grade water from Sigma was used for all solutions. Protease inhibitor was purchased from Roche (Complete Ultra Tablets Mini, Roche, Basel, Switzerland).

Animal housing and surgery

In-house bred C57BL/6J female mice were used. All animal experiments were carried out with the approval of the IACUC at the University of Vienna and of the Austrian Ministry for Education, Science and Research (BMBWF; license number 2021–0.138.925). All mice used in this study (four mice in each of the three experimental conditions) were group-housed in the same room, with a 12h light/dark cycle, and with water and food *ad libitum*. All mice were weaned between 22–24 days after birth. The spared nerve injury (SNI) paradigm was used to induce neuropathic pain at 4 weeks of age. Mice were anaesthetized using isoflurane (4% for induction first and 2% for maintenance in O₂). To expose the trifurcation of the left sciatic nerve, the skin and underlying biceps femoris muscle were incised. A ligature was placed around the common peroneal and tibial nerves with a 7–0 surgical braided silk (Vömel, Kronberg, Germany) below the bifurcation, followed by distal transection of both nerves. A 2 mm segment was excised from both nerves to impede regrowth. The skin was closed with one surgical clip (AutoClip®, Fine Science Tools, Heidelberg, Germany) and disinfected with povidone-iodine (Mundipharma, Frankfurt am Main, Germany). For analgesia mice were injected with carprofen (0.05 mL/10 g body-weight, Zoetis Österreich GmbH, Austria) immediately before surgery and on postoperative day 1. The clips were removed on postoperative day 5 with an AutoClip® remover (Mikron Precision Inc., Biel, Switzerland). In SHAM-operated mice, the same surgical procedure was carried out as in SNI but without ligating and transecting the branches of the sciatic nerve. All operated mice were weighed daily until postoperative day 7 and from then on, every second day until postoperative day 14 by female experimenters only (Sorge et al., 2014). Of note, the selection of which mice were used for SHAM or SNI surgeries was random and blinded to their microbiome profile, which was obtained only after the analysis of the fecal samples.

Mechanical sensitivity test

The test was performed 3–4 days before surgery (Pre) and on a postoperative day 14 (14D) using a dynamic plantar aesthesiometer (automated von Frey filament, Dynamic Plantar Aesthesiometer: 37450–001, Dynamic Plantar Aesthesiometer Touch Stimulator: 37400–002, Ugo Basile®, Gemonio, Italy). Mechanical force (Force Intensity: 10.0g, Ramp Time: 40s) was applied to the lateral side of the plantar hind paw and the withdrawal latency (in seconds) was measured five times for each hind paw, with at least a 2-min recovery period between each measurement. The test was done only by female experimenters (Sorge et al., 2014).

Fecal collection

Mice were placed into 10x10cm boxes covered with a lid for 15 min. After removing mice from the boxes, feces were collected

using forceps that were previously cleaned with 70% ethanol between each sample. Samples were transferred to precooled 2 mL autoclaved tubes (Eppendorf, Hamburg, Germany) and stayed on ice until stored at –80°C.

Protein extraction, SP3-assisted protein digestion, and peptide clean-up

For protein preparation, 30 mg of fecal samples were mixed with 200 µL of lysis buffer (5% SDS, 2 M Urea, 50 mM Tris–HCl, Protease Inhibitor 1x) and vortexed vigorously for 1 min. Following, samples were placed in a Thermomixer (Serial Number: 5382JR638726, Eppendorf, Hamburg, Germany) for 15 min at 1,200 rpm and 70°C. When finished, samples were ultrasonicated using a Bioruptor Pico (Diagenode, Seraing, Belgium; program: 15 cycles of 30 s “ON” and 30 s “OFF” frequency level: Low, water temperature: +20°C). Afterwards, tubes were centrifuged at 16,000 g for 5 min at room temperature. Supernatants were saved in new tubes and submitted to a new round of centrifugation. The saved supernatants were stored at –80°C until SP3-assisted protein digestion.

For protein clean-up and digestion, a modified version of the single-pot, solid-phase-enhanced sample preparation (SP3) method from Hughes et al. was used (Hughes et al., 2019). Briefly, 50 µg protein was taken into a 1.5 mL LoBind tube (Eppendorf, Hamburg, Germany), and the sample volume was added up to 50 µL with lysis buffer. The fecal sample was subjected to protein reduction (5 mM Dithiothreitol, DTT, 30 min incubation at +60°C) and alkylation (20 mM Iodoacetamide, IAA, 30 min at room temperature in the dark). The remaining IAA in the sample was quenched with the addition of DTT to a final concentration of 5 mM. 10 µL of pre-mixed Sera-Mag SpeedBead beads (50 mg/mL, Cytiva, Marlborough, Massachusetts) were added to 50 µg protein sample. To initiate the binding of proteins to the beads, one volume of absolute ethanol was added immediately, followed by incubation on a Thermomixer (Eppendorf) at 24°C for 5 min with 1,000 rpm agitation. The supernatant was removed after 2 min resting on a magnetic rack, and the beads were rinsed three times with 500 µL of 80% ethanol. Rinsed beads were reconstituted in 50 µL digestion buffer (50 mM ammonium bicarbonate, pH 8). Protein digestion was performed with 2 µg of sequencing-grade trypsin/LysC (Promega, Madison, United States) for 18 h at 37°C with 950 rpm agitation. After digestion, ACN was added to each sample to a final concentration of 95%. Mixtures were incubated for 8 min at room temperature and then placed on a magnetic rack for 2 min. The supernatant was discarded, and the beads were rinsed with 900 µL of 100% ACN. Rinsed beads were reconstituted in 20 µL LC–MS grade water to elute the peptides. The peptide concentration was measured in duplicate using NanoPhotometer N60 (Serial number: TG2022, Implen, Munich, Germany) at 205 nm. Peptide samples were acidified with FA to a final concentration of 0.1% and stored at –20°C until LC–MS/MS analysis.

LC–MS/MS setup

Nanoflow reversed-phase liquid chromatography (Nano-RPLC) was performed on a NanoElute system (Bruker Daltonik, Bremen, Germany). Peptides were separated with either a 70 min or 130 min

gradient on a 25 cm x 75 μ m column packed with 1.6 μ m C18 particles (IonOpticks, Fitzroy, Australia). Mobile solvent A consisted of 2% ACN, 98% water, 0.1% FA and mobile phase B of 100% ACN + 0.1% FA. For both gradient lengths, the flow rate was set to 400 nL/min for the first 2 min and the last 9 min of the gradient, while the rest of the gradient was set to 250 nL/min. In the 70 min separation, the mobile phase B was linearly increased from 0 to 20% from 3 min to 50 min, followed by a linear increase to 35% within 10 min and a steep increase to 85% in 0.5 min. Then a flow rate of 400 nL/min at 85% was maintained for 9 min to elute all hydrophobic peptides. In the 130 min separation, the mobile phase B was linearly increased from 0 to 20% from 3 min to 110 min, followed by a linear increase to 35% within 10 min and a steep increase to 85% in 0.5 min. Then a flow rate of 400 nL/min at 85% was maintained for 9 min to elute all hydrophobic peptides. NanoElute LC was coupled with a hybrid TIMS quadrupole TOF mass spectrometer (timsTOF Pro, Bruker Daltonik, Bremen, Germany) via a CaptiveSpray ion source. Samples were analyzed in both data-independent acquisition (DIA) and data-dependent acquisition (DDA) modes coupled with parallel accumulation serial fragmentation (PASEF) for methods comparison. The samples used for the neuropathic pain characterization (SNI, SHAM and Naive) were analyzed in DIA-PASEF. In both acquisition modes, the TIMS analyzer was operated in a 100% duty cycle with equal accumulation and ramp times of 100 ms each. Specifically, in DDA-PASEF mode (Meier et al., 2018), 10 PASEF scans were set per acquisition cycle with ion mobility range (1/k0) from 0.6 to 1.6, and singly charged precursors were excluded. Dynamic exclusion was applied to precursors that reached a target intensity of 17,500 for 0.4 min. Ions with m/z between 100 and 1700 were recorded in the mass spectrum. In DIA-PASEF mode (Meier et al., 2020), precursors with m/z between 400 and 1,250 were defined in 16 scans containing 32 ion mobility steps with an isolation window of 26 Th in each step with 1 Da overlapping for neighbouring windows. The acquisition time of each DIA-PASEF scan was set to 100 ms, which led to a total cycle time of around 1.8 s. In both DDA- and DIA-PASEF modes, the collision energy was ramped linearly from 59 eV at 1/k0 = 1.6 to 20 eV at 1/k0 = 0.6.

Protein database generation for metaproteome analysis

A metagenome-translated protein database (PD1) was downloaded from <http://gigadb.org/> containing 2.6 million protein sequences (Xiao et al., 2015). Due to the large size of the protein database, a two-step approach (Jagtap et al., 2013) was applied to generate a reduced and sample-specific protein database. Briefly, a fecal pooled peptide sample was recorded 10 times using DDA-PASEF and a 70 min chromatography gradient. The 10 raw data files were first converted into mgf format and then searched against the PD1 and a decoy database generated with reversed sequences using X!Tandem (Muth et al., 2010), a search engine integrated into SearchGUI (Barsnes and Vaudel, 2018) (Version 4.1.11). Trypsin was specified with a maximum of 2 missed cleavages allowed. The search included variable modifications of methionine oxidation and N-terminal acetylation and a fixed modification of carbamidomethyl on cysteine. The mass tolerances of 10 ppm for both precursor and fragment were used. The output of the X!Tandem search was further validated in PeptideShaker with 1% FDR at PSM, peptide and protein levels

(Vaudel et al., 2015). All validated proteins were exported as the reduced protein database (PD2) containing 9,750 protein sequences. PD2 was further used for the comparison of different workflows. Another pooled peptide sample made from Naive, SHAM and SNI mice was submitted to a 130 min gradient and analyzed in DDA-PASEF mode in 9 replicates. The 9 raw data files were subjected to the same aforementioned procedures to generate another reduced protein database (PD3) containing 10,859 protein sequences. PD3 was further used to analyze the metaproteome of Naive, SHAM and SNI mice. *Mus musculus* reference proteome (PD4) was downloaded from Uniprot¹ and used to identify host proteins from the fecal samples. To avoid misassignment of host- and microbiome-derived peptides, we used either PD2 or PD3 in combination with PD4 during the comparison of the different workflows and the analysis of neuropathic pain samples, respectively. To accurately compare the differences in performance across different acquisition modes (DDA- and DIA-PASEF) and the different data analysis solutions (MQ and DIA-NN), we used the same pooled fecal sample for all experiments shown in all figures except the last one. The use of this pooled sample prevents sample bias and ensures a generalized sample-independent representation of the mouse fecal microbiome.

Data processing using different workflows

DDA-PASEF raw data files were analyzed with MaxQuant (version 2.1.3.0) and searched with Andromeda against PD2 and PD4, at the same time. The search type was specified as “TIMS-DDA.” The minimal peptide length was set to 7 amino acids, and a maximum of 2 missed cleavages was allowed.

The search included methionine oxidation, asparagine and glutamine deamidation and N-terminal acetylation as variable modifications, as well as cysteine carbamidomethylation as fixed modification; a maximum of 5 modifications per peptide was allowed. The “Match between runs” function was checked within a 0.7 min retention time window and 0.05 ion mobility window. Mass tolerance for peptide precursor and fragments were set as 10 ppm and 20 ppm, respectively. The FDR was set to 0.01 at the precursor level and protein level. Label-free quantification algorithm with a minimum 1 LFQ ratio count was used to quantify identified proteins. The rest of the parameters were kept as default.

DIA-PASEF raw data files were analyzed with MaxQuant (version 2.1.3.0) and the search type was specified as “TIMS MaxDIA.” The data were searched against the spectral library generated with the DDA-PASEF data search in the MaxQuant workflow mentioned above using the Andromeda algorithm. Specifically, the output evidence.txt, peptide.txt and msms.txt files were used. The rest of the configurations were set as same as the search for DDA-PASEF data.

DIA-PASEF raw data files were also searched in DIA-NN (Demichiev et al., 2020). DIA-NN (version 1.8.1) was used to process DIA-PASEF data in library-free mode with PD2 or PD3 and PD4 to generate the predicted spectrum library. A deep learning-based method was used to predict theoretical peptide spectra along with their retention time and ion mobility. Trypsin/P was used for *in silico*

1 <https://www.uniprot.org/proteomes/UP000000589>

digestion with an allowance of a maximum of 2 missed cleavages. Variable modifications on peptides were set to methionine oxidation and N-term acetylation, while N-term methionine excision and carbamidomethylation on cysteine were fixed modifications. The maximum number of variable modifications on a peptide was set to 5. Peptide length for the search ranged from 7 to 52 amino acids. Aligned with the DIA-PASEF acquisition method, *m/z* ranges were specified as 400 to 1,250 for precursors and 100 to 1700 for fragment ions. Both MS1 and MS2 mass accuracy were set to automatic determination. Protein inference was set to “Protein names (from FASTA)” and the option of “Heuristic protein inference” was unchecked. Match-between-run (MBR) was checked for cross-run analysis in all analyses performed in this study. RT-dependent cross-run normalization and Robust LC (high precision) options were selected for quantification.

In addition, the same DIA-PASEF raw data files were processed with DIA-NN (version 1.8.1) using an experimental spectrum library generated in our lab with several metaproteome studies. The experimental library contains microbial and host proteins of in total 14,711 protein isoforms, 19,153 protein groups and 98,159 precursors. Besides the use of the experimental spectrum library, all other search parameters were the same as described above for the DIA-PASEF data search in library-free mode.

Metaproteome data batch correction and normalization

For the neuropathic pain experiment, precursor intensities (Normalized Intensity from the DIA-NN main report table) were submitted to median normalization and quantile batch correction using the proBatch R package (Cuklina et al., 2021). The resulting precursor intensities were further processed with the R package, DIA-NN,² to extract and calculate the MaxLFQ (Cox et al., 2014) quantitative intensity for all identified peptides and protein groups with *q*-value <0.01 as criteria at precursor and protein group levels.

Taxonomy and function annotation

iMetaLab (Cheng et al., 2020) (version 2.3.0) was used for taxonomy and function annotation. The MaxLFQ peptide data (microbial and host) were imported into iMetaLab, and the built-in taxonomy database was used for the mapping (Ignore blanks below rank: Superkingdom, Unique peptide count ≥3). MaxLFQ protein identifications with corresponding intensities were imported into iMetaLab for functional annotation (using default parameters). The K numbers (KEGG Orthology identifiers) of mapped proteins were extracted and analyzed online³ to obtain KEGG pathways and their corresponding number of mapped targets.

The analysis of the taxon-specific functions was performed using Meta4P (Porcheddu et al., 2023). The quantitative table of microbial

peptides (i.e., Supplementary Table 2) and the taxonomic annotation table (i.e., Supplementary Table 3) were imported into Meta4P. Proteins annotated with different function databases were generated *via* eggNOG⁴ using PD2. Taxa-specific KEGG pathways were exported and a total number of at least 3 peptides per taxon-KEGG pathway was set as the threshold to filter the results.

Taxonomic quantification strategy

The iMetaLab's taxonomy annotation output underwent further processing in R, where the following steps were taken to quantify the detected taxa in each sample. Firstly, peptides without annotations at a specific taxon level (e.g., Species) were removed. Next, taxa with at least three unique peptides annotated were retained. The peptide intensity in each sample was then extracted based on the retained taxa. Peptides that were not quantified in all samples were removed. Finally, the log2-transformed intensities of common peptides annotated in the same taxon were summed up in each sample. The intensities matrices of 14D and Pre in each condition were subjected to paired *t*-test in GraphPad Prism (version: 9.5.0(730)) using the “Benjamini and Hochberg” method for multiple comparisons.

Functional quantification strategy

The output of the function annotation in iMetaLab underwent further processing in R using the following steps to calculate the intensity of each KEGG pathway in each sample using proteins with a significance *p* < 0.005 when comparing 14D versus Pre within each condition (paired *t*-test in Limma package (Ritchie et al., 2015)). Firstly, significantly regulated proteins mapped with KEGG ortholog numbers (K numbers) were kept for the following analysis. Secondly, different pathways annotated with the same K number were separated into rows with duplicated intensities for this entry in all samples, as there is no evidence to suggest that the protein belongs to only one pathway. Thirdly, the intensities of annotated numbers mapped to the same pathway were summed up in each sample. Fourthly, the number of mapped K numbers to each pathway in each sample was counted. Finally, the intensity of each pathway was normalized by dividing the summed intensity by the total K numbers mapped for each pathway in each sample. The intensity of each pathway was log2-transformed before statistical analysis. The transformed intensities were tested by comparing 14D versus Pre in each condition (Naive, SHAM, and SNI) using the Limma (Ritchie et al., 2015) R package in a paired manner with the “Benjamini and Hochberg” method for multiple comparisons.

Pathway analysis of host proteome

The host protein identifications were analyzed in Metascape (Zhou et al., 2019) (<https://metascape.org/>, version 3.5.20230101, accessed on 2023.02.17) and searched against the Gene Ontology Molecular Function database with default settings.

² <https://github.com/vdemichev/diann-rpackage>

³ <https://www.genome.jp/kegg/ko.html>

⁴ <http://eggno-mapper.embl.de/>

Results

Development of a PASEF-based metaproteomic workflow

Current metaproteomic methods have reached their identification and quantification limits in complex microbial samples with high dynamic range (Duan et al., 2022) (e.g., feces). We speculated that the extra layers of information accessible by the PASEF technology (Meier et al., 2015, 2021) could offer significant improvements similar to what we demonstrated in our recent work on complex mouse tissue samples (Xian et al., 2022). PASEF creates an extra ion mobility dimension that increases the sequencing speed ten-fold and reduces spectral complexity. To this end, mouse peptide fecal samples were analyzed on a timsTOF Pro mass spectrometer (Bruker Daltonics) equipped with a dual TIMS cartridge and using the PASEF acquisition method (Meier et al., 2018) (Figure 1). In addition to implementing PASEF using a DDA acquisition scheme (DDA-PASEF), we also tested the potential advantages of the DIA-PASEF acquisition mode (Meier et al., 2020), which produces nearly complete datasets with peptide features defined in a four-dimensional data space (retention time, m/z, ion mobility, and intensity). Finally, we benchmarked two of the most commonly used publicly available software solutions for analyzing multi-dimensional datasets generated in DDA- and DIA-PASEF modes, MaxQuant (Cox and Mann, 2008) and DIA-NN (Demichev et al., 2022).

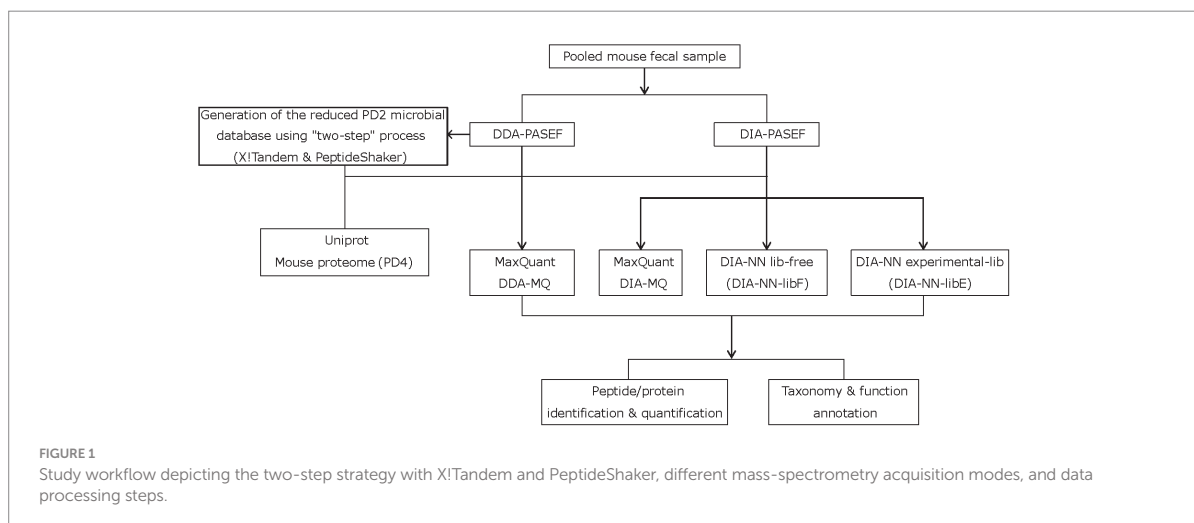
Identification and quantification performance

To compare the performance of different PASEF acquisition modes and data analysis software solutions, we first evaluated the total amount of microbial and mouse peptides and proteins, which were quantified in 10 technical replicates of a pooled mouse fecal sample (500 ng of total peptides per run). The data generated in DDA-PASEF mode were analyzed within MaxQuant (DDA-MQ), while DIA-PASEF data were analyzed using the TIMS MaxDIA module of MaxQuant (DIA-MQ) and DIA-NN. For the latter, we compared the performance

employing an experimentally generated spectral library (DIA-NN-libE) to the library-free mode (DIA-NN-libF). In all cases, we set a protein and precursor FDR cutoff of 1%, as calculated by each software.

We found that DIA-NN-libE yielded nearly two-fold improvements in the number of identified microbial peptides and proteins compared to DDA-MQ (Figure 2A). Due to the depth of the spectral library generated by DDA-MQ, DIA-MQ identifications were clearly limited (Figure 2A). In addition, the amount of identified host peptides and proteins were also increased two-fold (Figure 2A; Supplementary Table 1). Similar results were obtained when using the library-free mode (DIA-NN-libF; Figure 2A). This performance boost resulted in a fifteen-fold increase in sensitivity as highlighted by a higher detection of microbial and host peptides and proteins by DIA-NN-libE using 31.25 ng of peptides compared to 500 ng analyzed by DDA-MQ (Figure 2B; Supplementary Table 2). Importantly, all tested DIA approaches generated data matrices with considerably fewer missing identifications for both the microbial and host proteins, compared to DDA (Figure 2C). A comparison with representative studies using a classical DIA (Aakko et al., 2020) and a classical DDA methods (Kolmeder et al., 2016) revealed that the peptide overlap enabled by the DDA-PASEF vastly improved compared to classical DDA and reached similar performance as previously published classical DIA strategies. Further, DIA-NN-libE set new standards of reproducibility compared to the aforementioned methods (Figure 2D). This optimization of identification performance was not due to differences in FDR algorithms used by each software, as shown by a two-species strategy (Supplementary Figure 1). On the contrary, DIA-NN-libF detected significantly fewer false positive precursors than DDA-MQ at any given FDR level (Supplementary Figure 1).

We further critically evaluated the quantification performance of the DDA- and DIA-PASEF approaches. DIA-NN-libE quantified 12,700–28,487 peptides not detected by DDA-MQ at any injected amounts tested (Supplementary Figure 2A). Importantly, a detailed analysis showed that the quality score distribution of the peptides was similar between the ones uniquely identified by DIA-PASEF and the ones commonly identified by DDA- and DIA-PASEF (Supplementary Figure 2B). Notably, DIA-PASEF detected peptides



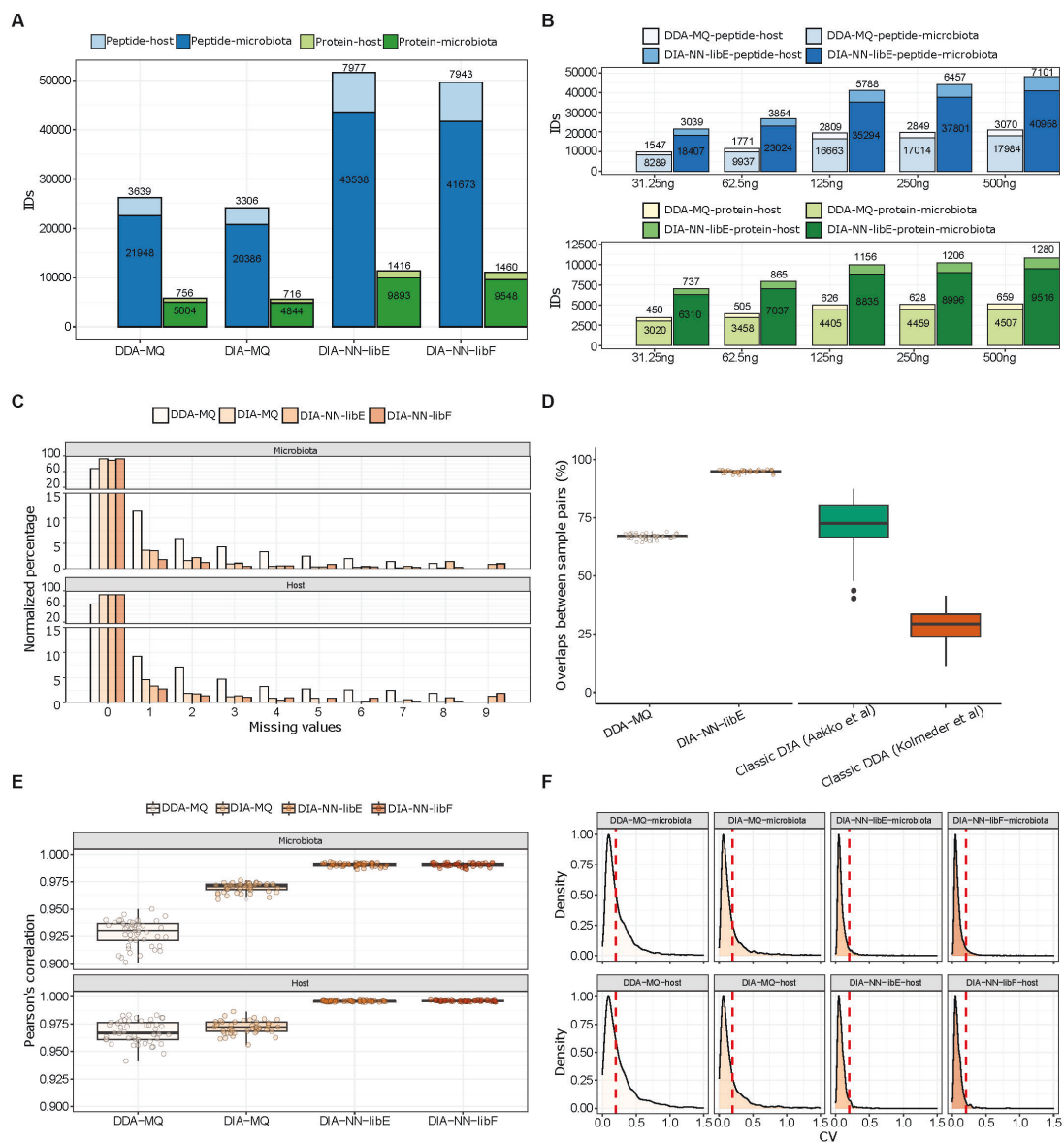


FIGURE 2
Performance evaluation of DDA- and DIA-PASEF analyzed with indicated software solutions. **(A)** Total amount of microbial and host peptides (dark and light blue, respectively) and proteins (dark and light green, respectively) identified in 10 technical replicates of a pooled mouse fecal sample in four different workflows. **(B)** Number of peptide (blue colors) and protein (green colors) identifications in either DDA-MQ or DIA-NN-libE workflows at different peptide injection quantities analyzed in triplicates. **(C)** Data completeness of microbial (upper) and host (lower) protein identifications in different workflows across 10 technical replicates. **(D)** Overlap of peptide identifications across all paired comparisons from 10 technical replicates in DDA-MQ and DIA-NN-libE workflows, and comparison with 2 previous studies. **(E)** Intra-group correlations of 4 workflows across all paired comparisons from 10 technical replicates. The dots indicate the Pearson's correlation for each possible paired comparison of proteins. **(F)** Coefficient of variation (CV) distribution of proteins in the microbiota (upper) and host (lower) across 10 technical replicates in 4 workflows. The red dotted line indicates the CV = 0.2.

covering over 4 orders of abundance magnitude, displaying the biggest gains, compared to DDA-PASEF, in the lower intensity ranges (Supplementary Figure 2C). These improvements were accompanied by better protein intensity correlations among all ten technical replicates in all three DIA-PASEF-based analyses (Figure 2E), which was preserved even at the lowest sample amount tested

(Supplementary Figure 3). As a result of this superior quantification accuracy, the number of proteins with a coefficient of variability (CV) < 20% significantly increased in DIA-PASEF (61% in DDA-MQ, 75% in DIA-MQ, and 94.49% in DIA-NN-libE; Figure 2F). In summary, our data clearly show the benefits of the fourth data dimension unlocked by PASEF highlighting the potential of

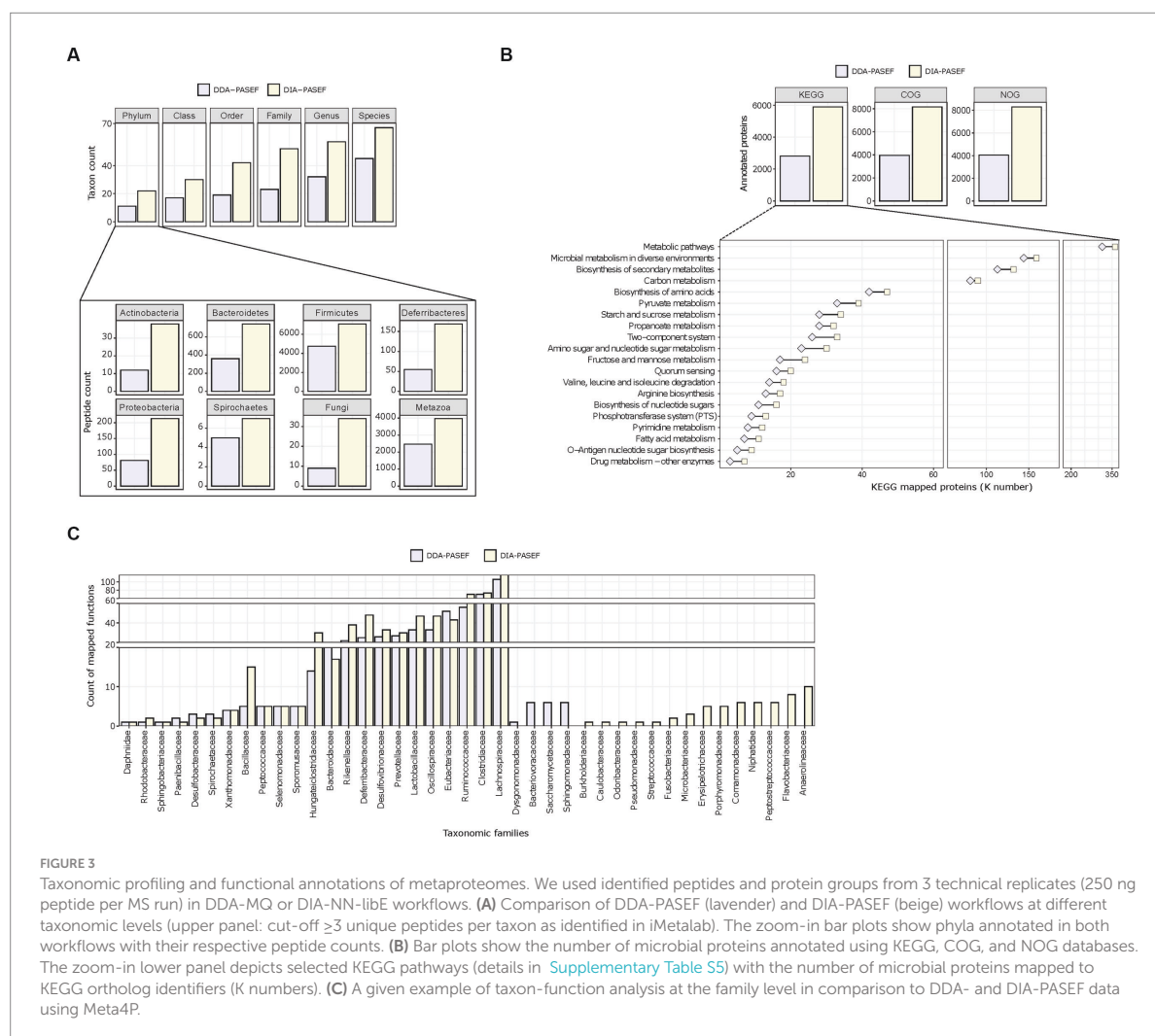
DIA-PASEF for serving as a new state-of-the-art acquisition method in metaproteomics.

Taxonomic and functional profiling

We next assessed the performance of DDA-PASEF and DIA-PASEF in identifying microbial taxa. Globally, DIA-PASEF identified organisms belonging to the four major kingdoms present in the mouse gut microbiome (Supplementary Figure 4A). Using a cut-off of at least three taxon-specific peptides, DIA-PASEF increased the identification performance from 1.48-fold (at the species level) to 2.26-folds (at the family level) compared to DDA-PASEF (Figure 3A; Supplementary Table 3). A detailed view revealed remarkable identification gains for several phyla such as *Actinobacteria*, *Bacteroidetes*, and *Firmicutes* in addition to fungi and metazoans (Figure 3A). DIA-PASEF detected more genera known to constitute the core gut microbiome of healthy mice reaching a lower limit of 0.04% relative abundance, as established by 16S rRNA sequencing (Wang et al., 2019) (genus: *Gordonia*; Supplementary Table 4).

Moreover, the peptide coverage was significantly increased for 34 of the 35 genera commonly detected by DIA- and DDA-PASEF (Supplementary Table 4).

Next, we investigated the annotation of detected proteins to functional pathways for both the microbiota and the host. The aforementioned improvements in protein quantification reached by DIA-PASEF translated to increased numbers of KEGG, COG, and NOG functional terms compared to DDA-PASEF (Figure 3B). A detailed analysis showed that DIA-PASEF increased the coverage of protein groups with general or unknown functions: DIA-PASEF enabled the quantification of 485 protein groups annotated with COG category R (General function prediction only) and category S (unknown function), in comparison to 220 protein groups annotated in these two categories with DDA-PASEF (Supplementary Table 3). DIA-PASEF data covered 100% of all KEGG pathways detected by DDA-PASEF (Figure 3B) and enabled the quantification of additional 11 pathways (Supplementary Table 5). Moreover, DIA-PASEF offered higher coverage in nearly 50% of commonly annotated pathways (Figure 3B and Supplementary Table 5).



Of particular interest is the fact that DIA-PASEF increased the detection of functions assigned to specific taxonomic families (Figure 3C). The number of KEGG entries assigned by peptides detected using DIA-PASEF increased for the majority of members at all taxonomic ranks (family level in Figure 3C, all other ranks in Supplementary Table 6), which highlights the benefits of the workflow.

As for the host biology, DIA-PASEF enabled the characterization of 77 unique protein functional categories (Supplementary Table 5), as well as an increased number of host proteins associated with Gene Ontology Molecular function terms (GO-MF) commonly detected by DDA-PASEF (Supplementary Figure 4B and Supplementary Table 5).

DIA-PASEF offers novel insights into host-microbiome interactions upon neuropathic pain

Chronic pain affects an estimated 20% of the world's population (Treede et al., 2015; Price and Gold, 2018). Nevertheless, current therapies are ineffective for many patients and exhibit significant side effects (Price and Gold, 2018). Initial studies utilizing 16S rRNA sequencing showed changes in the gut microbiome composition during the onset and regulation of chronic pain in humans and animal models (Morreale et al., 2022), suggesting the gut microbiome as a potential target for novel treatments. However, our knowledge is scarce and a thorough assessment of functional alterations of the gut microbiome associated with chronic pain is awaiting.

We set out to test our DIA-PASEF metaproteomic workflow in the validated spared nerve injury (SNI) mouse model of neuropathic pain (Decosterd and Woolf, 2000). We collected fecal samples from four aged-matched SNI, four SHAM (surgery without nerve injury), and four Naive female mice before (Pre) and 14 days (14D) post-surgery covering the critical period of chronic pain onset (Cobos et al., 2018; Segelcke et al., 2021) (Figure 4A). Analysis of mouse behaviours confirmed the expected development of neuropathic pain as indicated by mechanical allodynia (Cobos et al., 2018) (i.e., hypersensitivity to an innocuous tactile stimulus to the affected hind paw) in SNI but not in SHAM mice (Supplementary Figure 5). In total, our DIA-PASEF workflow detected 81,378 peptides corresponding to 12,239 and 2,267 microbial and mouse protein groups, respectively (Figure 4A; Supplementary Table 7). We quantified 29 phyla, 45 classes, and 119 species with at least 3 peptides per taxon (Supplementary Figure 6A and Supplementary Table 8). A comparison to two previously published classical DDA mouse gut metaproteomic studies (utilizing 16 times more injected peptides, and 4 times longer chromatography gradients per run (Tanca et al., 2017, 2018)), showed the ability of our DIA-PASEF workflow to profile up to 4 times more taxonomic units (Supplementary Figure 6B). Additionally, we harnessed a recent compilation of 2,446 global mouse gut metagenomes (Beresford-Jones et al., 2022) as a reference for microbiota abundance. Remarkably, DIA-PASEF exhibited high sensitivity and detected species with a mean abundance of 0.003 (when using the cutoff of 3 species-specific peptides, i.e., *Alistipes indistinctus*; Supplementary Table 4).

From the taxonomic point of view, we revealed that the bacterial classes *Actinobacteria* and *Gammaproteobacteria* were increased

during neuropathic pain in SNI animals at 14D (Figure 4B; Supplementary Table 9). Besides taxonomy, metaproteomics facilitates insights into host-microbiome interactions. To reveal functional changes in host-microbiome interactions upon nerve injury, we investigated proteins and their association with biological pathways. Pathway analysis using KEGG terms showed significant enrichment of distinct microbial pathways in each of the three experimental conditions when comparing 14D with “Pre” data (Figure 4C; Supplementary Table 9). With respect to the functional changes in the gut proteome of the host (i.e., in mice), there was only limited overlap across all three conditions; instead, we observed rather quite specific pathway alterations (Supplementary Figure 7A): while SNI animals exhibited upregulation of processes related to the immune response of the gut (Figure 4D), in SHAM animals the repertoire of altered proteins was distinct, and the extent of observed changes was lower except for the increase of several major urinary proteins (Supplementary Figure 7B). Notably, unsupervised clustering of protein intensities “Pre” surgery strongly suggests that observed condition-specific alterations were not due to baseline gut proteome differences between the mice (Supplementary Figure 7C; importantly, the mice were randomly assigned to the three experimental conditions before the surgeries, please see Methods for details). Taken together, our results provide a stepping stone for follow-up interventional studies investigating the mechanistic relevance of here discovered host-microbiome alterations for neuropathic pain.

Discussion

We present a novel metaproteomic workflow utilizing PASEF technology, as well as deep neural network-based data analysis offered by DIA-NN. The combination of both elements yielded very significant improvements compared to previous metaproteomic workflows and facilitated, for the first time, the global assessment of host-microbiome interactions in a mouse model of neuropathic pain.

We hypothesized that the features of the PASEF technology would be especially advantageous in a sample with high dimensionality like the fecal microbiome, where few species represent more than 80% of the total abundance (Duan et al., 2022). The sequential release of ion packages trapped in the tunnel of the TIMS mobility analyzer together with the synchronization of the quadrupole selection increases sensitivity and reduces the complexity of spectra generated by PASEF (Meier et al., 2015). Accordingly, we show that the reproducibility of detection between samples offered by DDA-PASEF doubled compared to classical DDA approaches and even matched the performance of classical DIA (Aakko et al., 2020). Adding a DIA mode to the PASEF scheme enabled us to even further increase the number of peptides and proteins quantified in comparison to DDA-PASEF. These gains became overtly evident in peptides at the lower abundance range, a fact that will greatly improve the detection limits of current metaproteomic workflows (Duan et al., 2022). The CScore distribution of the peptides was similar between the ones uniquely identified by DIA-PASEF and the commonly identified by DDA- and DIA-PASEF, which establishes high confidence in identification gains enabled by DIA-PASEF. Notably, DIA-PASEF reached similar or even better taxonomic depth than previous large-scale mouse metagenomic studies (using 16S rRNA). Upcoming DIA-PASEF schemes (Szyrwiel et al., 2022; Distler et al., 2023; Skowronek et al., 2023) as well as other

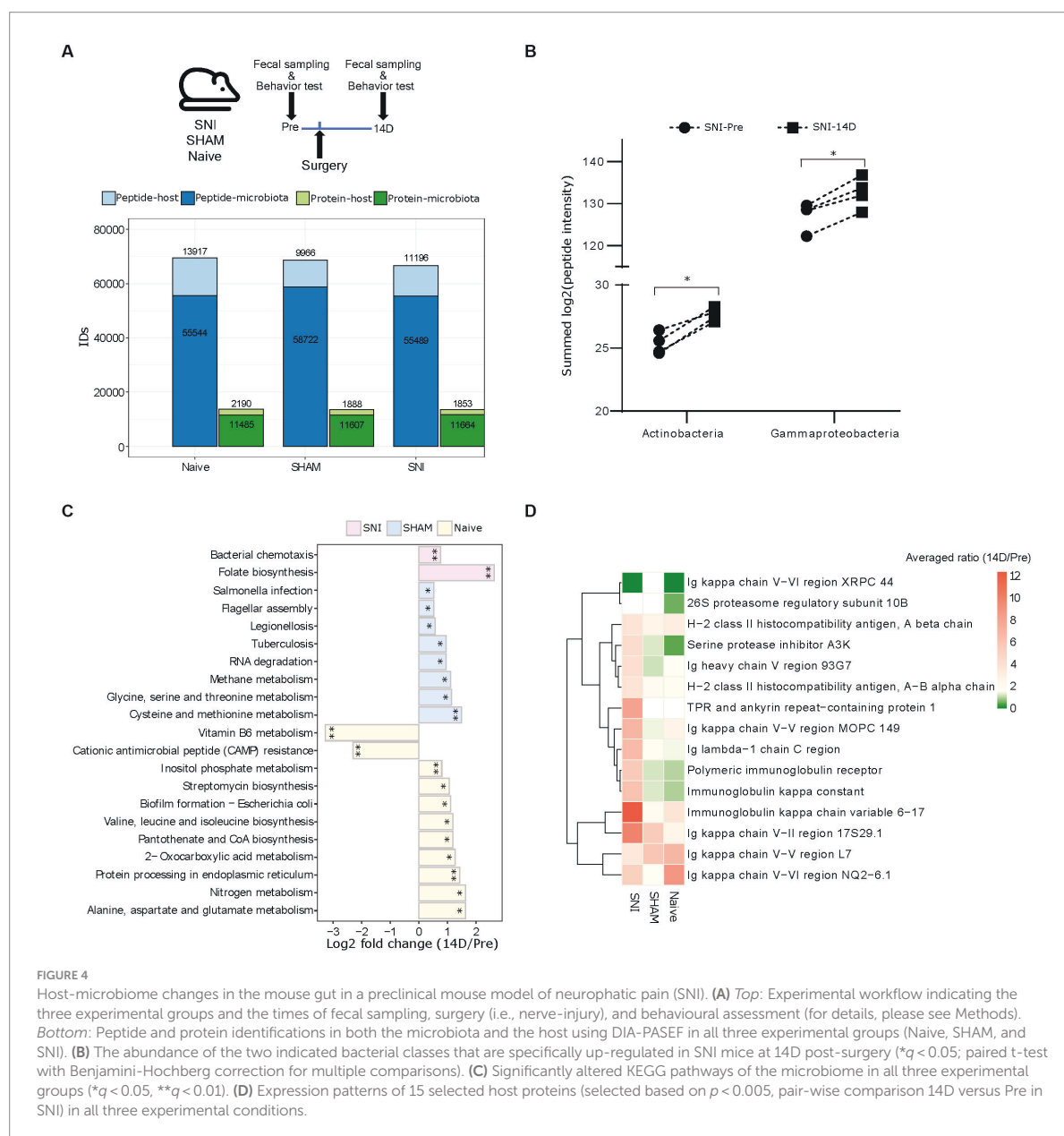


FIGURE 4

Host-microbiome changes in the mouse gut in a preclinical mouse model of neuropathic pain (SNI). **(A)** Top: Experimental workflow indicating the three experimental groups and the times of fecal sampling, surgery (i.e., nerve-injury), and behavioural assessment (for details, please see Methods). Bottom: Peptide and protein identifications in both the microbiota and the host using DIA-PASEF in all three experimental groups (Naive, SHAM, and SNI). **(B)** The abundance of the two indicated bacterial classes that are specifically up-regulated in SNI mice at 14D post-surgery (* $q < 0.05$; paired t-test with Benjamini-Hochberg correction for multiple comparisons). **(C)** Significantly altered KEGG pathways of the microbiome in all three experimental groups (* $q < 0.05$, ** $q < 0.01$). **(D)** Expression patterns of 15 selected host proteins (selected based on $p < 0.005$, pair-wise comparison 14D versus Pre in SNI) in all three experimental conditions.

DIA data analysis tools (Zhang et al., 2023) have the potential to further advance the impact of PASEF on metaproteomics that we pioneered in this study.

DIA-PASEF achieves a nearly complete record of all peptide fragments (Meier et al., 2020). Despite the significant improvements facilitated by DIA-PASEF, only 31,957 out of 66,748 microbial peptides (47%) identified during the analysis of the neuropathic pain samples were taxonomically annotated highlighting a limitation in the depth of currently available taxonomic databases. In addition, a large amount of peptide spectra likely remains unidentified. Nonetheless, this information is stored in very comprehensive digital MS/MS datasets. Thus, our workflow offers

the possibility to interrogate these data in the future by either using continuously improved microbiome-based databases or emerging AI-based algorithms. The use of these datasets in the metaproteomic field will offer extra advantages: community-shared data resources, optimization in the utilization of valuable microbiome samples (e.g., clinical human samples), and reduced animal experimentation for hypothesis testing.

The taxonomic and functional depths achieved by DIA-PASEF allowed us to directly interrogate, for the first time, the functional alterations in the gut ecosystem during neuropathic pain. Our findings suggest that *Actinobacteria* and *Gammaproteobacteria* are increased upon neuropathic pain in SNI animals. These data are in line with

previous genomic findings in SNI mice (Hua et al., 2021) and interstitial cystitis human pain syndrome (Braundmeier-Fleming et al., 2016).

Far beyond the extent of previous taxonomic studies, we detected an upregulation of the microbial KEGG pathway “folate biosynthesis” exclusively upon nerve injury. Folate is produced in the murine gut by several bacterial taxa including *Actinobacteria* (Engevik et al., 2019), and its deficiency has been associated with a greater risk of peripheral neuropathy in a retrospective cohort of more than 500,000 people (Tavernier et al., 2019). Remarkably, Botez et al. (1978) showed an improvement in clinical and electrophysiological measurements of five patients with polyneuropathy after 9–39 months of folate therapy. Therefore, the functional insights offered here might point towards an endogenous mechanism, by which the gut ecosystem aims at ameliorating neuropathic pain. This is an exciting hypothesis that may open new therapeutical avenues harnessing folate supplementation as already implemented for diverse autoimmune diseases (Mölzer et al., 2021). Folate was reported to stabilize and increases the abundance of T_{reg} lymphocytes in the colon (Mölzer et al., 2021). Interestingly, T_{reg} lymphocytes can alleviate chronic neuropathic pain by inhibiting the Th1 response via CD4+ helper cells (Bethea and Fischer, 2021). Emerging clinical evidence proposes an intricate interplay between the gut microbiome, neuroimmune signalling and neuropathic pain (Ustianowska et al., 2022), e.g., by influencing the balance between pro-inflammatory and anti-inflammatory T cells (Ding et al., 2021).

In addition to the potential role of bacterial folate production, we also discovered alterations of several host protein complexes, exclusively in SNI animals. For example, those known to be involved in the crosstalk between the host immune system and the gut microbiome. We observed an upregulation of two components of the Major Histocompatibility Complex II (MHC-II). MHC-II contributes to the maturation of B cells via the presentation of exogenous bacterial antigens and the production of secreted IgA (SIgA) (Jiang et al., 2019). Dysregulation of the IgA-microbiota axis affects multiple pathologies with an inflammatory component (Abokor et al., 2021), but its link to pain syndromes is unknown. Notably, we observed a significant upregulation of the Polymeric Immunoglobulin Receptor in SNI animals at 14D. Polymeric Immunoglobulin Receptor represents one of the two proteins that are necessary for the secretion of IgA from gut mucosal plasma cells into the gut lumen where it contributes to controlling the abundance of commensal microbiota (Weis and Round, 2021). A detailed analysis of our data shows that both the immunoglobulin J chain (J chain), also being part of this secretory complex, as well as IgA tend to be upregulated in SNI animals, even though they did not pass our statistical cutoff (Uniprot IDs: P01592 and P01878 in Supplementary Table 10). Furthermore, we observed a significant increase in *Gammaproteobacteria* in SNI mice. In this context, it is noteworthy that *Gammaproteobacteria* are known to produce gut inflammation in mice thereby triggering an IgA-dependent immune response via activation of B cells (Mirpuri et al., 2014). Thus, our findings open new avenues for future mechanistic studies aimed at deciphering the role of these microbiome-immune pathways for chronic pain. Besides, our data revealed prominent functional changes occurring in the gut of age-matched Naive mice over the two-week experimental period, which highlights the need to adequately control for purely age-induced changes in microbiome studies by including Naive non-treated mice.

In summary, we present the valuable potential of DIA-PASEF to provide in-depth and novel insights into host-microbiome interactions with a significant impact on our understanding of microbiological ecosystems across diverse biology disciplines.

Data availability statement

The data presented in the study are deposited in the PRIDE repository, accession number PXD040947.

Ethics statement

The animal study was approved by all animal experiments were carried out in strict accordance with institutional IACUC guidelines, international ARRIVE guidelines, and the principles of the 3Rs of animal research and were approved by BMBWF (Federal Ministry of Education, Science and Research, Austria). All experimental procedures were approved by the BMBWF (GZ 2020–0.592.919). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

DG-V: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. FX: Data curation, Investigation, Methodology, Resources, Software, Visualization, Writing – review & editing. SG: Data curation, Investigation, Methodology, Writing – review & editing. JS: Methodology, Writing – review & editing. GC: Data curation, Writing – review & editing. MS: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2023.1258703/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Identification performance of DIA-NN and MaxQuant software. Precursor identification numbers are plotted against the FDR, estimated using a two-species library method, searching the data against both the microbial protein database (PD2) and the *A. Thaliana* proteome (UP000006548). Each point in the graph corresponds to a decoy (*A. Thaliana*) precursor with the x-axis reflecting its estimated FDR and the respective score threshold and the y-axis representing the number of target microbial precursors at this threshold.

SUPPLEMENTARY FIGURE 2

Comparisons of peptide identifications. (A) Venn diagrams show the unique and overlapped number of identified microbial peptides between

the DIA-NN-libE and the DDA-MQ workflows, at increasing amounts of the injected peptides. (B) CScore (the score used to calculate precursor q-value in DIANN) distribution (histogram, 30 bins) of peptides uniquely identified in DIA-PASEF workflow (red) and commonly identified in DDA- and DIA-PASEF workflow (gray). (C) Left: Dynamic range of peptides uniquely identified using DIA-NN-libE (red symbols) or shared with DDA-MQ (grey symbols), when 31.25 ng of peptides were injected. Right: Number of detected peptides in each intensity quarter either exclusively identified using DIA-NN-libE (red-bordered boxes) or shared with DDAMQ (grey-bordered boxes).

SUPPLEMENTARY FIGURE 3

Correlation plots between 3 technical replicates at the lowest peptide quantity tested when using the DDA-MQ (A) and the DIA-NN-libE (B) workflow.

SUPPLEMENTARY FIGURE 4

(A) Hierarchical classification of annotated taxonomy from Unipept using DIA-NN-libE data. (B) Top 30 common GO-MF (Gene Ontology-Molecular Function) enriched in the host proteome (analyzed by Metascope using a cut-off of 3 proteins per function and an adjusted *p*-value < 0.01).

SUPPLEMENTARY FIGURE 5

Mechanical sensitivity tests of SNI mice (A) and SHAM mice (B) before (Pre) and after surgery (14D). Each data point on the left side of both graphs corresponds to the ratio of ipsilateral (operated) and contralateral (nonoperated) paw values of individual mice (two-tailed paired *t*-test; *N* = 4 mice/condition). The right side of both graphs shows the mean (SNI = 0.702, SHAM = 0.031) and individual differences at Pre and 14D with 95% confidence interval.

SUPPLEMENTARY FIGURE 6

(A) Taxonomic annotations in all three conditions at 6 taxa levels. Cutoff: at least 3 unique peptides per taxon (as identified by iMetablab). (B) Taxonomic comparison between our study using DIA-PASEF and two published studies using classical DDA. Peptide sequences of two published studies were retrieved from PRIDE using the identifiers and then subjected to iMetablab for taxonomic annotation (cutoff: at least 3 peptides per taxa). In the case of PXD004911 only peptides identified from fecal samples (but not caecal contents) were used for the comparison.

SUPPLEMENTARY FIGURE 7

Differential expression in the host proteome. (A) Shared and unique numbers of regulated host proteins after pairwise comparison (14D versus Pre) in each condition (*p*-value < 0.005). (B) The expression patterns of 32 selected host proteins (selected based on *p*-value < 0.005, pair-wise comparison, 14D versus Pre in SHAM) in all three experimental groups. The color code indicates the average ratio (14D/Pre) of each host protein. (C) PCA analysis of the protein intensities quantified in the feces of Naive, SHAM, and SNI mice before the surgery.

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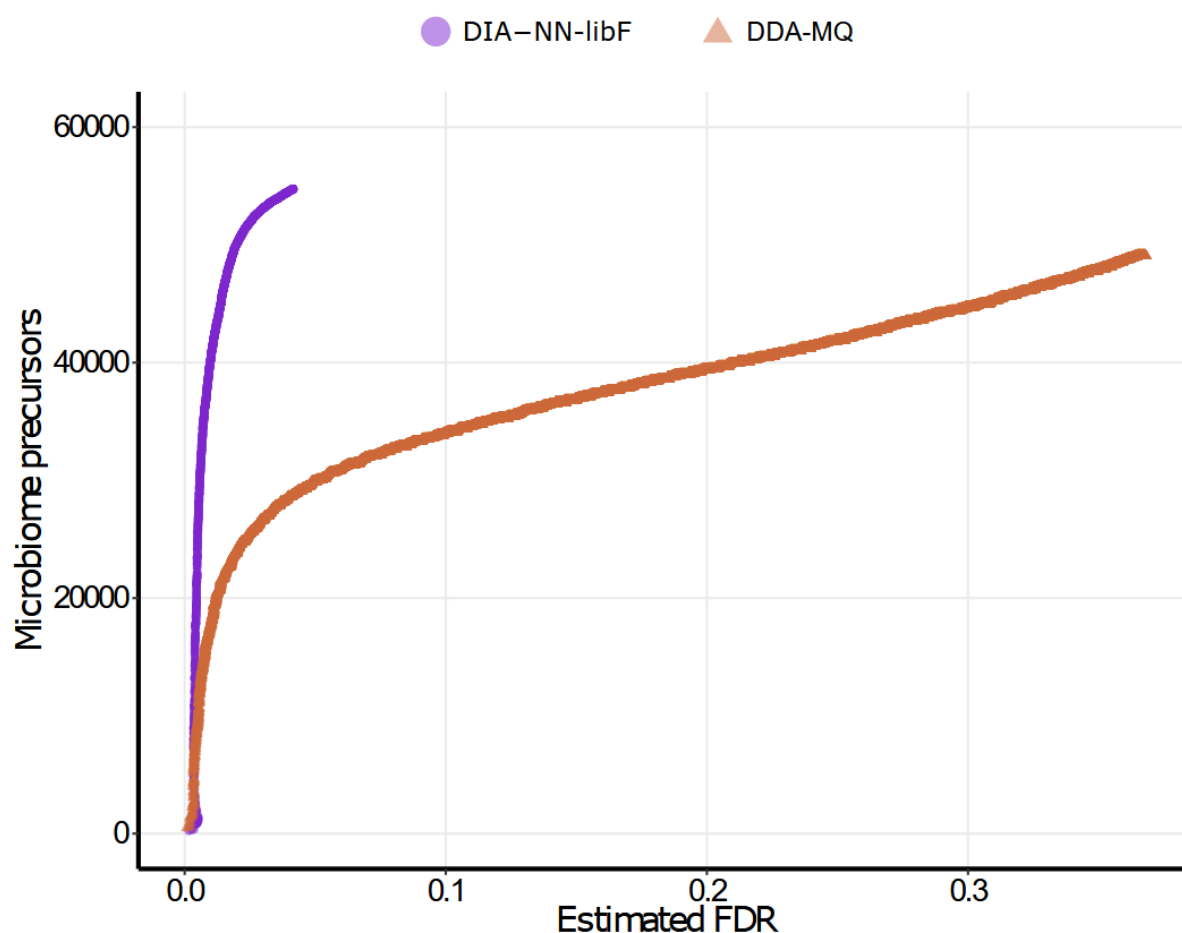
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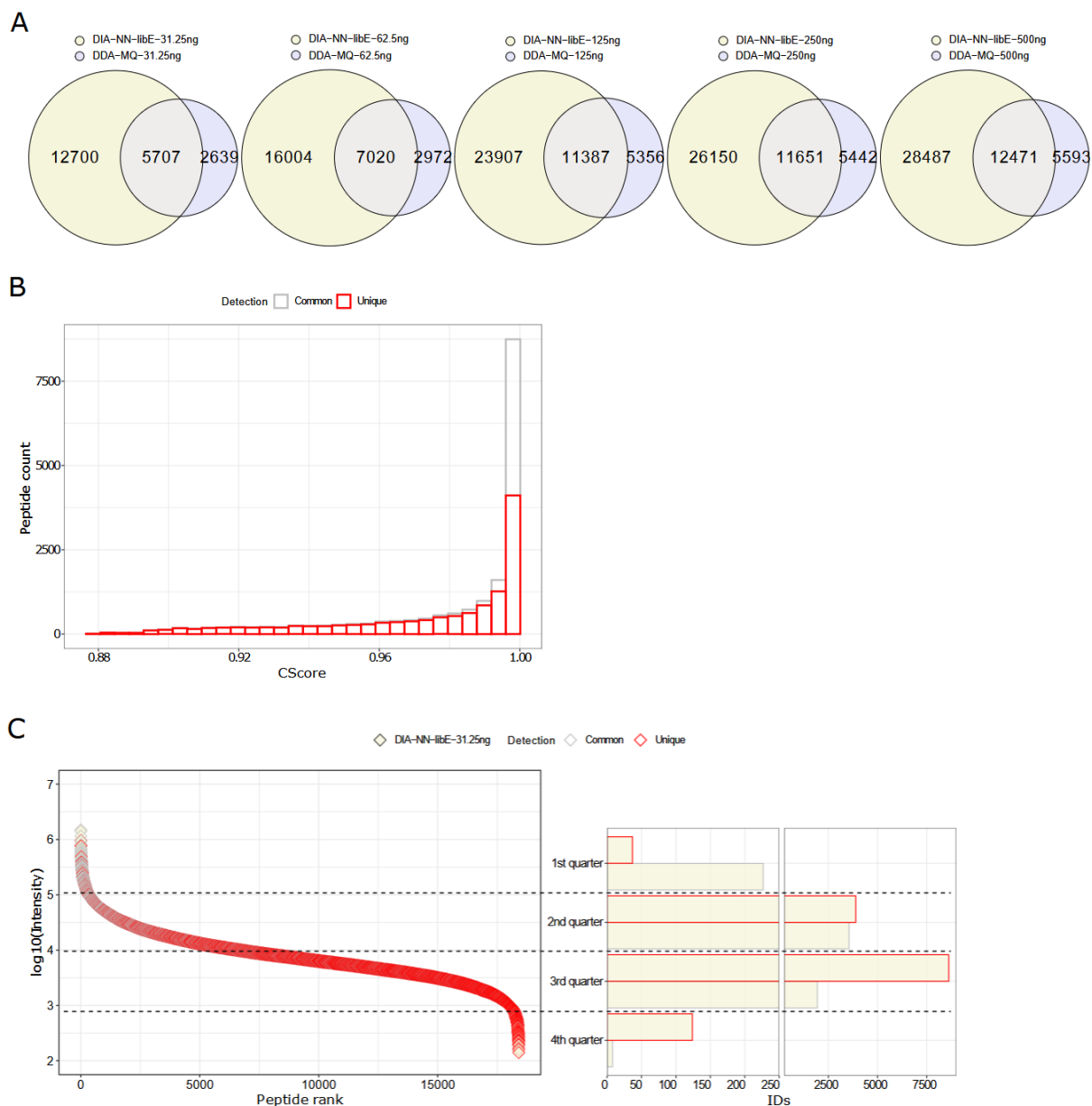
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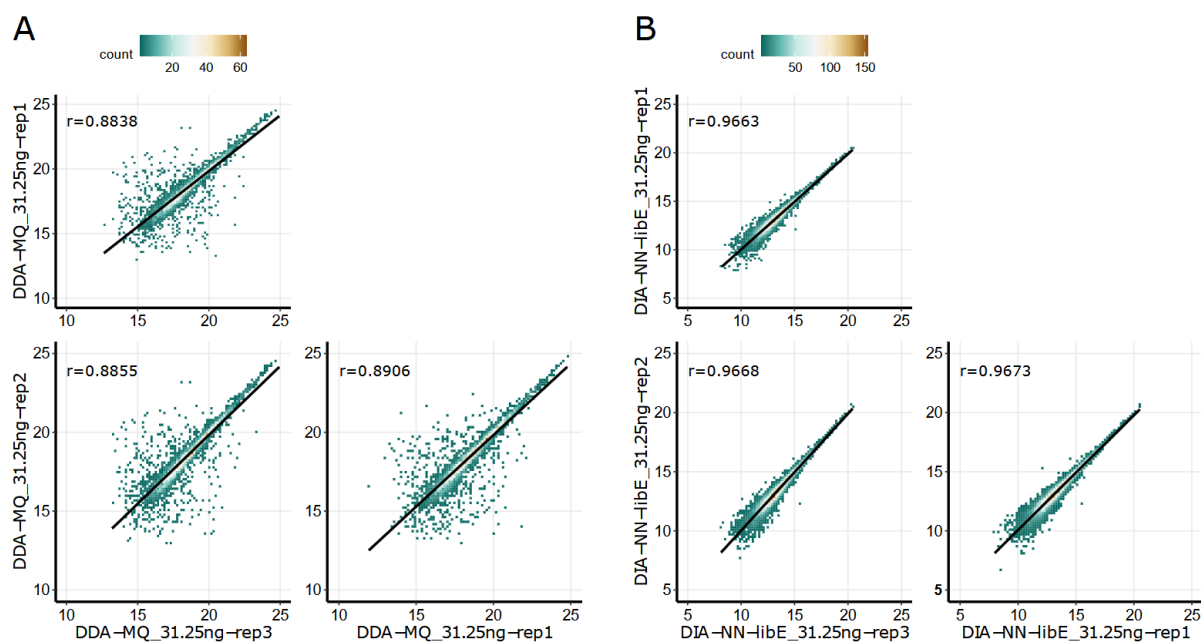
Supplementary Figure 1

Identification performance of DIA-NN and MaxQuant software. Precursor identification numbers are plotted against the FDR, estimated using a two-species library method, searching the data against both the microbial protein database (PD2) and the *A. Thaliana* proteome (UP000006548). Each point in the graph corresponds to a decoy (*A. Thaliana*) precursor with the x-axis reflecting its estimated FDR and the respective score threshold and the y-axis representing the number of target microbial precursors at this threshold.



Supplementary Figure 2

Comparisons of peptide identifications. **(A)** Venn diagrams show the unique and overlapped number of identified microbial peptides between the DIA-NN-libE and the DDA-MQ workflows, at increasing amounts of the injected peptides. **(B)** CScore (the score used to calculate precursor q-value in DIANN) distribution (histogram, 30 bins) of peptides uniquely identified in DIA-PASEF workflow (red) and commonly identified in DDA- and DIA-PASEF workflow (gray). **(C) Left:** Dynamic range of peptides uniquely identified using DIA-NN-libE (red symbols) or shared with DDA-MQ (grey symbols), when 31.25 ng of peptides were injected. **Right:** Number of detected peptides in each intensity quarter either exclusively identified using DIA-NN-libE (red-bordered boxes) or shared with DDAMQ (grey-bordered boxes).



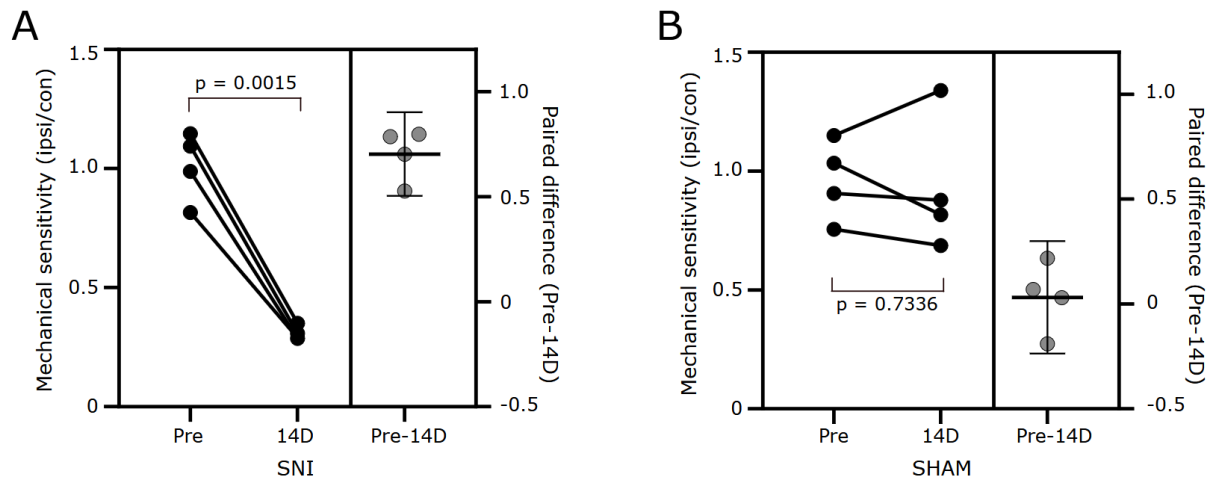
Supplementary Figure 3

Correlation plots between 3 technical replicates at the lowest peptide quantity tested when using the DDA-MQ (**A**) and the DIA-NN-libE (**B**) workflow.



Supplementary Figure 4

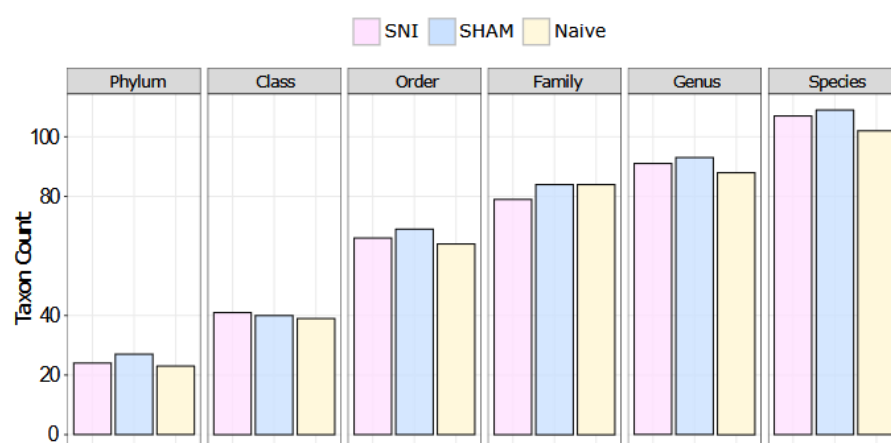
(A) Hierarchical classification of annotated taxonomy from Unipept using DIA-NN-libE data. **(B)** Top 30 common GO-MF (Gene Ontology-Molecular Function) enriched in the host proteome (analyzed by Metascape using a cut-off of 3 proteins per function and an adjusted p -value < 0.01).



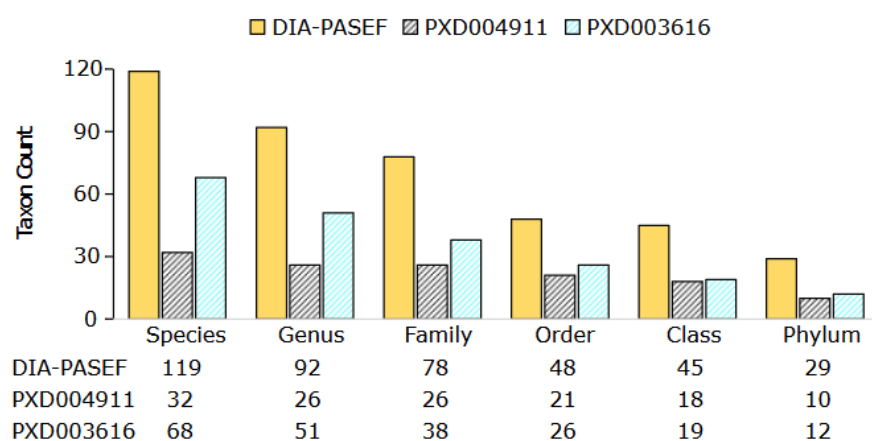
Supplementary Figure 5

Mechanical sensitivity tests of SNI mice **(A)** and SHAM mice **(B)** before (Pre) and after surgery (14D). Each data point on the left side of both graphs corresponds to the ratio of ipsilateral (operated) and contralateral (nonoperated) paw values of individual mice (two-tailed paired t-test; $N = 4$ mice/condition). The right side of both graphs shows the mean (SNI = 0.702, SHAM = 0.031) and individual differences at Pre and 14D with 95% confidence interval.

A

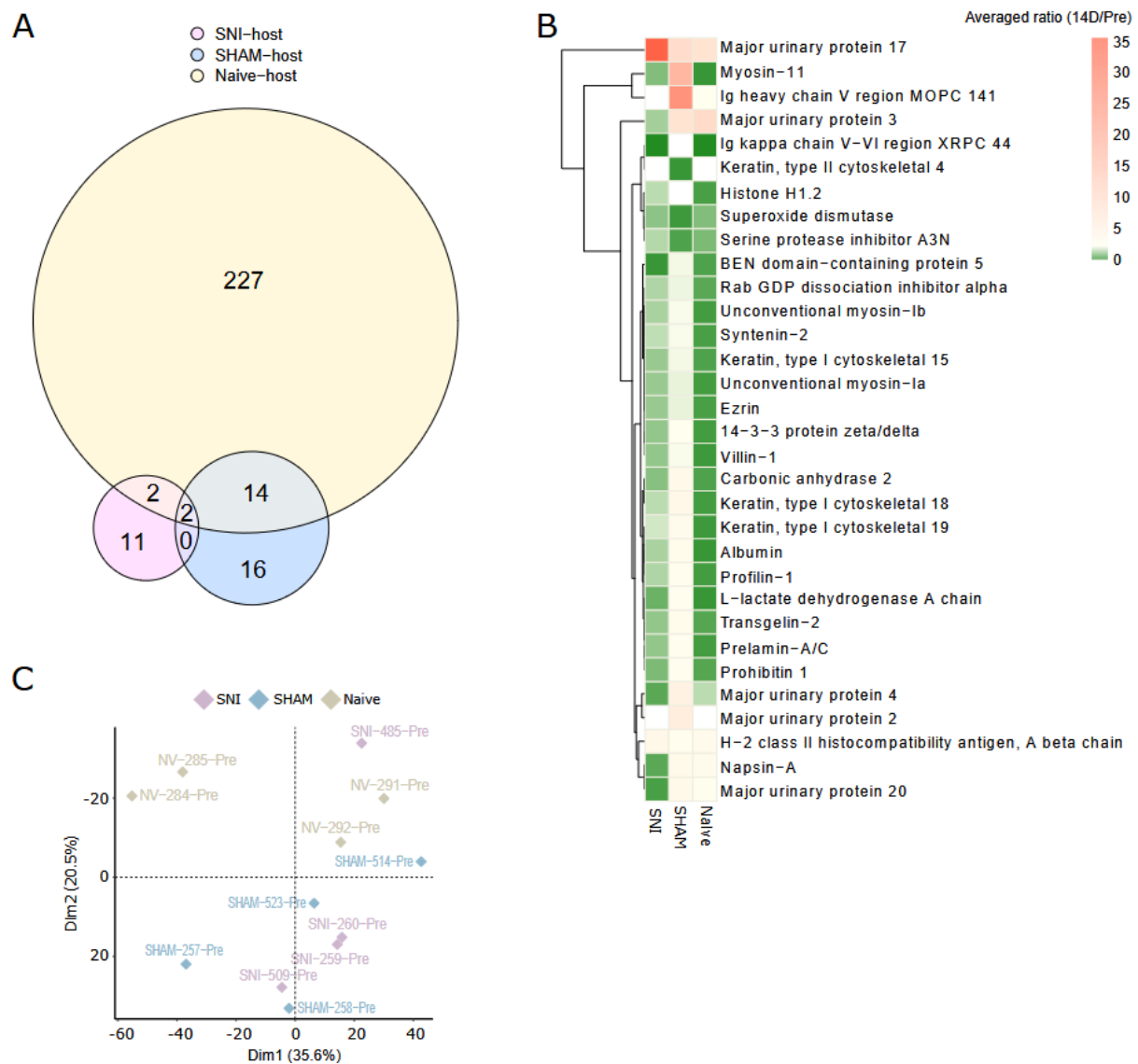


B



Supplementary Figure 6

(A) Taxonomic annotations in all three conditions at 6 taxa levels. Cutoff: at least 3 unique peptides per taxon (as identified by iMetalab). **(B)** Taxonomic comparison between our study using DIA-PASEF and two published studies using classical DDA. Peptide sequences of two published studies were retrieved from PRIDE using the identifiers and then subjected to iMetalab for taxonomic annotation (cutoff: at least 3 peptides per taxa). In the case of PXD004911 only peptides identified from fecal samples (but not caecal contents) were used for the comparison.



Supplementary Figure 7

Differential expression in the host proteome. **(A)** Shared and unique numbers of regulated host proteins after pairwise comparison (14D versus Pre) in each condition (p -value < 0.005). **(B)** The expression patterns of 32 selected host proteins (selected based on p -value < 0.005, pair-wise comparison, 14D versus Pre in SHAM) in all three experimental groups. The color code indicates the average ratio (14D/Pre) of each host protein. **(C)** PCA analysis of the protein intensities quantified in the feces of Naive, SHAM, and SNI mice before the surgery.

Chapter 4 - Discussion

4.1 Research gap

Neuropathic pain is a maladaptive disease, with a high prevalence [64-67]. Patients suffer from loss of, or increased, sensory sensitivity and spontaneous pain [71, 72], as well as many comorbidities [83-86]. Although pain has been extensively researched over the last years, its complexity and diversity makes it difficult to achieve a comprehensive understanding. Since current treatment options are inefficient, and often associated with many adverse effects, the need for new therapeutic tools is enormously high [60, 147, 149]. In order to develop those, the underlying molecular mechanisms of neuropathic pain have to be identified [18].

This dissertation aimed to investigate peripheral and systemic alterations of neuropathic pain, using proteome- and metaproteome-based techniques, thereby providing a comprehensive understanding of neuropathic pain pathophysiology and pave the way for the identification of potential new therapeutic targets.

4.2 Sex- and age-dependent impact on neuropathic pain

Sex has an immense influence on neuropathic pain, with women accounting for the majority of chronic neuropathic pain patients [179, 180, 466]. Although sex-specific pain mechanisms have already been identified [124, 238, 467], we still lack a comprehensive understanding. Historically, pain research predominantly focused on males and thus conclusions about pain mechanisms can also only be drawn for male subjects [68]. Another important variable in pain perception is age. Maturation processes of the body, including the pain-axis, take place, leading to significant changes in the sensory sensitivity across a lifespan [272, 296, 435, 468-472]. However, which molecular mechanisms underlie these age-dependent changes is still unclear. Therefore, it is of the utmost importance, to investigate male and female subjects, of different age groups. Only by investigating these variables simultaneously, can comprehensive conclusions be drawn and new (pharmacological) treatments be developed, which are suitable for males and females of different age groups. This can also lead to the further development of personalized medicine.

In chapter 2, we integrated sex and age as variables in our proteomic analysis, and could thereby capture the complexity of nerve injury-induced changes from dorsal

root ganglia (DRG) in mice. We employed two complementary approaches, differential expression analysis of single candidate proteins (DEPs), and multidimensional factor analysis (MEFISTO), revealing distinct and overlapping changes across sex and age groups. MEFISTO allowed us to investigate the data longitudinally, revealing changes in proteins associated with neuroimmune pathways, inflammatory signaling, and neuronal (dys)function. We focused on sex- and age-shared protein signatures, with the aim of identifying key pain players, important in chronic pain development in males and females of different age groups. With the aim to identify potential new targets for pain therapy, an important aspect is the translatability of preclinical data. In this study, we present evolutionary conserved proteins, from mice and humans, reflecting fundamental processes induced by nerve injury [39, 55, 56, 252, 444]. These protein candidates have an increased likelihood to successfully translate into clinical application as targets for novel pharmacological treatments. The seemingly most promising pain targets from the above work are *Itgam*, *Ptprc* and *Vav1*. These sex- and age-shared proteins form a subnetwork of immune and inflammatory pathways. *Ptprc* is known to influence B- and T-cell activation, nervous system development, and axon guidance [473, 474]. Furthermore, previous transcriptomic studies could already show alterations of *Ptprc* in neuropathic pain in rodent and human studies [252, 475, 476]. *Vav1*, a guanosine diphosphate/triphosphate (GDP/GTP) exchange factor (GEF) [477, 478], is relevant for microglial activation and T-cell-derived signal transduction, whereby it influences development, proliferation, and activation of T-cells [479, 480]. Additionally, analysis of drug-gene interactions showed targeting of *Ptprc* and *Vav1* via Epoetins. Epoetins are known to promote neuronal plasticity and regeneration [481], which could be of interest for nerve injury-induced neuropathic pain. The role of protein tyrosine phosphatases (Ptp)-modulators has been investigated in autoimmune disorders and various tumor types with promising results [482].

Since our proteome data cannot be assigned to certain cell types or neuronal subpopulations harbored in DRG, we decided to use the currently most comprehensive single-cell RNA-sequencing database of mouse and human DRG (<https://painseq.webflow.io>). Thereby, we could show an enrichment of immune cells in mouse and human DRGs, as well as species-specific differences in neuronal subtypes. The promising targets, *Ptprc* and *Vav1*, seem to be expressed by neuronal and immune cells in human DRG. However, these assignments to certain cell types

are only rough estimations. Single-cell mass spectrometry will improve our understanding, however, this technique still requires further development.

Overall, this study shows the importance of integrating sex and age as variables in pain research, to get a comprehensive picture of neuropathic pain mechanisms. Investigations at the protein level, including their networks and dynamics are needed for a system-wide understanding of the somatosensory system [483]. The identification of key pain players promotes future functional validations.

4.3 The microbiome in neuropathic pain

Chapter 3 of this thesis investigated changes of the gut microbiome during neuropathic pain. Many diseases have been associated with gut microbiome disbalances [337, 347-350], including visceral and abdominal pain [351, 352, 484], with increasing evidence for an impact in neuropathic pain [372, 373]. A rapidly advancing technology in this field is metaproteomics [450, 485, 486], with its advantage of exploring host-microbiome interactions, as well as functional insights of microorganisms [452]. However, the complexity of microbial samples [453, 454], such as feces, pushes current technologies to a detection limit [453]. Therefore, improved techniques are necessary to enable complex analysis of the microbiome and its host.

In our metaproteomic workflow, we used a novel combination of data-independent acquisition (DIA) with Parallel Accumulation-Serial Fragmentation (PASEF). This methodology afforded us the first global analysis of host-microbiome interactions in neuropathic pain. We achieved increased peptide and protein identifications even with reduced injection amount and chromatography gradient time, especially at the lower abundance range. In addition, we could show an improvement of the consistency and reproducibility from a quantitative point of view. Moreover, this method deepened the taxonomic and functional detection of the gut microbiome greatly, compared to data-dependent acquisition (DDA) workflows. Applying this combined technique, we observed an increased abundance of *Actinobacteria* and *Gammaproteobacteria*, in neuropathic pain mice, consistent with findings from a previous metagenome study on neuropathic pain [487]. Notably, *Gammaproteobacteria* have been previously implicated in gut inflammation in mice through an immunoglobulin A (IgA)-dependent immune response [488]. Furthermore,

our analysis revealed the upregulation of the folate biosynthesis pathway upon neuropathic pain in mice, which can be produced by *Actinobacteria* [489]. Evidence linking folate levels to peripheral neuropathy has already been demonstrated, suggesting a potential endogenous role of the gut microbiome in alleviating neuropathic pain [490, 491]. This mechanism may involve the stabilization and expansion of T_{reg} lymphocytes, facilitated by folate, which is known to suppress the pro-inflammatory Th1 response, thereby contributing to neuropathic pain reduction [492]. An increasing body of evidence supports a causal relationship between the gut microbiome, neuroimmune signaling, and neuropathic pain modulation [341], mediated through the interplay of pro- and anti-inflammatory T-cells [373]. Moreover, our study uncovered interactions between the microbiota and the host. Following nerve injury, we discovered upregulation of immune-related proteins, including Major Histocompatibility Complex II (MHC-II) and Polymeric Immunoglobulin Receptor (PIGR), suggesting an interaction of the gut microbiota with the host immune response, further emphasizing its role in neuropathic pain. Both, MHC-II and PIGR are involved in the production and secretion of IgA [493, 494], a key player in inflammatory diseases [495], however, its connection to pain regulation remains unclear. The observed increase in *Gammaproteobacteria* and their role in IgA-dependent immune responses further underscores the need for continued investigation in neuropathic pain. Additionally, we highlighted the importance of using age-matched controls, as we identified significant functional changes in the gut of naïve mice, reinforcing the impact of age as a variable in neuropathic pain research.

To conclude, this exploratory study provides novel insights into the microbiological ecosystem in neuropathic pain, shedding light on the involvement of microbiome-immune pathways. These findings pave the way for mechanistic studies that can further elucidate the complex interactions within the gut microbiome in neuropathic pain conditions.

4.4. Future directions

4.4.1 Pain axis

Despite the progress gained through the studies summarized in this thesis, a broader scope is required for a comprehensive understanding of neuropathic pain. Our research highlights the complexity of neuropathic pain at the DRG and microbiome

level. However, the entire pain axis, including tissues of both, the peripheral and central nervous system, should be investigated using high-throughput approaches and examining variables, such as sex and age. In this dissertation, the spared nerve injury (SNI) model was used to induce neuropathic pain. This model is based on the ligation and transection of two branches of the sciatic nerve, the largest peripheral nerve. A comprehensive proteome analysis of this tissue, as the origin of neuropathic pain and an important part of the pain axis that bundles axons of sensory neurons [496], would therefore be of huge interest. It is commonly known that after nerve injury, axon degeneration processes are activated. Schwann cells, which are the glial cells of peripheral nerves, are suggested to play a role, specifically a subtype, which are called repair Schwann cells. Those cells seem to become activated post nerve injury, contributing to nerve repair and myelinophagy, i.e., the clearance of myelin debris [497, 498]. If the formation of repair Schwann cells is disrupted, axon regeneration is significantly reduced, which is followed by neuronal death and persistent impairment of repair processes of the peripheral nervous system [499]. This highlights the dynamic nature of peripheral nerve injury, however, a deeper understanding of the involved processes is needed.

4.4.2 Longitudinal approach

The here presented data investigate neuropathic pain in the initial phase of establishment (up to 14 days post injury). However, since neuropathic pain is a form of chronic pain and patients suffer from long-lasting symptoms, a longitudinal approach is highly relevant. Investigations on the behavioral level could already show sex-dependent longitudinal changes [160], yet, similar studies on the molecular level remain lacking. A comparison of proteome changes at the initial phase versus longitudinal phases could help us understand mechanisms responsible for the chronification of neuropathic pain and help identify protein targets, which potentially could lead to the prevention of this transition. Hereby, a focus on different age groups is necessary, as it is known that the timepoint of an injury significantly influences pain perception and progression later in life [294-296].

For a comprehensive understanding of pain chronification, longitudinal changes should be investigated along the entire pain axis, as well as the microbiome. This approach may allow us to uncover systemic changes and key drivers in the transition from acute to chronic pain.

4.4.3 Functional validation

Although we identified potential drug targets and matched them to FDA-approved drugs, further research is needed to confirm their functional relevance. Testing the impact of proposed drug candidates, such as Epoetins or Ptp-modulators *in vitro*, on cultured DRGs, would be of interest. Cultured DRGs from naïve mice have been suggested to exhibit an injury-like phenotype. A transcriptome study from Wangzhou *et al.*, 2020 [500], found upregulation of inflammatory genes in cultured DRGs from naïve mice and human organ donors, resembling changes observed after axonal injury. Thus, cultured DRGs from naïve mice can provide a useful *in vitro* model to study neuropathic pain mechanisms and discover drug potential, while reducing the need for surgical procedures and post-operative care. Despite this, one should take into account that cultured DRGs do not fully reflect the characteristics of native DRGs. Particularly, the levels of gene expression seem to differ between the two conditions, which may be accounted for by changes in the cell type proportion. Furthermore, although the conservation of DRGs from mouse to humans is high, small differences exist which may limit the translatability [500]. Nevertheless, testing Epoetins and Ptp-modulators on cultured DRGs, and investigating their impact on the proteome level could be a crucial next step. Additionally, their therapeutic potential could be tested by *in vivo* administration to mice with neuropathic pain, to investigate phenotypical changes.

Moreover, promising protein candidates could be investigated through conditional or global knock-out experiments *in vivo*, to assess their phenotypic impact on neuropathic pain behavior, as well as potential sex- and age-dependent differences.

4.4.4 The impact of the gut microbiome on neuropathic pain

Investigating feces gives valuable insights into the microbial composition and function of the gut, as well as its interaction with the host. Furthermore, the sampling method is non-invasive, making it useful for both preclinical and clinical approaches. Despite that, feces may represent a snapshot of the gastrointestinal tract. The microenvironment of the gastrointestinal tract changes along rostral-caudal axis, in terms of pH, oxygen saturation, mucus thickness, etc., which has an impact on the microbiome and its host [501-504]. Direct investigation of the gut itself, including its mucosa and the cecum, could provide deeper insights into further mechanisms of the

gut-brain axis in neuropathic pain, such as inflammatory response, microbial colonization patterns, and altered integrity of the gut barrier.

Furthermore, our microbiome study represents only an exploratory study. As a next step, since our study of the DRG proteome showed the significant impact of sex and age during neuropathic pain, these variables should be investigated on the microbiome level. Under healthy conditions, it is known that males and females have already different nutritional and energetic demands, leading to adaptations of the gut microbiome to fulfill these requirements [505]. Furthermore, the composition of the microbiome changes throughout life, correlating with sex- and age-specific risks of various diseases. Especially, childhood and puberty seem to underlie compositional and functional changes of the gut microbiome [506]. Given these facts, and the interactions between the gut microbiota and the host immune system, it is plausible that sex- and age-related differences affect the gut microbiome under painful conditions. Further investigations could be justified in identifying their roles in pain mechanisms.

Importantly, the human gut microbiome is highly diverse, making it difficult to generalize findings from mice to humans. Nonetheless, our study demonstrated alterations in the gut microbiota of mice with neuropathic pain. Such changes in microbial composition could influence drug absorption, metabolism, and efficacy, highlighting the importance of considering the microbiome when developing or repurposing pain therapeutics. Further research in this area is essential to advance personalized and more effective pain therapeutics.

4.4.5 Heterogeneity

Importantly, sex and age represent only two of many factors which could influence pain. To provide a more comprehensive understanding of mechanistic differences in pain pathways, and identify variables that modulate pain differently, more heterogeneous cohorts in preclinical studies are needed. As pain is a very complex disease, its complexity may be reduced, leading to reduced reproducibility, by relying on homogenous rodent models. Incorporating and investigating biological variables can potentially overcome this problem and will help us to understand this maladaptive disease [507-514]. Furthermore, other factors, such as gender, ethnicity, genetic predisposition and lifestyle should be incorporated into future research.

Gender, which is not to confused with sex, is shaped by social and cultural factors. Given the fact that pain is a highly subjective experience, influenced by sensory, cognitive and emotional components, it is reasonable that gender can have a major impact on pain perception, however little attention is given to this topic in pain research. Moreover, cultural norms and bias still exist in medical practice, further exacerbating sex and gender inequalities. Women often have to face greater scepticism with regard to the severity or justification of their pain symptoms and are more likely attributed to psychological factors or hormonal fluctuation compared to men, leading to the fact that medical conditions are less likely addressed [177]. Future research should investigate sex and gender, and incorporate gender-based analyses when possible. Recognizing and addressing both biological and sociocultural factors is crucial for understanding the multi-faceted impact on neuropathic pain and enabling more efficient and personalized pain treatment strategies.

4.4.6 Behavioral limitations

The studies presented here in this thesis, mainly focus on neuropathic pain on the molecular level. Behavioral assessment in the form of mechanical hypersensitivity (and non-evoked pain, measured by paw print area in the study from chapter 2) were conducted as proof for the induction of neuropathic pain by the SNI model. The clinical translatability of evoked pain behavior has been questioned recently, as neuropathic pain patients suffer primarily from spontaneous pain [73, 79, 166-173]. Although we implicated a first attempt of measuring non-evoked spontaneous pain in the study from chapter 2, further approaches could be useful here. Since SNI causes a slight crook of the ipsilateral hind paw, a comparison of ipsilateral versus contralateral paw print area as measurement of weight distribution, is limited. Furthermore, the whole print area, was taken into account for the measurement, but not the intensity of individual areas. This could lead to a bias, as on the ipsilateral hind paw smaller areas showed greater intensity.

Further development in behavioral tests, investigating non-evoked pain are needed. Ideally, those tests should be performed in home cages, to reflect the natural behavior of the testing subject and reduce its stress, which also influences pain behavior.

4.4.7 Summary

In summary, this dissertation extends the current understanding of neuropathic pain, using proteomic and metaproteomic approaches. The identification of evolutionary conserved protein networks, drug-targets, novel microbial pathways, and an interplay of the host immune system with the gut microbiome, provides valuable opportunities for translational research and therapeutic applications, with the aim to enable more precise and efficient treatment options for neuropathic pain. However, future research needs to investigate the entire pain axis, including the microbiome, and consider key variables, such as sex and age, in a longitudinal manner. Thereby, we might gain a system-wide perspective and can uncover interconnected mechanisms underlying neuropathic pain.

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