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Susceptibility and Resilience Factors for Chronic Pain Development in Experimental Models and Human Pain Conditions

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ARE	antioxidant response element
ATF-3	activating transcription factor 3
BL	Baseline
BNDF	brain-derived neurotrophic factor
CCI	chronic constriction injury
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CPSP	chronic post-surgical pain
CSF	cerebrospinal fluid
CSI	Children Somatization Inventory
DAWBA	Development and Well-Being Assessment
DMF	Dimethyl fumarate
DRG	dorsal root ganglia
DSM	Diagnostic and Statistical Manual of Mental Disorders
EPM	Elevated Plus Maze
Follow-up	FU
GABA	Gamma-aminobutyric acid
GAD	generalized anxiety disorder
GWAS	Genome-wide association studies
Ho-1	Heme Oxygenase 1
HSCL	Hopkins Symptom Checklist
IASP	International Association for the Study of Pain
ICD	International Classification of Diseases
IFN-γ	interferon-gamma
IL	Interleukin
IMAGEN	Imaging Genetics (IMAGEN) study
LBP	low back pain
LDB	Light-Dark Box
LTP	long-term potentiation
MDD	major depressive disorder
mGluR	metabotropic glutamate receptor subtypes

MIA	monosodium iodoacetate
MRI	magnetic resonance imaging
MS	multiple sclerosis
NF-κB	nuclear factor-κB
NMDA	N-methyl-D-Aspartate
NOR	Novel Object Recognition
Nrf2/NRF2	nuclear factor erythroid 2-related factor 2
ns	not significant
NTG	nitroglycerin
PRRs	pattern–recognition receptors
ROS	Reactive oxygen species
Sal	Saline
SCI	spinal cord injury
SD	Sprague-Dawley
SNI	spared nerve injury
SNL	spinal nerve ligation
SNPs	single-nucleotide polymorphisms
SOD	superoxide dismutase
SPT	Sucrose Preference Test
Th1	T-helper 1
TNF-α	tumor necrosis factor-alpha
Tri	Trigonelline
TRPM	transient receptor potential cation channel subfamily M
TRPV	transient receptor potential cation channel subfamily V
VEH	Vehicle

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FOREWORD

Pain is defined as ‘an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage’ (Raja et al., 2020). As a natural protective mechanism, the ability to feel pain is critical for survival (Lischka et al., 2022). In certain conditions, however, pain might persist, losing its protective function. Chronic pain can severely impair life quality, often accompanied by comorbid conditions such as anxiety and depression (Cohen et al., 2021; Raja et al., 2020). Curiously, predisposition to such states varies between individuals, even if the pain-triggering events are alike. For example, among people who underwent major surgery, nearly 50% of them were resilient to developing chronic pain (Rabbitts et al., 2017). This number in spinal cord injury is 30% (Hunt et al., 2021) and in traumatic brain injury is 50% (Nampiaparampil, 2008). Evidence indicates that certain biological factors and psychological traits are predictors of chronic postsurgical pain progression (Liu et al., 2022; Pinto et al., 2018; Sluka et al., 2023). Among these, the immune system has been increasingly demonstrated as a key factor (Fragiadakis et al., 2015; Gaudillière et al., 2014; Iordanova Schistad et al., 2020; Parisien et al., 2022; Souquette and Thomas, 2024). Considering the lack of reliable clinical indicators for predicting chronic pain and the substantial burden it imposes, identifying novel susceptibility and resilience factors is essential. Such efforts are critical not only for developing preventive strategies but also for elucidating the underlying mechanisms.

The general aim of this thesis is to identify susceptibility and resilience factors for the development of chronic pain and associated comorbidities, with a focus on the immune system. This thesis starts with a general introduction to pain, chronic pain, associated comorbidities, heterogeneity in its development, and current state-of-the-art literature on susceptibility/resilience factors. Next, the relevant immune system’s roles, with potential markers and targets for modulation, are presented. Based on this, hypotheses are formulated and tested in two studies. The first study examines how modulating the immune system at the onset alters pain trajectories in a rat model of chronic neuropathic pain, while the second study, leveraged data from a longitudinal cohort, examines the contributions of immune-driven genetic variants to the development of pain symptoms and to the predictive relationship of anxiety and depression to pain. The thesis closes with an overall discussion including limitations and suggested directions for future research.

1. INTRODUCTION

1.1. Pain

1.1.1. Historical background

The evolution of pain concepts is an intriguing chapter in the history of medicine. Back in ancient times (*c*. 428 to 347 *B.C.*), Plato and his pupil Aristotle proposed pain as an emotion, rather than a physical experience, which was controlled by the heart. This concept prevailed for over 2000 years until 1644, when a breakthrough occurred as René Descartes introduced the Cartesian dualism theory (Duncan, 2000). The French philosopher believed that the experience of pain was controlled by the pineal gland in the brain. Pain not only results from psychological injury but also from physical injury; in other words, the biological dimension of pain has started to be acknowledged. However, according to Descartes, these two dimensions are completely independent and have no synergistic effects on pain. From 1811 to 1894, the specificity theory was introduced with important discoveries on brain structure, sensory receptors, and somatosensory modalities (Handwerker and Brune, 1987; Pearce, 2006; Perl, 2011). This theory greatly elaborates the biological construct but was contested by the pattern theory, which suggested that pain perception arises from the pattern and intensity of neural activity rather than being linked to specific receptors. These competing concepts highlighted the complexity of pain mechanisms and set the stage for more integrative models. In 1965, Patrick David Wall and Ronald Melzack introduced the gate control theory, revolutionizing pain research. This concept postulates that signals from stimuli are not projected directly to the brain but are first controlled by a neural gate in the dorsal horn of the spinal cord (Melzack and Wall, 1965). Melzack and Wall further suggested the role of cortical regions in the context of pain. Accordingly, pain is viewed as a multidimensional experience shaped by cognitive and emotional factors. In the most comprehensive model of pain, the biopsychosocial model, introduced by Roy Grinker in 1954, first utilized by John Joseph Bonica and John David Loeser (Lugg, 2022), the biological, psychological, and social constructs are all recognized as key contributors to pain experience.

1.1.2. A modern Pain definition

The biopsychosocial model underpins the current definition of pain and accompanying notes, developed by the International Association for the Study of Pain (IASP).

Accordingly, pain is defined as ‘an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage’ (Raja et al., 2020). Additionally, pain is always a personal experience, shaped by the interplay between biological, psychological, and social factors. Not only do these aggregate the knowledge compiled in the prior decades, but they also provide important information that helps explain one of the most puzzling observations – the variability in pain experience and in the development of chronic pain.

1.1.3. Anatomophysiology of pain

Pain is a complex process involving multiple regions from the periphery to the brain (Woolf, 2010). In the periphery, noxious stimuli, including mechanical, heat, and cold, are sensed by primary afferent neurons, thanks to receptors and ion channels on their nerve endings. Those proteins, including transient receptor potential cation channel subfamily V member (TRPV1-4) (González-Ramírez et al., 2017), transient receptor potential cation channel subfamily M member 8 (TRPM8) (Julius, 2013), and voltage-gated sodium channels Nav 1.3, 1.7, 1.8, and 1.9 (Bennett et al., 2019), are specifically sensitive to adequate stimuli and, upon reaching threshold, initiate the generation of action potentials. These action potentials, as nociceptive information, are then conducted to the grey matter of the dorsal horn of the spinal cord.

Primary afferent neurons that convey pain information include Aδ groups of myelinated axons with velocities ranging from 5 to 30m/s and C fiber groups of unmyelinated axons with velocities less than 2m/s (Basbaum et al., 2009; Scholz and Woolf, 2002). Centrally, these neurons, when reaching the dorsal horn, branch into ascending and descending collaterals, forming the dorsolateral tract of Lissauer. Axons in this tract penetrate the gray matter of the dorsal horn before connecting with second-order neurons located in laminae I, II, and V. Aδ terminate in laminae V, C terminate in laminae I and II. Laminae I and V contain neurons whose axons project to brainstem and thalamic targets, while in laminae II, these interneurons show morphological and histochemical differences (Basbaum et al., 2009). The axons of the second-order neurons in laminae I and V cross the midline and ascend toward the brainstem and thalamus via the anterolateral tract of the contralateral side of the spinal cord (Basbaum, 2022; Motzkin et al., 2024; Schmidt and Willis, 2007; Willis and Westlund, 1997). This is referred to as the anterolateral system, conveying pain and temperature information. The anterolateral system has parallel pain pathways. One is the pathway,

in which second-order neurons travel to the ventral posterior lateral nucleus of the thalamus, and then to the somatosensory cortex via third-order neurons. This mediates the sensory-discriminative aspects of pain. The other pathway conveys information about the affective-motivational aspects, with the axons projecting to different regions such as the reticular formation, the periaqueductal gray, the amygdala, and the hypothalamus (Basbaum, 2022; Leite-Almeida et al., 2006; Motzkin et al., 2024; Schmidt and Willis, 2007; Willis and Westlund, 1997). These ascending pain pathways are modulated by supraspinal descending pathways, also known as the endogenous analgesic system, originating in the midbrain periaqueductal grey and then projecting to the dorsal horn of the spinal cord via the rostral ventromedial medulla (Lau and Vaughan, 2014). The ascending and descending pathways cooperate to control our perception of pain.

1.2. Chronic pain

1.2.1. Classification and mechanisms

As discussed above, pain serves as a protective signaling mechanism. However, when pain persists or recurs for more than 3 months, it is classified as chronic pain, which exerts detrimental effects (Cohen et al., 2021; Raja et al., 2020). Chronic pain affects around 20.4% of the global population and can adversely influence virtually every aspect of an individual's life (Cohen et al., 2021). For example, Chronic pain financially costs more than most other medical conditions (Phillips, 2009). According to the U.S. Pain Foundation in 2010, the nation lost up to $635 billion each year to this condition for medical care, disability payments, and lost productivity. In Sweden, the loss of productivity resulting from chronic pain was around $10 billion in 2003 (Swedish Council on Technology Assessment in Health Care, 2006), while the number for the UK was $14 billion annually, as reported in 1998 (Maniadakis and Gray, 2000).

Chronic pain can be divided into three main categories – nociceptive, neuropathic, and nociplastic (Cohen et al., 2021) – with specific pathophysiology. Nociceptive pain, which is the most common form of chronic pain, arises from real tissue or potential tissue damage, located in the somatic system, such as joints (osteoarthritis) and muscles (muscle spasm), and the visceral system, such as mucosal injury (peptic ulcer) and ischaemia (angina) (Cohen et al., 2021; DiBonaventura et al., 2017). Neuropathic pain, which accounts for 15-25% of chronic pain, is associated with damage or disease affecting the somatosensory nervous system (Treede et al., 2008).

Subtypes include peripheral pain, such as diabetic neuropathy, or central pain, like postsurgical trauma in spinal cord injury (Cohen, 2014). Nociplastic pain, previously known as central sensitization, is caused by abnormal sensory processing and diminished descending inhibition pathways in the absence of tissue damage and nerve injury (Fitzcharles et al., 2021). This type can occur in conditions such as fibromyalgia, tension-type headache, and non-specific back pain – see for a recent review (Kaplan et al., 2024).

Mechanisms of all three types of chronic pain, especially nociceptive and neuropathic pains, are closely connected with the immune system activity. In nociceptive pain, following tissue damage, leukocytes such as macrophages, along with glial cells like microglia and astrocytes, are rapidly recruited to the site. These immune cells release pro-inflammatory cytokines that activate their receptors on the nociceptive neurons. As a result, the neurons in the periphery become hyperexcited and sensitized to subsequent inputs, a phenomenon known as peripheral sensitization (Basbaum et al., 2009; Littlejohn, 2015; Scholz and Woolf, 2007). Next, those hyperexcited nociceptors, in turn, release neurotransmitters like glutamate, which bind to receptors such as N-methyl-D-Aspartate (NMDA) and metabotropic (G-protein coupled) glutamate receptor subtypes (mGluR) expressed on postsynaptic neurons in the dorsal horn of the spinal cord. The activation of those receptors is pivotal in triggering and maintaining another important phenomenon, central sensitization. Central sensitization leads to an exaggerated response to painful stimuli, known as hyperalgesia, and causes pain in response to stimuli that are normally not painful, which is called allodynia (Scholz, 2014). Central sensitization is the key mechanism underlying the transition from acute to chronic pain (Latremoliere and Woolf, 2009), not only in nociceptive but increasingly evident in all three forms of pain.

Neuropathic pain has a different pathophysiology, resulting from lesions in the peripheral and central nervous systems (Costigan et al., 2009). However, well-established evidence indicates that this type of pain is a neuroimmune disorder featuring the interplay of not only neuronal but also immune pathways – please see review (Scholz and Woolf, 2007). After peripheral nerve injury, local macrophages secrete proteases that degrade the surrounding blood vessels. The vascular changes at the blood-nerve barrier compromise its integrity, facilitating the invasion of circulating immune cells, including macrophages, T lymphocytes, and mast cells

(Campbell and Meyer, 2006; Shubayev et al., 2006) , which release a variety of mediators, including prostaglandins, pro-inflammatory cytokines, interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF), and anti-inflammatory cytokines such as Interleukin-10 (IL-10). These molecules are involved in a complex network, modulating primary sensory neurons' activity and stimulus sensitivity distal to the lesion site (Chen et al., 2015; Hu and McLachlan, 2003) . In the dorsal root ganglion (DRG), apart from macrophages and T lymphocytes, satellite glia cells are also present to regulate the immune response that can persist for months. Dominant players are pro-inflammatory cytokines IL-1, IL-6, and TNF, as well as ion channels, such as the P2X family (Ding et al., 2000; Richards et al., 2019), whose receptors are expressed on nociceptors and immune cells (Di Virgilio et al., 2001; Kobayashi et al., 2005) . The resulting immune activity in the DRG subsequently modulates synaptic input to the spinal cord (Scholz and Woolf, 2007), where microglia are the main players, critical not only to the response to peripheral nerve injury but also in the context of central nervous system injury (Inoue and Tsuda, 2018). At the dorsal horn of the spinal cord, microglia activation releases chemical mediators, such as brain-derived neurotrophic factor (BDNF), which reduces the inhibitory effects of Gamma-aminobutyric acid (GABA)ergic and glycine, and leads to central sensitization (Scholz, 2014).

Peripheral and central sensitizations are also commonly observed, although not specific, in nociplastic pain (Kosek, 2024). While alterations in nociceptive processes for this type remain largely unknown, one of the mechanisms involves the immune response, with glial cells themselves directly modulating the excitability of nociceptors in the central nervous system (CNS) (Hore and Denk, 2019; Latremoliere and Woolf, 2009; Wieseler-Frank et al., 2005; Yang et al., 2022). Protein that promotes glia activation, such as fractalkine (Bäckryd et al., 2017), and glia downstream effectors, including IL-8 (Kadetoff et al., 2012) and TNF-α (Ohgidani et al., 2017), were found elevated in the cerebrospinal fluid (CSF) and blood of patients with nociplastic pain condition, fibromyalgia – for more examples, please see the review (Kaplan et al., 2024). Another mechanism of nociplastic pain that demonstrates neuroimmune involvement is the neurogenic neuroinflammation. In this process, the inflammatory cascade is triggered by neuropeptides such as substance P and calcitonin gene-related peptide (CGRP), which are released upon nerve activation, particularly the C-fibers – please see for review (Cordero, 2015; Littlejohn, 2015).

Overall, the mechanisms underlying chronic pain are highly complex. This contributes to the significant therapeutic challenges we are facing and therefore emphasizes the need to identify predictive markers to develop preventive strategies (Scholz, 2014). Another important point is that despite having certain distinct aspects regarding the pathophysiology, three types of chronic pain's mechanisms commonly involve the immune system activity, regulating peripheral and central sensitizations. This suggests an implication that immune modulation might be an effective treatment for chronic pain (Conaghan et al., 2019; Rodríguez-Palma et al., 2024; Scholz and Woolf, 2007; Shraim et al., 2024; Zhao et al., 2023) .

1.2.2. Chronic pain comorbidities

In addition to its varied pathophysiology, the frequent comorbidity of chronic pain with other conditions warrants attention. Indeed, the co-occurrence between chronic pain and anxiety and depression has been well documented in both rodent and human studies (Bair et al., 2003; Flor et al., 1993; Kremer et al., 2021; McWilliams et al., 2003). Epidemiological studies show that the prevalence of depression in individuals with fibromyalgia, chronic spinal pain, and chronic abdominal pain can be up to 50%. The number for neuropathic pain is 12% - see for review (Hooten, 2016). Similarly, the prevalence of chronic pain that co-occurs with anxiety is high, with 35% to 40% in migraine headache (Oedegaard et al., 2006; Zwart et al., 2003), up to 60% in fibromyalgia (Arnold et al., 2006; Häuser et al., 2012; Uguz et al., 2010), and 26% in chronic spinal pain (Demyttenaere et al., 2007; McWilliams et al., 2004; Von Korff et al., 2005). Meanwhile, the number for neuropathic pain varies from 5% to 27% - see for review (Hooten, 2016). In both adult and pediatric populations, comorbidity between pain and mental health conditions is considered a clinical reality (Bateman et al., 2023; Lundqvist et al., 2023; Soltani et al., 2019; Vinall et al., 2016; Voepel-Lewis et al., 2023).

Animal models have long been developed to model different types of chronic pain, such as the spared nerve injury (SNI) model of peripheral neuropathic pain (Decosterd and Woolf, 2000), the spinal cord injury (SCI) model of central neuropathic pain (Sharp et al., 2012), nitroglycerin (NTG) – induced migraine (Chou and Chen, 2018), and monosodium iodoacetate (MIA) – induced osteoarthritis (Burma et al., 2017). Assessment of pain includes evoked measurements of mechanical allodynia (e.g., von Frey), thermal hypersensitivity (e.g., acetone drop test, hot-cold plate, Hargreaves

apparatus), and spontaneous measurements (the grimace scale, weight bearing, home cage monitoring for abnormal behaviors) – please see for review (Burma et al., 2017). Alongside a good face and construct validity for chronic pain, the animal models have been widely reported to show anxiodepressive and cognitive-like behaviors, which reflect the comorbidities seen in humans – please see for review on pain-depression comorbidity (Li, 2015), on pain-affective disorder, or -cognitive deficit comorbidities (Liu and Chen, 2014). Therefore, in addition to pain measurements, growing attention is being directed toward assessing the affective and emotional dimensions of pain experience in animals. These measurements include assessment of anxiety-like behaviors using, for instance, the elevated plus maze, light-dark box test, open field test, assessment of depression-like behaviors using the sucrose preference test, forced swimming test, and assessment of cognitive dysfunction-like behaviors using the novel object recognition test – please see for review (Kremer et al., 2021; Leite-Almeida et al., 2015).

1.2.3. Heterogeneity in the development of chronic pain

Chronic pain comorbidities highlight its multidimensional nature, which is further evidenced by the substantial individual variability in its development. An intriguing observation is that chronic pain susceptibility varies significantly even if the pain-triggering events are alike. For example, up to 50% of adults and 80% of children who underwent major surgery were resistant to developing chronic postsurgical pain (Rabbitts et al., 2017). Even with spinal cord injury, where the likelihood of persistent pain development is often high, more than 30% of patients recovered fully from pain (Hunt et al., 2021). In another example, regarding individuals with traumatic brain injury, the chance of progressing to chronic pain was almost equally balanced at 50% (Nampiaparampil, 2008). This observation is not only seen in humans but also in rodent studies. Using rats with spinal nerve ligation (SNL) as a model for neuropathic pain, De Felice reported a variability of chronic pain that resided in the model. Among Sprague-Dawley rats (SD) with SNL, 15% of them exhibited no sign of allodynia, while almost half of the Holtzman rats were classified as non-allodynic (Felice et al., 2011). In the spared nerve injury, which is a well-established and robust model of neuropathic pain (Bourquin et al., 2006; Decosterd & Woolf, 2000), there were still around 13% of SD injured rats that did not manifest mechanical allodynia (Guimarães et al., 2019). Those examples emphasized the heterogeneity in chronic pain development, which

has drawn great attention in the field to provide explanations. De Felice believed that it is the descending inhibition from the rostral ventromedial medulla that protects the rats and leads to variability in persistent pain (Felice et al., 2011). Others reported that the difference in genetic traits of nociception, or environments like rearing conditions and diets, or the divergence of strains or sub-strains of rats, resulted in dissimilar degrees of pain expression (Mogil et al., 1999; Yoon et al., 1999). Particularly, increasing evidence indicates that the immune system, a critical contributor in chronic pain pathogenesis, may play an essential role in determining susceptibility and resilience (Fragiadakis et al., 2015; Gaudillière et al., 2014; Iordanova Schistad et al., 2020; Parisien et al., 2022; Souquette and Thomas, 2024). While much work is required to find out the mechanisms, the obvious dichotomy in chronic pain development raises an interesting question: can we separate individuals who are susceptible to chronic pain development from those who are resilient? The identification of susceptibility and resilience factors would be critical, not only for the prediction, but also it may offer a promising avenue for prevention through targeted modifications. The latter is especially important given the highly frequent co-occurrence between chronic pain and related comorbidities, as well as the difficulties we have been encountering in treating chronic pain (Cohen et al., 2021; Finnerup et al., 2015; Shoji Yabuki et al., 2019).

1.2.4. Susceptibility and resilience factors

Susceptibility and resilience factors to the development of chronic pain can be classified into four broad categories, including psychosocial, blood-based, neuroimaging, and functional measures – please see for review (Sluka et al., 2023). Among these, psychosocial, especially psychological markers, are probably the most widely studied and acknowledged. In contrast, blood-based and neuroimaging factors, which reflect biological processes, are relatively under-investigated. It is worth noting that despite substantial efforts, there is still a lack of reliable markers that can be used effectively in clinics to predict chronic pain and related comorbidities (Sluka et al., 2023). Additionally, modulation targeting those factors to prevent the development of chronic pain has not yet been explored.

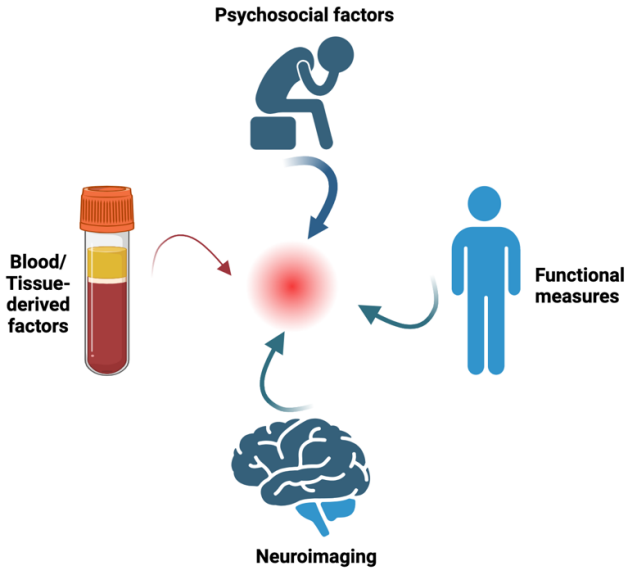

Figure 1. Susceptibility and resilience factors for chronic pain development. These factors can be classified into four broad categories, including psychosocial, blood-based factors, neuroimaging, and functional measures (Sluka et al., 2023). Blood-based markers encompass genetics, proteins such as inflammatory molecules, lipids, and metabolites. Psychosocial factors, on the other hand, include anxiety, depression, or working environments. Alongside these, neuroimaging and functional measures such as quantitative sensory testing and conditioned pain modulation can be used to predict chronic pain development.

1.2.4.1. Psychological factors

As mentioned above, psychological factors have been increasingly recognized as important determinants of chronic pain development, with much evidence obtained from chronic post-surgical pain (CPSP) studies. The noticeable markers identified include pain catastrophizing, fear of movement, past trauma, and especially depression and anxiety – see reviews (Fregoso et al., 2019; Glare et al., 2019; Pergolizzi et al., 2023; Rosenberger et al., 2023; Sluka et al., 2023). Beyond clinical pain research, findings from population-based studies also highlight the involvement of psychological factors in susceptibility and resilience to pain symptoms. For example, on a community sample of adolescents, it is reported that depression at age 13.7 significantly predicted headache frequency 14 years later (Larsson et al., 2018). Similarly, sleep problems, anxiety, and depression were found as predictors of musculoskeletal conditions in children and adolescents (Andreucci et al., 2020). Early-in-life anxiety and depression have been shown as determinants for pain symptoms in

numerous studies (Bondesson et al., 2024; Eckhoff et al., 2017; Murray et al., 2024; Noel et al., 2016). Despite the substantial body of evidence, psychological predictors alone demonstrated limited predictive value. A more comprehensive and potentially effective approach involves integrating them with biological factors (Fillingim et al., 2025), which to date have been comparatively underexplored.

1.2.4.2. Biological factors

Identifying biomarkers for chronic pain development encounters certain challenges, such as the severity of injury not being a reliable indicator of pain progression (Yusuf et al., 2011), studies with small sample sizes (Button et al., 2013), limited availability of biological features and pain phenotypes (Steingrímsdóttir et al., 2017), and the condition's varied aetiology (Fitzcharles et al., 2021). This partly explains why these factors are underexplored. Among a limited number of studies available, important findings can be seen in brain imaging research. For example, Apkarian et al. found that pre-surgery measurement of white matter fractional anisotropy, gray matter volume of medial prefrontal cortex, and functional connectivity in regions such as corticostriatum and hippocampus can be used as predictors for the development of chronic pain one year later (Apkarian et al., 2013; Baliki et al., 2012, 2010; Mansour et al., 2013; Musto et al., 2015; Vachon-Presseau et al., 2016). Additionally, over the past two decades, genetic association studies have significantly advanced our understanding of the key contributors to the initiation and persistence of chronic pain. A variety of related genes have been implicated, including genes from catecholaminergic, serotonergic, glutamatergic, GABAergic, and cytokine pathways (Zorina-Lichtenwalter et al., 2016). Notably, there seem to be differences in the types of predisposition genes between pain categories grouped by etiology. For example, in conditions such as nociplastic pain, arising from altered nociception, studies showed nearly half of the identified genetic variants are linked to neurotransmission pathways (Diatchenko et al., 2013; Zorina-Lichtenwalter et al., 2016). Meanwhile, variability in neuroimmune interaction-related genes appears to be associated with neuropathic pain susceptibility and resilience, with leading effectors being *IL-10* genetic polymorphisms (Zorina-Lichtenwalter et al., 2018). Despite all these findings, it has yet to be confirmed if these candidate genes can predict the development of chronic pain and associated comorbidities. Genome-wide association studies (GWAS), testing single-nucleotide polymorphisms (SNPs), have become a popular tool in recent years

in the pain prediction context. However, there is a great need for more related SNPs to be identified in order to increase the predictive power of polygenic scores (van Reij et al., 2020).

In addition to brain structure and functions and genetics, inflammatory molecules have increasingly been suggested to play a role in determining the susceptibility and resilience of chronic pain development. Among them, cytokines have probably been the most extensively studied markers, with important effectors including TNF-α, IL-6, IL-12, IL-1β, IL-10, and leptin (Chidambaran et al., 2024; Gandhi et al., 2013; Klyne et al., 2018; Schrepf et al., 2018; Singh et al., 2017; Stannus et al., 2013). Compared to brain-based data and genetics, these immune factors become of great interest not only because they potentially hold predictive value, but also because they are modifiable – an important quality for prevention strategies.

1.3. The immune system in chronic pain heterogeneity

Building on the understanding of susceptibility and resilience factors, increasing evidence points to the immune system as a central player associated with chronic pain development heterogeneity. Pathophysiologically speaking, this aligns with our knowledge of this medical condition. Chronic pain, in all forms, has a strong neuroinflammatory component (Ji et al., 2016). As outlined in section 1.2.1, the immune system is particularly involved in the mechanisms underlying not only nociceptive and neuropathic pain but also nociplastic pain. This system is also a critical contributor to the development of chronic pain comorbidities, such as anxiety and mood disorders (Khandaker et al., 2017; Köhler et al., 2014). Furthermore, growing evidence suggests that the immune system might play a role in the complex interplay between negative affect, like anxiety, and brain plasticity that contributes to the development of chronic pain. For instance, anxiety has been shown to increase glutamatergic synaptic activity in the anterior cingulate cortex, resulting in long-term potentiation (LTP), a mechanism underlying central sensitization (Bliss et al., 2016; Koga et al., 2015; Zhuo, 2016). Meanwhile, cytokines and other immune components are widely reported as critical modulators of LTP – see reviews (Joanna L. Jankowsky and Patterson, 1999; Prieto and Cotman, 2017), therefore, might influence the development of pain. Such evidence reinforces the centrality of this system in determining the susceptibility and resilience to chronic pain and associated comorbidities.

1.3.1. Impacts of baseline immune profile on chronic pain development

Variations in the basal immune state, which happen across the human population, have been shown to affect the acute immunity landscape and potentially predispose individuals to severe diseases (Souquette and Thomas, 2024). In fact, genetic association studies proved that immune response is a key determinant of pain chronicity, with evidence pointing to genes encoding for neutrophil-attracting chemokine CXCL8, COX-2 enzyme, ion channel P2X7, nuclear factor-κB (NF-κB) and various cytokines, including IL-1β, IL-6, TNF, IL-10, IL-4 receptors, and IL-18 receptors – please see the works from Luda Diatchenko group for comprehensive reviews (Diatchenko et al., 2007; Zorina-Lichtenwalter et al., 2018, 2016). On a molecular level, following patients undergoing hip arthroplasty, Fragiadakis et al. reported two key findings: first, the pre-surgical immune states, assessed by the activation of relevant signaling pathways in $CD14^+$ cells, varied significantly between patients, and second, one of the variations – TLR4 receptor signaling responses – was correlated with postoperative pain resolution (Fragiadakis et al., 2015). Additionally, changes in NF-κB signaling between baseline and 1 hour after surgery in $CD14^+$ cells were also positively associated with time to reduce pain (Gaudillière et al., 2014). Meanwhile, following patients with acute low back pain (LBP) (time point t0) for 3 months (time point t1), Parisien et al. found that the inflammatory response at t0 and its changes from t0 to t1 differ between pain-persistent and resolved subjects, emphasizing that the start of the inflammatory process programs pain resolution (Parisien et al., 2022).

1.3.2. Potential targets for modulating the basal immune response

What makes the immune markers particularly appealing is their modifiability, offering potential targets for intervention. Our literature review (manuscript in preparation) shows that (1) studies investigating how immunomodulation affects susceptibility and resilience to chronic pain and associated comorbidities remain scarce, and (2) the strongest evidence that support preventive use of immune-targeted drugs involves modulating glial cells, in particular microglia, cytokines such as interleukin 10, and transcriptional factors such as the nuclear factor erythroid 2-related factor 2 (Nrf2/NRF2).

1.3.2.1. Microglia and cytokines

Microglia have specific properties that make them a promising target for pre- and early treatment, in addition to their critical involvement in mechanisms underlying peripheral and central sensitizations. First, these glial cells become activated and their proliferations occur swiftly within hours following the inciting events (Gu et al., 2022; Na et al., 2008) – see also for review (Inoue and Tsuda, 2018). Second, *in vivo* experiments showed that microglia are highly dynamic and active even during the resting state within the intact brain (Nimmerjahn et al., 2005). These suggest that the period surrounding the injury might be a critical window for modulating these cells. In fact, there are reports showing the potential to target microglia for preventive pharmacological approaches against persistent pain. For example, minocycline – an immunomodulator known for its microglia inhibition properties (Tikka et al., 2001) – when administered around the surgery time, provided protection against hypersensitivity for 3 to 4 weeks after injury in different models of neuropathic pain, including SCI and chronic constriction injury (CCI). Molecular analysis shows that the preemptive treatment altered immune response, increasing cytokines such as TNF-α, IL-6, and IL-1β (Afshary et al., 2020; Padi and Kulkarni, 2008). Additionally, pretreatment with lidocaine, bupivacaine, or amitriptyline, agents whose efficacy is associated with the activation time course of microglia, attenuated the development of long-term mechanical allodynia for up to 21 days post-surgery in rodent pain models (Cheng et al., 2012; Fu et al., 2000; Linl et al., 2011).

In our recent publication, we administered minocycline – a widely known inhibitor of microglial activity – in a preemptive regimen on the rat SNI model of neuropathic pain. The results demonstrated that the treatment conferred protective effects against mechanical allodynia, which sustained for 10 days following the final dose, and attenuated depressive-like behaviors at 4 weeks after SNI (Le et al., 2024). Although the protective effects were still modest, our results support the assertions that (1) it is possible to prime the system prior to pain installation to change the pain trajectories, and (2) the neuroimmune system may be an important target for this purpose.

Other components in the system, such as cytokines, may also be potential targets, with IL-10 as the leading candidate due to its potent anti-inflammatory effects (Moore et al., 2001). However, intrinsic challenges make it difficult to employ cytokines in treatment. These include their rapid clearance from the circulatory system (short half-

life) (Eijkelkamp et al., 2016), acting coordinated as a network, and their pleiotropic effects (Cui et al., 2024).

1.3.2.2. Transcriptional factor Nrf2

One important regulator of the immune response is the oxidative system, and the interaction between these systems appears to be critical in chronic pain development (Morris et al., 2022). Reactive oxygen species (ROS), a product of oxidative stress, can be produced by immune cells in the event of inflammation and, in turn, induce the release of cytokines via activating NF-κB – see for review (Mittal et al., 2014). In human chronic pain and rodent models, elevated levels of ROS have been found in various pain-processing-related cells and tissues, including DRG, spinal cords, glia, and immune cells (Gamper and Ooi, 2015; Kim et al., 2010a; Lee et al., 2012; Meeus et al., 2013; Scholz and Woolf, 2007; Schwartz et al., 2009, 2008; Yousuf et al., 2020). Scavenging ROS by antioxidants has been shown to attenuate chronic pain (Kallenborn-Gerhardt et al., 2013; Kuthati et al., 2019; Siniscalco et al., 2007; Yoshizawa et al., 2021). However, pretreatment strategies targeting ROS remain limited despite evidence supporting their potential (Kwak et al., 2009; Mao et al., 2009). Nrf2 is considered a master regulator of the antioxidant and anti-inflammatory pathway, appearing therefore in this context as an important target. Inducers of this factor reversed pain-related behaviors in various rodent pain models (Chen et al., 2019; Lee et al., 2021; Li et al., 2020a; Zhou et al., 2020) – for a review, please see (Zhou et al., 2021). Downstream effectors of Nrf2 include various cytokines, produced through the interplay with NF-κB, and antioxidant enzymes, which are all critically involved in the mechanisms underlying both pain and anxiety and depression (Basu et al., 2022; Wardyn et al., 2015). Nrf2 also plays an essential role in modulating microglia dynamics (Rojo et al., 2010; Saha et al., 2022). Additional important evidence is found in physical exercise, a well-known protective factor against chronic pain development. Preconditioning rodents with exercises before either nerve injury or chemotherapy induction prevented the initiation of neuropathy, and the prevention was maintained for weeks (Grace et al., 2016; Suzanne M. Green-Fulgham et al., 2022; Slivicki et al., 2019). Mechanistic analysis showed that activating the Nrf2 pathway before nerve injury is required to prevent the development of persistent neuropathy, while interestingly, the downstream effects, including microglia, NF-κB, and cytokines, were also involved (Suzanne M. Green-Fulgham et al., 2022). On the genetic aspects,

although not yet studied in the context of pain, single nucleotide polymorphisms in the *NRF2* gene, such as rs35652124 and rs6721961, have been identified as being associated with a higher risk of various medical conditions, including cancer, diabetes, neurodegenerative, and cardiovascular diseases (Ikejiri et al., 2024), those that are highly comorbid with pain (Barrett and Kohut, 2024; Copenhaver et al., 2021; Cruz et al., 2022; Fayaz et al., 2016; Lawn et al., 2021; Reichling and Levine, 2011). SNP rs6721961, which affects NRF2 protein expression (Yamaguchi et al., 2019), is also proposed as a potential predictor of pain (Smith, 2010). Despite all the mentioned evidence, Nrf2's role in susceptibility and resilience to chronic pain and relevant comorbidities remains to be elucidated. Since the immune mediators always function in a coordinated network and pathways leading to pain vary, modulating those upstream effectors, such as Nrf2, which then lead to a cascade of biological changes, could be a viable option for modulation.

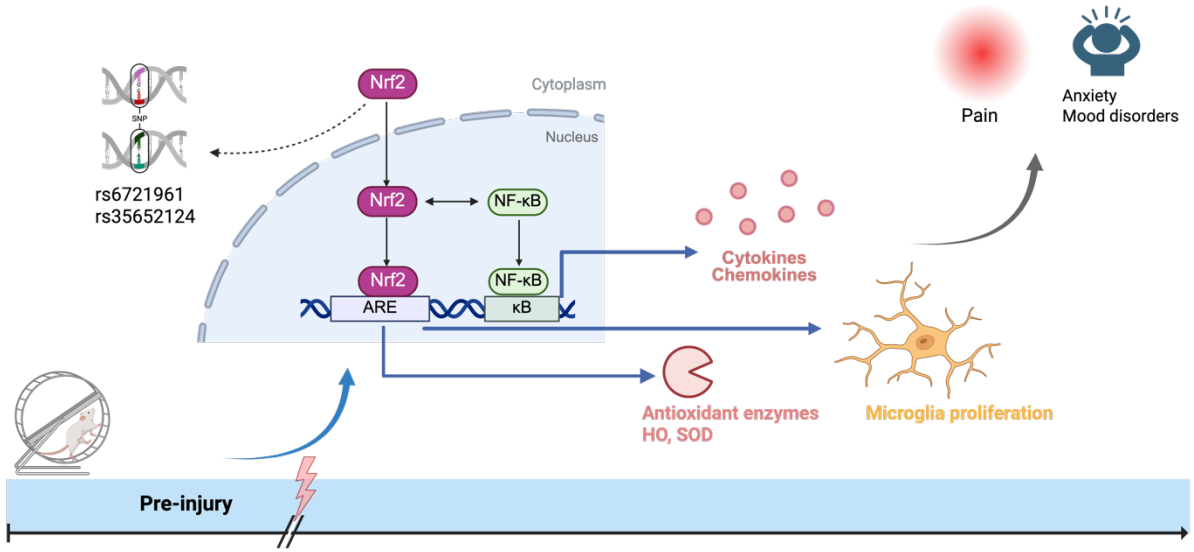

Figure 2. Schematic representation of the potential involvement of Nrf2 in susceptibility and resilience to chronic pain and related comorbidities. Nrf2 can be activated by pre-injury exercise (Suzanne M. Green-Fulgham et al., 2022), which may lead to a post-injury cascade of biological changes, including production of anti-inflammatory cytokines (independently or via interaction with NF-κB) and antioxidant enzymes, and inhibition of microglia activation (Basu et al., 2022; Rojo et al., 2010; Saha et al., 2022; Wardyn et al., 2015). These downstream effects can protect against the development of chronic pain and associated comorbidities, including anxiety and mood disorders. On the genetic level, the SNPs rs6721961 and rs35652124 are

considered the most clinically important SNPs of *NRF2* (Ikejiri et al., 2024). These polymorphisms can affect NRF2 protein expression (Yamaguchi et al., 2019), potentially influencing the development of pain (Smith, 2010). HO: Heme Oxygenase; SOD: superoxide dismutase; Nrf2: nuclear factor erythroid 2-related factor 2; ARE: antioxidant response element; NF-κB: nuclear factor-κB; SNP: single-nucleotide polymorphism.

1.4. Aims and hypotheses

The identification of susceptibility and resilience factors in chronic pain development has important clinical implications, not only for the prediction, but also for the prevention through targeted modifications. Given that chronic pain remains a challenge to treat, preventive approaches are of obvious interest, especially when chronic pain is a potential outcome. This includes, for instances, patient undergoing surgical approaches (Glare et al., 2019), chronic exposure to drugs like platins or antiretrovirals – see (Brozou et al., 2018) and (Madden et al., 2020), respectively – or having conditions of a (neuro)degenerative nature that eventually will culminate in pain – diabetes (Feldman et al., 2019) or Parkinson's disease (Marques and Brefel-Courbon, 2021), and also individuals who are on a certain extend a potential chronic pain patient, due to professional activities or unhealthy habits.

The general aim of this thesis is to identify susceptibility or resilience factors to the development of chronic pain and its associated comorbidities. Given the evidence presented above, we focused on the immune system, particularly on the transcriptional factor Nrf2.

To achieve that, the first study was conducted in a rat model of chronic neuropathic pain, examining the effects of activating Nrf2 around pain-triggering events on pain manifestation. The second study leveraged data from a longitudinal adolescent cohort, investigating the contribution of *NRF2* genetic polymorphisms to pain development and to the relationship of pain with anxiety and depression.

1.4.1. Study 1

This study has been published: 'Le, A.C.T., Fiuza-Fernandes, J., Silva, J.M., Sampaio, M.T., Texeira-Castro, A., Duarte-Silva, S., Leite-Almeida, H., 2025. Pretreatment with dimethyl fumarate prevents chronic pain and its comorbidities via Nrf2 pathway in a rat model of neuropathic pain. Brain Behav Immun 128, 725–736. https://doi.org/10.1016/J.BBI.2025.05.003.'

1.4.1.1. Summary of the research background

Despite substantial efforts to seek effective treatments, chronic pain remains a therapeutic challenge with detrimental impacts on individuals' life quality (Cohen et al., 2021; O'Connor, 2009). Identifying susceptibility and resilience factors for chronic pain might offer a potential avenue for preventing the condition through targeted

modifications. In human studies, preoperative anxiety, depression, and past experiences were reported as predictors of chronic postsurgical pain (Liu et al., 2022; Pinto et al., 2018) – see also for review (Sluka et al., 2023). Meanwhile, preclinical experiments showed diet, exercise, age, and sex might affect rodents' susceptibility to developing chronic pain (Da Silva et al., 2020; Ferrari et al., 2018) – see for review (Marques Miranda et al., 2021). Among factors, the immune system appears critical as neuroimmune interaction is one of the most significant culprits in chronic pain pathogenesis, with both adaptive and innate immune systems playing substantial roles in peripheral and central sensitization (Hore and Denk, 2019; Hutchinson et al., 2013; Scholz and Woolf, 2007; Wieseler-Frank et al., 2005). Importantly, evidence from both preclinical and clinical studies shows that variations in baseline immune profiles are associated with differences in pain sensitivity and symptoms following pain-evoked events (Fragiadakis et al., 2015; Gaudillière et al., 2014; Iordanova Schistad et al., 2020; Parisien et al., 2022; Souquette and Thomas, 2024). This suggests that the immune system is a potential area for identifying susceptibility and resilience factors in pain chronicity, and a preemptive modification to this system might alter subsequent pain trajectories. Additional evidence comes from studies involving physical exercise, which is a well-known protective factor against chronic pain development. Indeed, it has been demonstrated that such beneficial effect is associated with the involvement of various components of the immune system, including microglia, nuclear factor-κB (NF-κB), anti- and pro-inflammatory cytokines, as well as Tumor Necrosis Factor α (TNF-α) (Bobinski et al., 2015; Chhaya et al., 2019; Cho and Seo, 2022; Cobianchi et al., 2013; Grace et al., 2016). In particular, nuclear factor E2-related factor 2 (Nrf2), a major transcriptional factor that regulates the antioxidant and anti-inflammatory response, has emerged as one of the most important molecular pathways with its activation by exercise before nerve injury linked to greater resilience against subsequent chronic pain (S M Green-Fulgham et al., 2022). This evidence suggests that pre-injury immune modulation, targeting the Nrf2 factor, which results in changes in the complex of downstream effectors, could prime pain pathways and protect against the chronic process of pain.

Dimethyl fumarate (DMF) is a well-known Nrf2 inducer with antioxidant effects and a large-spectrum immunomodulator that can direct the immune response toward an anti-inflammatory state (Cuadrado et al., 2019; Grace et al., 2021; Wardyn et al., 2015).

The drug has been shown to have anti-allodynic effects by activating Nrf2 in different sites, including both peripheral, like the DRG, and central nervous system tissues, such as the spinal cord, amygdala, cerebellum, and cortex (Gao et al., 2022; Li et al., 2020b; Suárez-Rojas et al., 2023; Zhang et al., 2024). In those examples, DMF efficacy in alleviating chronic pain has been demonstrated, but the drug is only administered after the pain has already developed. Data for the effectiveness of pretreatment, however, is missing.

1.4.1.2. Hypotheses

Based on the above-mentioned evidence, this study tested the hypothesis that modulating the immune response by activating Nrf2 around pain-triggering events can protect spared nerve injury rats against the development of chronic pain and comorbid anxiety, depression, and cognitive dysfunction. DMF was used as an Nrf2 inducer. The outcome of the study will help elucidate the susceptibility and resilience related to the immune system and shed light on the targets for preventive approaches.

1.4.2. Study 2

1.4.2.1. Summary of the research background

Adolescence is a sensitive period of brain development characterized by significant structural and functional changes, especially in regions essential for behavior and emotion regulation (Fuhrmann et al., 2015; Steinberg, 2005). These changes are critical for behavioral adaptations and health. Studies show maladaptations can render adolescents more susceptible to negative affect (Crone and Dahl, 2012; Dow-Edwards et al., 2019; Giedd et al., 2008), which contributes to long-term health outcomes (Sawyer et al., 2012), including both mental such as anxiety and depression (Murray et al., 2024; Senger-Carpenter et al., 2025), and physiological outcomes like pain (Murray et al., 2024). When looking at the prevalence of pain symptoms, it is stated that there is an overall high prevalence during adolescence. For example, more than 60% of pupils report experiencing headaches in the past year (Miao et al., 2019; Philipp et al., 2019; Wöber et al., 2018) – see for review (Nieswand et al., 2020), while conditions like recurrent musculoskeletal pain, including low back pain or, in general, chronic pain conditions, affect up to 33.6% of adolescents (Calvo-Muñoz et al., 2013; Chambers et al., 2024; Gassmann et al., 2008; Larsson et al., 2018; Laurell et al., 2006). Research indeed indicates that such pain has often been linked to earlier life events, which include psychological stressors (Bondesson et al., 2018; Chang et al., 2015). It is therefore not surprising that comorbidities of affective symptoms and pain are commonly observed. Moreover, a previous existence of affective symptoms also seems to determine the development of pain symptoms. While its importance in identifying individuals at risk and preventing the high prevalence of pain is well-recognized, research on the relationship between negative affect, such as anxiety and depression during adolescence, and pain outcomes later in life remains limited.

Not only since COVID-19 but also before, changes in the immune system have been highlighted as an important health factor for adolescents, particularly in relation to neurodevelopment and sensitivity. Adolescence is a critical period for immune system maturation, during which neural immune signaling plays a key role in shaping neurodevelopment – please see for review (Brenhouse and Schwarz, 2016). Additionally, a study published in the journal *Psychiatric Genetics* discusses the role of genetic and gene-environment interactions in mental health disorders, emphasizing the importance of immune system changes during adolescence (Maier et al., 2017).

The link between inflammation and anxiety and depression is well-documented, with increased levels of pro-inflammatory cytokines such as interleukin 1 (IL-1), IL-6, Tumor Necrosis Factor (TNF), and interferon (IFNs) associated with symptoms of those disorders (Howren et al., 2009; Milaneschi et al., 2021; Mostafavi et al., 2014; Vogelzangs et al., 2013; Ye et al., 2021) – see for review (Goldsmith et al., 2023). Modulating the immune system towards anti-inflammatory phenotypes has shown efficacy in ameliorating depression and anxiety (Beurel et al., 2020; Miller et al., 2017). Not only with mental health disorders, but the role of the immune system in pain, particularly regarding the mechanisms of neuropathic and nociceptive pain (Cohen et al., 2021), is also critical (Hore and Denk, 2019; Scholz and Woolf, 2007; Wieseler-Frank et al., 2005). This suggests potential involvement of this system in the mechanisms underlying the relationship between affective and pain symptoms during adolescence. For example, anxiety has been shown to increase glutamatergic synaptic activity in the anterior cingulate cortex (ACC), resulting in long-term potentiation (LTP), a mechanism underlying pain sensitization (Bliss et al., 2016; Koga et al., 2015; Zhuo, 2016). LTP is widely reported to be modulated by cytokines and other immune components – see reviews (Joanna L. Jankowsky and Patterson, 1999; Prieto and Cotman, 2017). Therefore, it can be speculated that changes in the immune system may play a role in determining the risk of developing pain from anxiety.

Within the immune system, one molecule of particular interest is the nuclear factor erythroid 2-related factor 2 (NRF2), which is widely recognized as the master regulator of the antioxidant and anti-inflammatory pathway. NRF2 is normally located in the cytoplasm and translocated to the nucleus under stress conditions, where it activates the transcription of various cytokines and antioxidant enzymes, which are all critically involved in the mechanisms underlying both pain and anxiety and depression (Basu et al., 2022; Wardyn et al., 2015). On the molecular level, inducers of NRF2 reversed pain-related behaviors in various rodent pain models (Chen et al., 2019; Lee et al., 2021; Li et al., 2020a; Zhou et al., 2020) – for a review, please see (Zhou et al., 2021). Important evidence from animal studies linked this factor to the determining role in susceptibility and resilience to the development of chronic pain, as well as comorbid anxiety and mood disorders (S M Green-Fulgham et al., 2022; Le et al., 2025). On the genetic level, although not yet studied in the context of pain, variations in single-nucleotide polymorphisms (SNPs) in the *NRF2* gene, such as rs35652124 and

rs6721961, have been found to be associated with a higher risk of various medical conditions, including cancer, diabetes, neurodegenerative, and cardiovascular diseases (Ikejiri et al., 2024), those that are highly comorbid with pain (Barrett and Kohut, 2024; Copenhaver et al., 2021; Cruz et al., 2022; Fayaz et al., 2016; Lawn et al., 2021; Reichling and Levine, 2011). SNP rs6721961 has also been proposed as a potential predictor of pain (Smith, 2010). Given the NRF2's regulating role in coping with stress and immune modulation, variations in its SNPs, which may result in relevant molecular changes (Yamaguchi et al., 2019), can influence the affect-pain association, which remains to be elucidated.

1.4.2.2. Hypotheses

Examining the relationship between affective symptoms and pain outcomes in adolescence, while considering the contribution of changes in the immune system, will provide novel avenues for preventive approaches and help elucidate the biopsychological mechanisms underlying pain development, which is also important in the context of chronic pain. Based on the evidence outlined above, we hypothesize that anxiety or depression during adolescence is expected to predict higher pain complaints in later years. Additionally, we tested the contribution of the *NRF2* polymorphisms rs35652124 and rs6721961 to these relationships. *NRF2* polymorphisms are also expected to affect the magnitude of pain complaints.

2. MATERIALS AND METHODS

2.1. Study 1

2.1.1. Animals

2–3-month-old Wistar Han male rats (Charles River Laboratories, Barcelona, Spain) were used in all experiments, while female rats were employed in one line of experiments – see below for more details. Animals were same-sex paired and housed in standard plastic cages with food and water available *ad libitum* in a light-controlled (12 hours light-dark cycle; lights on at 08:00 $_{AM}$) and temperature-controlled (22ºC±1ºC) room. Experiments were performed by FELASA category C researchers. All procedures involving animals were approved by the respective local organizations, and the experiments were performed according to the European Community Council Directive 2010/63/EU guidelines.

2.1.2. Experimental design

To avoid stress induced by drug administration, one week before experiments, all rats were carefully handled and habituated to oral gavage with sterile water. Subsequently, animals were treated with either dimethyl fumarate (DMF) or vehicle (VEH) for 7 days before being subjected to SNI surgery. After that, half of the animals in each group maintained the treatment while the other half switched to the other treatment for an additional 7 days, resulting in 4 groups: the continuous treatment (DMF-DMF), the pretreatment (DMF-VEH), the early treatment (VEH-DMF) and the control (VEH-VEH) (n = 8 males per group). Blood was collected for cytokines and chemokines protein expression analyses at day 7 after SNI. This time point corresponds to immediately after the final DMF dose in the DMF-DMF and VEH-DMF groups, and 7 days following the final DMF dose in the DMF-VEH group. Meanwhile, mechanical allodynia and depression-like behavior tests were used as treatment behavioral outcomes (Figure 1A).

In the following study, trigonelline, an Nrf2 inhibitor, was used to block DMF's downstream effects. Mechanical allodynia and cold allodynia were recorded weekly using von Frey and acetone drop test until 7 weeks after SNI, where a battery of behavioral tests for depression-, anxiety- and cognitive-like behaviors was applied (Figure 1B) (n = 8-10 males/group). Ipsilateral dorsal root ganglia (DRG) L4-6 at day 3 and 49 after treatment cessation were collected for molecular analysis (Figure 1C)

(n = 8-12 males/group, which becomes n = 4-6 samples/group. DRG from 2 animals were pooled as 1 sample). After the first experiment, the best outcome treatment, rather than all regimens, was selected for testing on female rats (n = 8/group). This is to reduce the number of animals used.

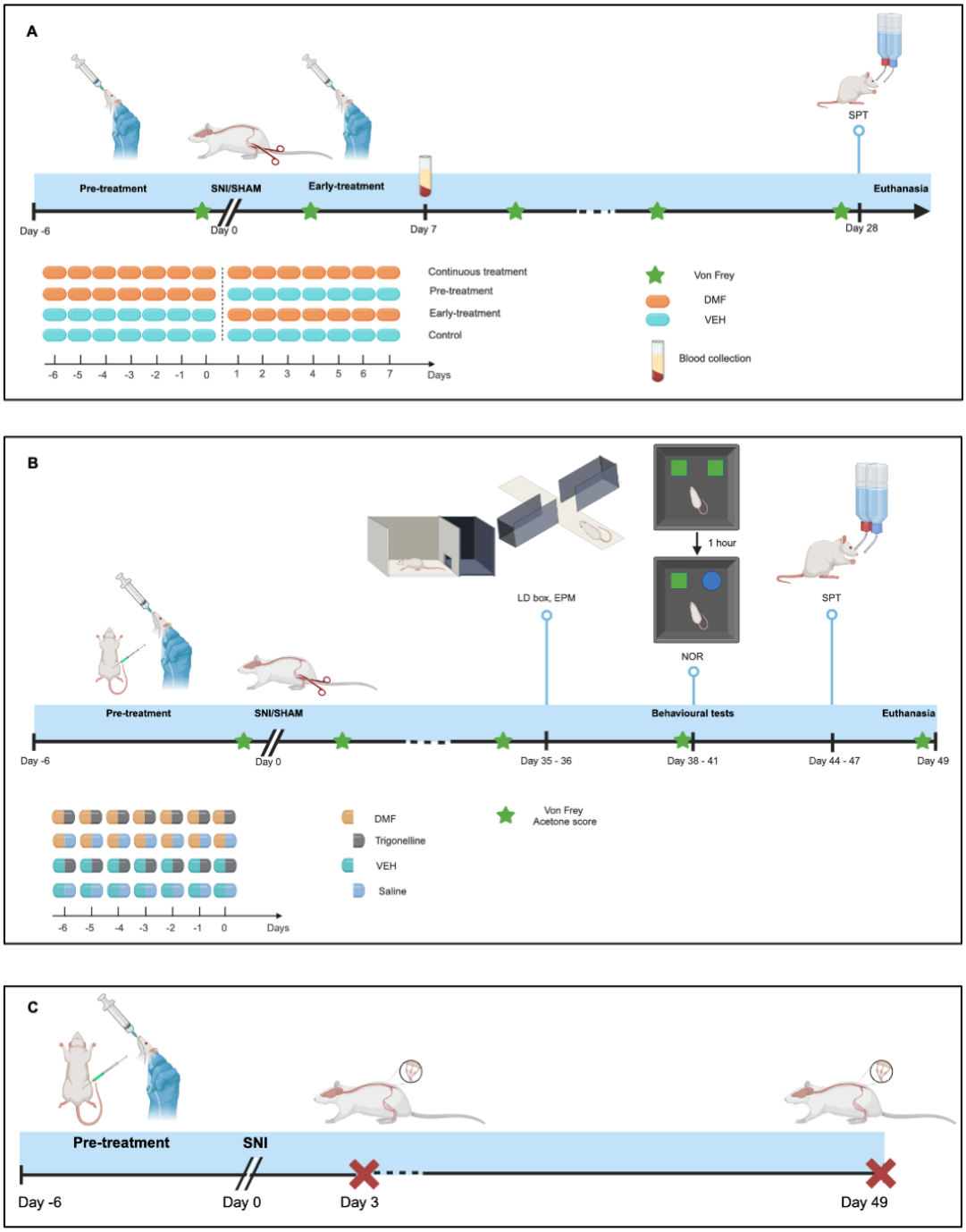

Figure 1. Experimental designs. (A) Effects of preemptive treatment with DMF on chronic neuropathic pain and its comorbidities. SNI was used to model chronic neuropathic pain. DMF and VEH were orally administered daily at 300mg/kg. DMF was administered 7 days before SNI (Day 0), while control animals received VEH.

After SNI, half of the animals in each group shifted treatments for an additional 7 days, resulting in 4 groups: continuous treatment (DMF-DMF), pretreatment (DMF-VEH), early treatment (VEH-DMF), and control (VEH-VEH). Mechanical allodynia was measured using the von Frey test, while depression-like behaviors were assessed using the SPT. **(B)** Exploring the protective mechanism of DMF. Trigonelline, given i.p. daily at 20mg/kg, was added to block DMF's effects. In addition to mechanical, cold allodynia was also measured using Acetone drop tests. Depression-, anxiety- and cognitive-related were assessed using SPT, LDB, EPM, and NOR tests, respectively. **(C)** Molecular analysis. The protein level of ATF-3 was measured using Western blot at days 3 and 49 after SNI/treatment cessation. All tissue was collected at the ipsilateral DRG L4-6. SPT: Sucrose Preference Test, LDB: Light-Dark Box, EPM: Elevated Plus Maze, NOR: Novel Object Recognition, DRG: Dorsal Root Ganglia.

2.1.3. Drug administration

Dimethyl fumarate (300 mg/kg Sigma-Aldrich, Spain) was suspended in methylcellulose (viscosity 15cP, 2% w v^{-1} in water; Sigma-Aldrich, Spain) and administered by oral gavage (daily 10:00). This DMF dose, route, and duration of administration were selected based on ref. (Li et al., 2020b) where the authors have tested different doses (30, 100, and 300 mg/kg) in various durations and found that 300 mg/kg daily for 5 - 7 days attenuated SNI-induced neuropathic pain – see also ref. (Gao et al., 2022). Trigonelline (20 mg/kg; Sigma-Aldrich, Spain) was given by intraperitoneal injection (i.p.) 30 minutes before DMF or VEH (please see ref. (Gao et al., 2022)). It is worth mentioning that in ref. (Li et al., 2020b), trigonelline was used at 300 mg/kg twice daily by oral gavage. Instead, we chose 20 mg/kg given by i.p. based on ref. (Gao et al., 2022), which demonstrated that this dose and route of trigonelline administration effectively counteracted oral DMF at 300 mg/kg. This selection is expected to reduce the stress caused by gavage and high doses of drugs and to minimize drug adverse events for the animals. A corresponding volume of methylcellulose 2% and saline 0.9% was administered as vehicle controls for DMF and trigonelline, respectively (Gao et al., 2022; Li et al., 2020b).

2.1.4. Spared nerve injury model

The spared nerve injury was performed as previously described by our group (Cunha et al., 2019) with minor changes from the original report (Decosterd and Woolf, 2000).

Briefly, rats were anesthetized with a dosage of 1ml/kg of a 1,5:1,0 mixture of ketamine (Imalgene, 100 mg/mL; Merial, Lyon, France) and medetomidine (Dormitor, 1 mg/mL; Orion Pharma, Espoo, Finland), administered intraperitoneally. The right sciatic nerve was exposed, and the tibial and common peroneal nerves were ligated. The axotomy was performed distal to the ligation site, leaving the sural nerve spared. SHAM animals underwent the same procedures but without the axotomy. At the end of the procedure, the animals were administered a subcutaneous injection of atipamezole hydrochloride (Antisedan®, Esteve Farma; 0.35 mg) and monitored closely until fully ambulatory before returning to their home cage.

2.1.5. Behavioral readouts

2.1.5.1. Mechanical allodynia

The von Frey test was performed to assess mechanical allodynia before SNI as baseline values and then weekly (starting from day 3) after SNI until the end of the experiments. Before the surgery, rats were habituated to the apparatus, a plastic enclosure on an elevated grid, for 5 minutes on two occasions. In subsequent sessions, after 10 minutes of habituation, allodynia was examined with the up-and-down method previously described by our group (Cunha et al., 2020a, 2019). Briefly, the sural dermatome was provoked with monofilaments, including 15.0, 8.0, 6.0, 4.0, 2.0, 1.0, 0.6, and 0.4 g (North Coast Medical Inc., USA). The test was started with the 2.0g filament. Next, subsequent stronger or weaker filaments would be used depending on whether no response (=O) or a withdrawal of the limb (=X) was recorded, respectively. The test was terminated either when six measurements were already obtained around (including) the threshold point or when animals showed no response with the maximal force (15.0 g) or response with the minimal force (0.4 g). The 50% response threshold was interpolated using the following formula: 50% threshold = $\frac{10^{X_f+k\delta}}{10000}$, where Xf = value (in log units) of the final von Frey filament, k = tabular value for the pattern X/O responses and δ = mean difference (in log units) between stimuli (0.224).

2.1.5.2. Cold allodynia

Cold allodynia was assessed using the acetone drop test. This test was performed after each von Frey test in the same apparatus (Bravo et al., 2012). Following the habituation phase, a drop of 100 μL acetone was applied 3 times at 5-minute intervals

to the sural dermatome of the ipsilateral paw regarding the injury. The responses were observed over 60 seconds and scored as 0: no response, 1: quick withdrawal or flicking, 2: prolonged withdrawal or repeated flicking of the paw, and 3: repeated flicking and licking the paw. The acetone score for each rat was calculated by summing the scores of 3 trials and dividing by 3.

2.1.5.3. Sucrose preference test (depressive-like behavior)

The SPT was used to indicate anhedonia in the animals (Cunha et al., 2020b). A 4-day protocol was applied with minor adjustments (Liu et al., 2018). Briefly, on day 1 (09:00), two bottles (polycarbonate, neck-covered silicone gasket) with 300,0 mL of a 2% sucrose solution and 300,0 mL of sterile water were placed in the animal's home cage. The next day (09:00), the bottles' position was switched to minimize side-related preference. Finally, on day 3 (09:00), rats were weighed and moved to the test room in a new cage, single-housed, and were put on food and water deprivation for 12 hours. At 21:00, two previously weighed bottles – 2% sucrose and sterile water, approximately 300,0 mL each – were placed in each cage (random position). Bottles were weighed 1 hour and 12 hours later. Rats were then returned to their original cages. Sucrose preference (%) was calculated using the formula:

$$\text{Sucrose preference} = \frac{Sucrose\ intake}{Total\ intake\ (sucrose+water)} \times 100.$$

2.1.5.4. Light-dark box test (anxiety-like behavior)

The Light-dark box test (LDB) protocol was described by our group (Guimarães et al., 2019). Briefly, LDB was performed in a square open field arena (43.2 x 43.2 cm) with transparent acrylic walls and a white floor (model ENV-515; Med Associates Inc, St. Albans, Vermont), which was divided into two equal compartments. The dark side was a black acrylic box connected to the light side, illuminated by a 235-lx light by an aperture. On the test day, animals were moved and acclimatized to the test room for 10 minutes. After that, each rat was placed in the center of the light side facing the aperture. The test lasted for 5 minutes. The time spent in the light chambers was used to index anxiety-like behaviors.

2.1.5.5. Elevated plus maze (anxiety-like behavior)

The EPM apparatus, elevated 72.4 cm from the floor, was plus-shaped and made of polypropylene plastic. The setting contained two opposed open arms and two opposed

closed arms with walls (Med Associates Inc., St. Albans, Vermont, USA) (Leite-Almeida et al., 2009). At the beginning of the test, each animal was placed in the center of the maze, facing one of the open arms. Their movements were recorded for 5 minutes, with particular attention given to the time spent in the open arms. A 10% alcohol solution was used to clean the apparatus between animals.

2.1.5.6. Novel object recognition test (cognitive dysfunction-like behavior)

The protocol for the novel object recognition test (NOR) was previously described by others (Machado-Santos et al., 2022). The test was performed on a black acrylic box 50(W) x 50(L) x 150(H) cm. On day 1, rats were acclimatized to the arena without objects for 8 minutes. On day 2, two identical objects were placed in the arena, and rats were given 8 minutes for exploration. One hour later, one of the objects was replaced by a novel object, and rats were allowed 3 minutes to explore. The familiar (f.o) and novel objects (n.o) differed in color, size, shape, and texture. All sessions were videotaped while data was analyzed manually. The formula calculated the discrimination index (d.i):

$$\text{d.i.}=\frac{time\ spent\ on\ n.o - time\ spent\ on\ f.o}{time\ spent\ on\ n.o + time\ spent\ on\ f.o}$$

2.1.6. Tissue collection

On days 3 and 49 after SNI, animals were euthanized by decapitation under deep anesthesia. Dorsal root ganglia L4-6 were quickly collected and snap-frozen in liquid nitrogen before storing at – 80^{o}C. The time points were selected based on the observed anti-allodynia effects – the highest and lowest – and the known time course of ATF-3 expression following axotomy, which peaks within the first week after the sciatic nerve cut (Tsujino et al., 2000).

2.1.7. Molecular analysis

2.1.7.1. Tissue preparation

The radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA with protease and phosphatase inhibitors) was used as the homogenization buffer (Silva et al., 2019). The resulting samples were centrifuged for 10 minutes at 13,000 rpm at 4^{o}C, and the supernatant was collected. The protein quantification in the present study was conducted using Bradford Assay.

2.1.7.2. Western Blot

After reconstitution in Laemmli buffer (Bio-Rad, USA, prepared with 2% β-mercaptoethanol), the samples were electrophoresed using SDS-PAGE gels (10% acrylamide) and wet-transferred onto PVDF membranes (GE Healthcare). Total protein normalization was performed using TotalStain Q fluorescent (Azure Biosystems, USA). Membranes were then blocked in Tris-buffered saline, including 5% non-fat milk and 0.2% Tween-20, before being incubated overnight with the following antibodies: β-actin (1:2000, Abcam, ab8226), activating transcription factor 3 (ATF-3) (1:500, Santa Cruz, sc-518032), Heme Oxygenase 1 (Ho-1) (1:1000, Abcam, ab13243), Nrf2 (1:500, Santa Cruz, sc-722), Histone H3 (1:300, Proteintech, 17168-1-AP). Next, the membranes were incubated with the corresponding Mouse/Rabbit secondary antibodies (1:5000, Bio-Rad, 170-6515 & 170-6516). The signals were then revealed by enhanced chemiluminescence (Clarity, Bio-Rad, USA). The blot imaging was obtained using Sapphire Biomolecular Imager (Azure Biosystems, USA), while the signals were quantified by AzureSpot Analysis Software (Azure Biosystems, USA). β-actin was used to normalize values.

2.1.7.3. Multiplex Bead-based Immunoassay

Cytokines/chemokines were quantified using A Rat Cytokine/Chemokine Magnetic Bead Panel kit (Millipore, Sigma-Aldrich, Spain) according to the manufacturer's instructions. Briefly, rat blood was collected following the last dose (day 7 after SNI). The blood was allowed to clot for 30 minutes before centrifugation for 10 minutes at 1000 x g. The resulting supernatant (serum) was collected and stored at -20^{o}C until use for analysis. In the immunoassay, 25 μl of Standard, Control, or Sample were incubated in a 96-well plate with multi-cytokine beads for 2 hours at room temperature, with agitation. After washing well contents, biotinylated reporter (Detection Antibodies) was added, followed by 1 hour of incubation. Next, Streptavidin-Phycoerythrin was added and incubated for 30 minutes. After the final wash, the beads were read on a MAGPIX® instrument and analyzed using BioPlex-Manager software (BioRad Laboratories).

2.1.8. Data analysis

Statistical analyses were conducted using JASP version 0.18.1, GraphPad Prism version 9.5.1, Microsoft Office Excel, and Python 3.11.4, with packages including

pandas, seaborn, matplotlib, and scipy.stats. To analyze mechanical and cold allodynia, we performed ANOVA with repeated measures and a post-hoc comparison using the Holm-Bonferroni method. We performed two-way or three-way ANOVA to analyze the results of the behavior tests (EPM, LDB, SPT, NOR) and molecular analysis. When all Main Effects and Interactions were significant, the Tukey test was used for multiple comparison tests. If only specific Main Effects were found and/or without significant Interaction Effects, independent t-tests or Mann-Whitney U-tests with Bonferroni corrections were applied (Wei et al., 2011). The correlation between pain hypersensitivity and anxiety-like behaviors was obtained using the Pearson Product-Moment Correlation test. Grubbs's test with the threshold α=0.05 was used to define significant outliers. A p-value < 0,05 was considered statistically significant.

2.2. Study 2

2.2.1. Participants

We leveraged data from the Imaging Genetics (IMAGEN) study (Schumann et al., 2010), where healthy adolescents were recruited from the general population via school visits, flyers, and registration offices in Germany, the United Kingdom, Ireland, and France. Exclusion criteria for study participation were: contraindications for magnetic resonance imaging (MRI) examinations, pregnancy, severe medical conditions, and previous head trauma with unconsciousness. For the present analyses, a subset of participants with complete baseline (BL) (age 14-15) and follow-up 1 (FU1) (age 16-17) data was used.

The study was approved by the local ethics committees and adhered to the Declaration of Helsinki. After a complete study description, written informed consent was obtained from both adolescents and their parents.

Table 1. Participants' characteristics

Characteristics	Sample
N	724
Gender (m/f)	312/412
Age BL	14.439 ± 0.380
Anxiety BL	1.233 ± 0.549
Depression BL	0.928 ± 1.006
Age FU1	16.097 ± 0.085
Sum pain score FU1	3.301 ± 3.308
Data presented as mean ± standard deviation	

2.2.2. Assessment of anxiety and depression

The Development and Well-Being Assessment (DAWBA) (Goodman et al., 2000) was utilized to assess major depressive disorder and generalized anxiety disorder, with definite symptoms obtained by structured questions to the child and parent. The answers to those questions were fed into a computerized diagnostic algorithm, which predicts the likelihood that an experienced clinical rater would assign the child operationalized Diagnostic and Statistical Manual of Mental Disorders (DSM) DSM-IV and International Classification of Diseases (ICD) ICD-10 diagnoses. The computer

prediction provides output as a 6-point scale, ranging from less than 0.1% likely to more than 70% likely. Data on GAD and MDD were used at BL.

2.2.3. Assessment of pain symptoms

The participants completed the Children Somatization Inventory (CSI) (Walker et al., 2008) to assess the severity of somatic symptoms, including pain. The CSI consists of 35 items, including the somatic symptoms defined by DSM-III-R (American Psychiatric Association, 1987), the Hopkins Symptom Checklist (HSCL) (Friedman et al., 2007), and additional symptoms common in functional gastrointestinal disorders. The response format is a 5-point scale ranging from "*not at all*" (0) to "*a whole lot*" (4), which results in a range of sum scores from 0 to 140, and the standard period for symptom reports is 2 weeks. Items that are related to pain and motor weakness symptoms were selected based on ref.(Heukamp et al., 2024). Participants completed the CSI online from home using Psytools software (Delosis Ltd, London, United Kingdom). In this study, we included data on pain symptoms at FU1.

2.2.4. DNA extraction, genotyping, and quality control

Whole blood samples (~10mL) were collected and stored in BD Vacutainer EDTA tubes (Becton, Dickinson and Company, Oxford, United Kingdom). DNA extraction was performed using Gentra Puregene Blood Kit (QIAGEN Inc, Valencia, CA), while genotyping was done at 582,982 markers within the context of the IMAGEN study (Schumann et al., 2010) using the Illumina HumanHap610 Genotyping BeadChip (Illumina, San Diego, CA). Single nucleotide polymorphisms were excluded if call rates were below 98%, minor allele frequency under 1%, or significant deviation from Hardy-Weinberg equilibrium ($P \leq 1 \times 10^{-4}$). Additionally, individuals with excessive missing genotypes (failure rate > 2%), outlying heterozygosity (more than three standard deviations from the mean), or an ambiguous sex code were also excluded. Before the following analysis, closely related individuals with an identity-by-descent greater than 0.1875 were excluded. Population stratification for the GWAS was examined by principal component analysis with EIGENSTRAT software (Pritchard et al., 2000) and the four HapMap II populations as references. Additionally, individuals with divergent ancestry (from CEU [Utah residents of northern and western European ancestry]) were also excluded (Price et al., 2006).

For this study, we report data on 589 GG genotype carriers of rs6721961, 130 TG, and 5 TT genotype carriers. Due to a limited number of the TT allele, TG and TT will be merged (N=135). Meanwhile, the SNP rs35652124 consists of 337 TT, 312 CT, and 75 CC. Please refer to Table 2 for more details on the SNPs.

Table 2. Details on SNPs rs6721961 and rs35652124 of the human *NRF2*

SNP_ID	Position	Localization	Allele (minor/major)	SNP regions
rs6721961	177265309	GRCh38, Chr 2	T/G	Promoter
rs35652124	177265345	GRCh38, Chr 2	C/T	Promoter

2.2.5. Data analysis

Association between anxiety/depression and pain complaints during adolescence

For the prediction of pain complaints, ordinary least-squares regression was used, with anxiety/depression measured at BL as a regressor for painful symptoms at FU1. Gender was added as a covariate (Equation 1a-b).

$$sum\ pain\ (FU1) \sim GAD\ (BL) + sex$$ (Equation 1a)

$$sum\ pain\ (FU1) \sim MDD\ (BL) + sex$$ (Equation 1b)

The contributions of SNP rs35652124 and rs6721961 to the associations

The prediction models were tested in the whole group and separately for the allele groups of rs6721961 and rs35652124. Moreover, we analyzed possible differences in pain complaints and early-in-life anxiety/depression symptoms between the allele groups using either analysis of variance (ANOVA) or independent t-tests if the data met parametric assumptions. Otherwise, the Kruskal-Wallis test or the Mann-Whitney U test was employed.

Statistical significance, multiple comparison correction, and identified outliers

All tests were performed with a significant level of α = 0.05, two-tailed. Bonferroni correction was applied for multiple comparisons. In this study, significant outliers were defined using Cook's distance of 4/(n – k – 1), where n is the sample size and k is the number of independent variables. We employed Python 3.11.4 with packages including pandas and statsmodels, RStudio 2024.09.0+375 with library ggplot2 for the analyses.

3. RESULTS

3.1. Study 1

3.1.1. Results

3.1.1.1. Preemptive treatment with DMF attenuated SNI-induced mechanical allodynia and related mood disorders

Rats were treated with DMF in 4 different regimens: DMF-VEH, DMF-DMF, VEH-DMF, and VEH-VEH. Regarding the von Frey test, no group differences in thresholds were found before SNI (Figure 2A). In the male subset, repeated measure ANOVA showed the main effects of Treatment [$F(7,55) = 105.940$, $p<0.001$], Treatment x Time [$F(21,165) = 1.975$, $p =0.010$], but no main effects of Time [$F(3,165) = 1.313$, $p=0.272$]. Post hoc comparisons test showed that DMF-DMF SNI vs VEH-VEH SNI $p <0.001$, DMF-VEH SNI vs VEH-VEH SNI $p <0.001$, and VEH-DMF SNI vs VEH-VEH SNI $p = 0.007$. DMF did not affect mechanical allodynia in SHAM-operated animals. Although no significant differences in effects between DMF regimens over the course of four weeks, the pre-, and continuous treatments demonstrated efficacy in the first week ($p<0.05$), whereas the early treatment did not ($p>0.05$). Regarding the impact on depressive-like behaviors measured by SPT, a three-way ANOVA showed effects of post-injury Treatment [$F(1,53) = 4.304$, $p<0.05$], Pre-injury Treatment [$F(1,53) = 5.323$, $p <0.05$], and Surgery [$F(1,53) = 4.225$, $p<0.05$] while Tukey's multiple comparisons revealed DMF, in each of all three forms of preemptive treatments, prevented anhedonia developed in SNI rats, compared to VEH-treated animals, with DMF-DMF SNI vs VEH-VEH SNI $p <0.01$, DMF-VEH SNI vs VEH-VEH SNI $p <0.01$, VEH-DMF SNI vs VEH-VEH SNI $p < 0.01$ (Figure 2B). Body weight reduction was recorded after the treatment period (14 days). Within the SNI animals, in comparison to the weight change (percentage) for VEH-VEH 7.943 ± 3.251, DMF-DMF -5.455 ± 4.536 ($p<0.0001$), DMF-VEH 0.568 ± 5.510 ($p<0.01$), VEH-DMF -1.586 ± 4.051 ($p<0.001$). Within the SHAM animals, DMF-DMF -0.721 ± 4.510, DMF-VEH 0.556 ± 8.106, VEH-DMF -0.869 ± 3.990, compared to VEH-VEH 12.744 ± 3.204 ($p<0.001$).

Given that there were no significant differences in effects between different DMF regimens shown in male rats overall and the body weight loss observed, we explored the protection of DMF in female rats only using the pretreatment approach (treatment ended before SNI). As a result, the effects observed were limited and not as sustained as in male rats (Figure S1A-D). In detail, for the von Frey test, repeated measure

ANOVA showed main effects of Treatment [F (3,27) = 322.093, p<0.001], Treatment x Time [F (15,135) = 2.910, p <0.001] but no main effects of Time [F (5,135) = 0.853, p=0.514]. Post hoc comparison tests showed no further differences. In the acetone drop test, the post hoc comparisons test showed DMF only exerted protective effects in the first two weeks post-surgery (p<0.05), while there were main effects of Treatment [F (3,27) = 116.507, p<0.001], Treatment x Time [F (15,135) = 3.177, p <0.001], and Time [F (5,135) = 2.565, p=<0.05]. In terms of the anhedonia test, a two-way ANOVA showed the effect of Surgery [F (1,27) = 4.284, p<0.05] but no impact of Treatment [F (1,27) = 0.024, p>0.05]. Similarly, in the NOR test, there were effects of Surgery [F (1,26) = 8.728, p<0.05] but not of Treatment [F (1,26) = 0.123, p>0.05]. After the treatment period (7 days), the body weight change for the DMF group was -5.773 ± 3.751, VEH group 1.299 ± 1.987 (p<0.0001).

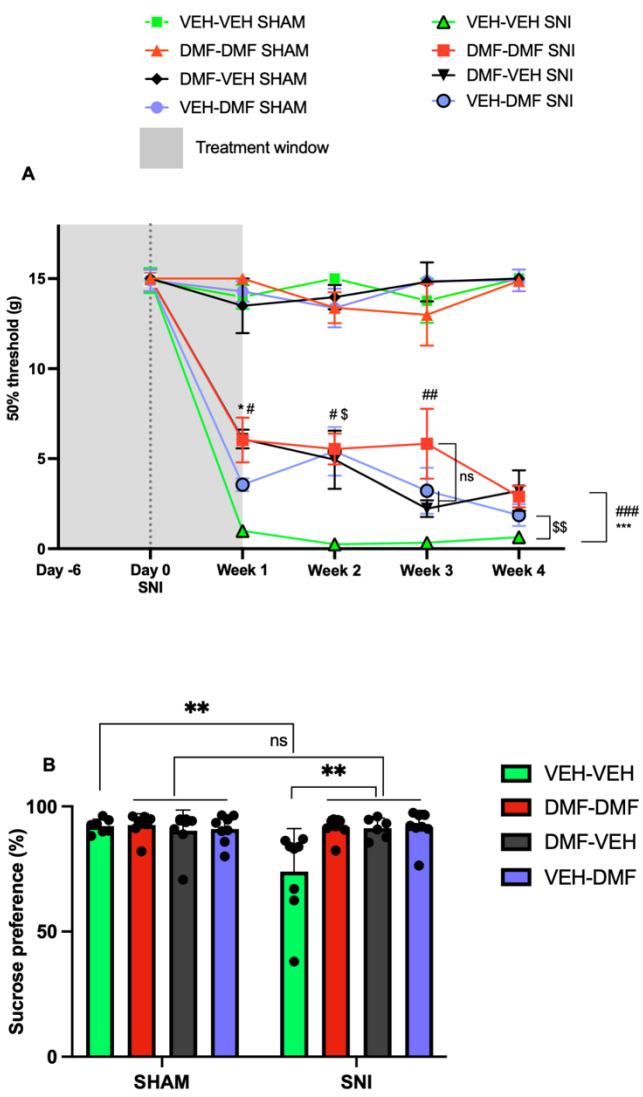

Figure 2. Preemptive treatment with DMF attenuated the severity of SNI-induced allodynia and related mood disorders. (A) Mechanical allodynia was assessed using the von Frey test. **(B)** Effects of DMF preemptive treatments on depressive-like behaviors were assessed using the sucrose preference test. Data was analyzed using ANOVA repeated measure and three-way ANOVA with Tukey's multiple comparisons. Data are presented as mean ± SD. n=8 male rats per group. *,#,$ p<0.05, **,##,$$ p<0.01, ***,###,$$$ p<0.001. ns: not significant. (A) Brackets to the right of the plot indicate statistical differences between the two groups across the entire period.

3.1.1.2. Preemptive treatment with DMF modulated the immune response

After treatment cessation, blood was collected for cytokines and chemokines examination. As a result, compared to the control group SNI VEH-VEH, treatment with VEH-DMF increased the protein levels of anti-inflammatory cytokines, including IL-10 (Treatment effect [$F(3,24) = 3.210$, $p < 0.05$], with Dunnett's post hoc test showed $p < 0.05$), G-CSF ([$F(3,23) = 3.115$, $p < 0.05$]), pleiotropic cytokines such as IL-6 ([$F(3,23) = 3.140$, $p < 0.05$]), interferon-gamma (IFN-γ) ([$F(3,23) = 4.348$, $p < 0.05$]), IL-17A ([$F(3,24) = 3.190$, $p < 0.05$]), and chemokine CXCL2 ([$F(3,25) = 4.582$, $p < 0.05$]) (Figure 3A-F). Meanwhile, treatment with DMF-VEH significantly reduced leptin ([$F(3,26) = 4.366$, $p < 0.05$]), with post hoc test showing $p < 0.05$, whereas treatment with DMF-DMF exhibited a trend toward reduction ($p=0.0587$) (Figure 3G). Regarding proinflammatory cytokines such as IL-1β and TNF-α, there were no effects in all DMF treatments (Figure S2). Although not statistically significant, other anti-inflammatory cytokines, including IL-4 and IL-13, increased in rats treated with VEH-DMF with relatively large effect sizes (0.76 and 0.99 – Figure S2). In order to maintain a broad spectrum of potential cytokine markers, the false discovery rate was not applied, instead, the effect sizes of all 27 cytokines/chemokines were provided in the supplementary material (Figure S2).

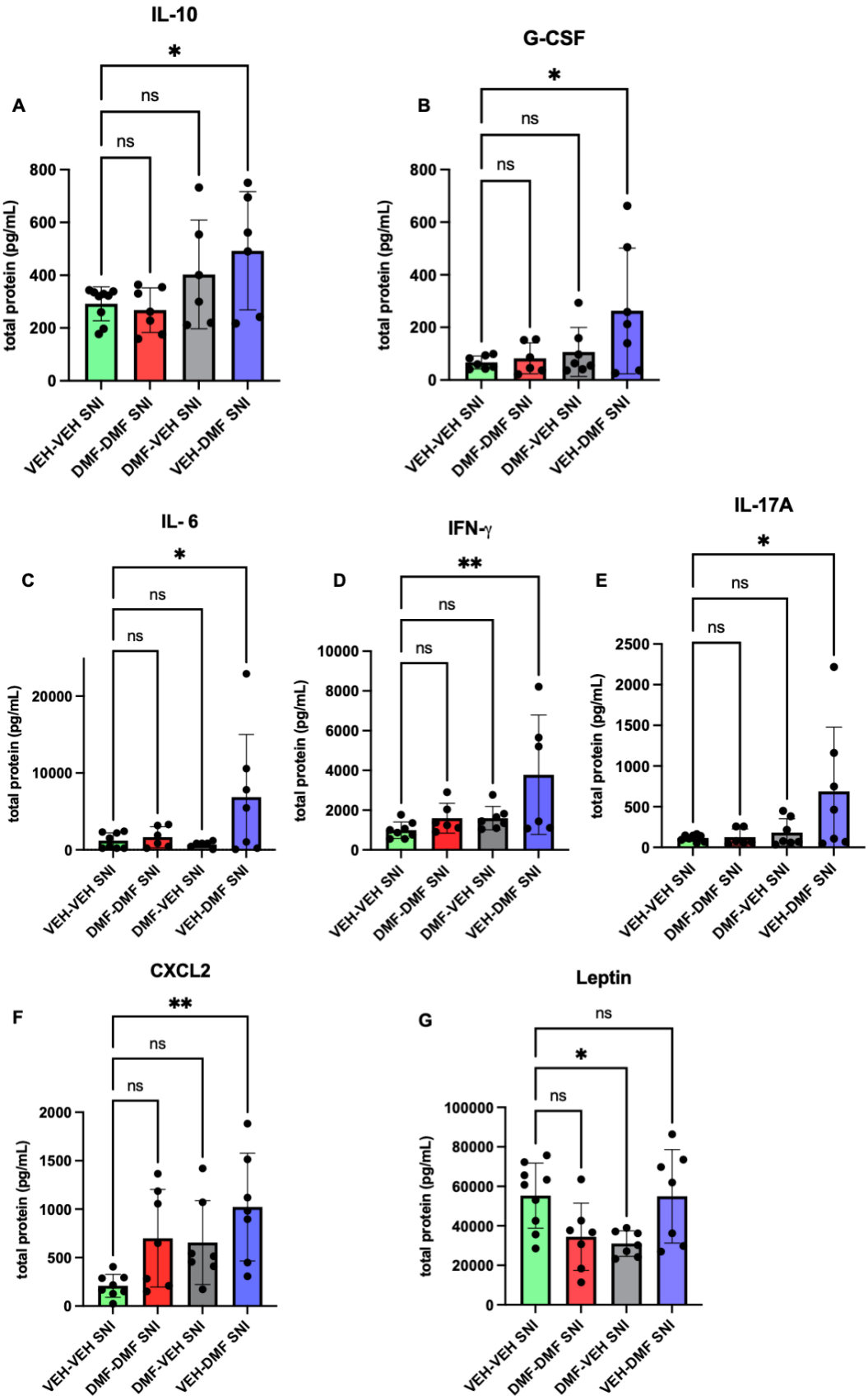

Figure 3. Effects of preemptive treatment with DMF on serum cytokines and chemokines. Inflammatory marker protein levels in serum were measured using the

Multiplex Bead-based Immunoassay. DMF treatment increased anti-inflammatory cytokines, including IL-10, G-CSF, and those with profound anti-inflammatory effects, such as IL-6, IFN-γ, IL-17A, and chemokine CXCL2. Leptin, a stimulator of pro-inflammatory markers, was found to be reduced. Samples were diluted 1:2. Data were analyzed using one-way ANOVA with Dunnett's multiple comparisons. Data are presented as mean ± SD**.** n=7-9 male rats per group. * p<0.05, ** p<0.01, *** p<0.001. ns: not significant.

3.1.1.3. Protective effect of DMF preemptive treatment is associated with the Nrf2 pathway

Following the first experiments, the pretreatment using male rats was selected for further analysis. This regime was preferred because it offers protection as early as the first week after surgery (Figure 2A). Moreover, it aligns with the pre-surgery phase, where at-risk individuals can be identified in clinical practice based on predisposing factors, including the basal immune state. In this experiment, trigonelline – an Nrf2 inhibitor – was used to examine the protective pathways of DMF, while the protein level of ATF-3 was assessed.

3.1.1.3.1. Nrf2 activation before injury is related to the protective effects of DMF against chronic pain development.

The baseline thresholds for mechanical and cold allodynia presented no differences prior to SNI. Neither DMF nor trigonelline influenced allodynia in the SHAM-operated rats compared to the control rats (Figure 4A-B). Pretreatment with DMF attenuated the severity of SNI-induced mechanical and cold allodynia over the course of 7 weeks after SNI. Noticeably, protection was abolished in the presence of trigonelline. In detail, in terms of mechanical allodynia, ANOVA repeated measures revealed that there were Main effects of Treatment [$F(7,66) = 759.775$, $p<0.001$], Treatment x Time [$F(35,330) = 8.210$, $p < 0.001$], and Time [$F(5,330) = 11.744$, $p < 0.001$]. Post hoc comparisons test indicated that DMF-Saline SNI vs VEH-Saline SNI $p <0.001$, while DMF-Tri SNI vs VEH-Saline SNI $p =1.000$ and VEH-Tri SNI vs VEH-Saline SNI $p = 1.000$ (Figure 4A). Regarding cold allodynia, there were Main effects of Treatment [$F(7,66) = 121.594$, $p<0.001$], Treatment x Time [$F(35,330) = 2.550$, $p < 0.001$], Time [$F(5,330) = 2.865$, $p = 0.015$]. Post hoc comparisons test showed DMF-Saline SNI vs VEH-Saline SNI $p <0.001$, DMF-Tri SNI vs VEH-Saline SNI $p =1.000$, and VEH-Tri SNI vs

VEH-Saline SNI p = 1.000 (Figure 4B). Additionally, we did not observe any impact of trigonelline on animal weights, DMF-Saline vs DMF-Tri (p>0.05), VEH-Saline vs VEH-Tri (p>0.05).

3.1.1.3.2. Nrf2 activation before injury is related to the protective effects of DMF against chronic pain comorbidities.

Anxiety-like behaviors were examined at week 5 after SNI using the time the animals spent in the light chamber of the Light-Dark box test and the time they spent in the open arm of the Elevated Plus Maze test as parameters. In the LDB, there were main effects of Surgery [F (1,60) = 29.98, p < 0.0001] and DMF vs VEH Treatment [F (1,60) = 4.029, p < 0.05], but no effect of Trigonelline vs Saline Treatment [F (1,60) = 0.9042, p > 0.05] (Figure 4C). Pairwise comparison of the main effects using an independent t-test with Bonferroni correction showed SNI-induced anxiety-like behaviors in rats [t (18) = 4.106, p < 0.01], which was attenuated by DMF treatment [t (14) = 0.4194, p > 0.05] (for the rationale of post hoc tests used, please see the materials and methods section). In the presence of Trigonelline, DMF failed to show its protective effects [t (14) = 0.9784, p > 0.05]. However, the inhibitor did not completely abolish the effect of DMF [t (15) = 2.824, p = 0.1024] (Please see Table S1 for details on statistical outputs).

A similar outcome was obtained in the EPM. Pretreatment with DMF was able to prevent anxiety-like behaviors developed in SNI rats at 5 weeks after injury. Trigonelline diminished, although not entirely, the effect of DMF. In detail, there were Main effects of Surgery [F (1,63) = 17.70, p < 0.0001], DMF vs VEH Treatment [F (1,63) = 4.904, p < 0.05], but no effect of Trigonelline vs Saline Treatment [F (1,63) = 0.4017, p > 0.05] (Figure 4D). For post hoc test outputs, please see Table S1. Interestingly, we found a positive correlation between mechanical allodynia measured on Day 3 after SNI and anxiety-like behaviors assessed on Day 35 with r = 0.5530, p < 0.001 (Figure 4E).

Next, at 6 weeks after SNI, cognitive dysfunction was assessed using the NOR test with a focus on the short-term memories of the animals. Here, we found the Main effects of Surgery [F (1,62) = 31.85, p < 0.0001], DMF vs VEH Treatment [F (1,62) = 13.71, p < 0.001], but no effect of Trigonelline vs Saline Treatment [F (1,62) = 0.8120, p > 0.05] (Figure 4F, Table S1). Post-hoc tests showed SNI rats developed cognitive dysfunction-like behaviors [t (18) = 3.443, p < 0.05], which was attenuated by DMF

treatment [t (14) = 0.0281, p > 0.05]. In the presence of Trigonelline, there was no significant difference between DMF-treated and VEH-treated SNI rats [t (15) = 1.784, p > 0.05], although the effect of DMF was not totally canceled by trigonelline [t (16) = 2.915, p = 0.0808].

Following the NOR test, SPT was conducted to examine depressive-like behaviors. Compared to the results obtained from 4 weeks after SNI, at this time point (6 weeks after SNI), there were no differences, either in terms of the sucrose preference or the sucrose consumed, between SHAM VEH Saline vs SNI VEH Saline (p > 0.05), which hindered our conclusions on the effects of Trigonelline (Figure S3A-B). However, when assessing the sucrose preference, there were Main effects of DMF vs VEH Treatment [F (1,65) = 5.776, p < 0.05], besides Surgery [F (1,65) = 4.003, p < 0.05], but no effect of Trigonelline vs Saline Treatment [F (1,65) = 0.6837, p > 0.05]. Meanwhile, regarding the amount of sucrose consumed per weight, there was also a Main effect of DMF vs VEH Treatment [F (1,65) = 8.675, p < 0.01], but no effect of Trigonelline vs Saline Treatment [F (1,65) = 0.2266, p > 0.05] nor Surgery [F (1,65) = 3.916, p > 0.05] (Figure S3A-B).

3.1.1.3.3. DMF pretreatment significantly reduced ATF-3 protein level

We examined the protein level of ATF-3, a neuronal injury marker, in the ipsilateral DRG. As a result, remarkably, the protective effects of DMF persisted at day 49 with ATF-3 level in DMF Saline treated being significantly lower than that in the control group (two-way ANOVA showed effects of DMF vs VEH [F (1,20) = 6.055, p=0.0231], Trigonelline vs Saline [F(1,20) = 0.0561, p=0.8153], Interaction [F (1,20) = 0.3622, p=0.5540], independent t-test with Bonferroni corrections [t (10) = 2.701, p < 0.05]) (Figure 4G). Trigonelline significantly canceled the effects of DMF (independent t-test with Bonferroni correction for DMF Trigonelline vs VEH Trigonelline [t (10) = 1.128, p > 0.05]). Additionally, ATF-3 protein level at day 3 was also measured. VEH Saline-treated rats exhibited significantly higher levels of the marker compared to DMF Saline (a one-way ANOVA [F (2,9) = 8.941, p < 0.01], with Bonferroni's post hoc test showing p < 0.05) (Figure S4A). Meanwhile, trigonelline could not completely abolish the effects of DMF (DMF Tri vs VEH Saline p < 0.05) at this time point. The protein levels of Nrf2 and Ho-1 at day 3 were measured, but no significant differences between treatments were found (Figure S4B-C).

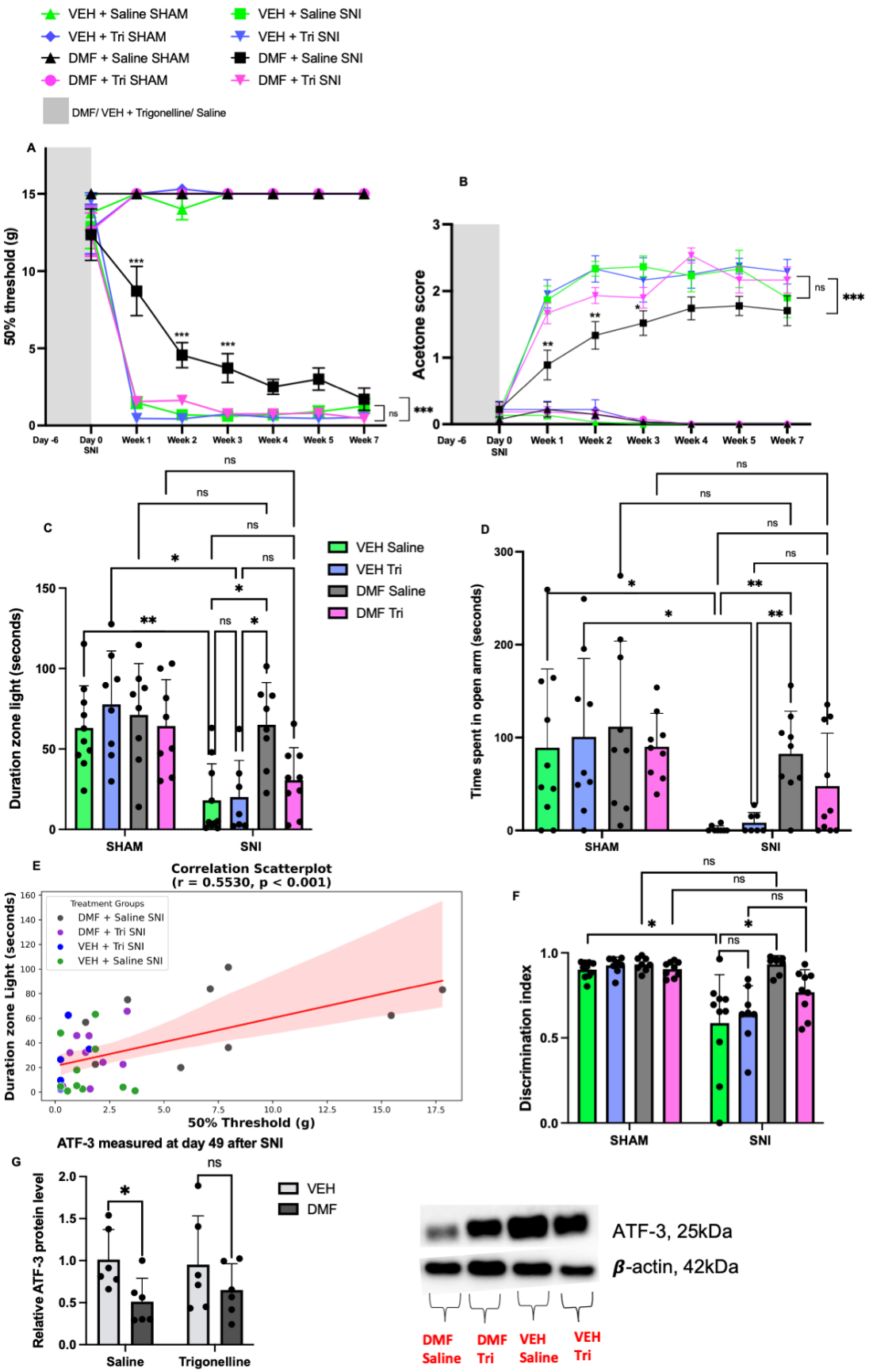
VEH + Saline SHAM
VEH + Tri SHAM
DMF + Saline SHAM
DMF + Tri SHAM
VEH + Saline SNI
VEH + Tri SNI
DMF + Saline SNI
DMF + Tri SNI
DMF/ VEH + Trigonelline/ Saline
A
50% threshold (g)
Day -6
Day 0 SNI
Week 1
Week 2
Week 3
Week 4
Week 5
Week 7
B
Acetone score
C
Duration zone light (seconds)
SHAM
SNI
D
Time spent in open arm (seconds)
E
Correlation Scatterplot
(r = 0.5530, p < 0.001)
Treatment Groups
Duration zone Light (seconds)
50% Threshold (g)
F
Discrimination index
G
ATF-3 measured at day 49 after SNI
Relative ATF-3 protein level
Saline
Trigonelline
ATF-3, 25kDa
β-actin, 42kDa
DMF Saline
DMF Tri
VEH Saline
VEH Tri

Figure 4. Effects of pretreatment with DMF on chronic pain and its comorbidities in the presence of Nrf2 inhibitor. (A, B) Mechanical and cold allodynia were assessed using the von Frey and acetone drop tests, respectively. **(C)** Time spent in the light zone in the LDB. **(D)** Time spent in the open arm in EPM was assessed at 5 weeks after SNI. **(E)** Correlation between mechanical allodynia measured at day 3 after SNI and anxiety-like behaviors evaluated at day 35. **(F)** Short-term memories measured by the NOR test were assessed 6 weeks after SNI. **(G)** DMF pretreatment significantly reduced ATF-3 protein levels. **(A-F):** n=8-10 male rats/group. Data was analyzed using ANOVA repeated measure and three-way ANOVA with Tukey's multiple comparisons, or independent t-test, or Mann-Whitney U-test with Bonferroni correction. **(G):** n = 6 samples/group. All tissue was ipsilateral DRG L4-6. Data was analyzed using two-way ANOVA with Bonferroni's post hoc test. All data are presented as mean ± SD except for (A) and (B), where data are mean ± SEM, *p<0.05, **p<0.01, *** p<0.001. Tri: Trigonelline, Sal: Saline. ns: not significant. (A, B) Brackets to the right of the plot indicate statistical differences between the two groups across the entire period.

3.1.2. Interim discussion

DMF is an immunomodulatory drug approved by the Food and Drug Administration for multiple sclerosis (MS) treatment (Gold et al., 2012). The drug impacts the immune system both on peripheral and central levels, skewing the immune response towards an anti-inflammatory phenotype (Ghoreschi et al., 2011; Peng et al., 2012; Yadav et al., 2019). For instance, treating with DMF for 3 months or more led to a significant reduction of circulating CD8(+), CD4(+) T cells, especially T-helper 1 (Th1) T-helper 17 (Th17), CD19(+) B cells, $CD56^{dim}$ NK cells and plasmacytoid dendritic cells in MS patients (Longbrake et al., 2016). These cells, in turn, release pro-inflammatory cytokines and chemokines such as IL-6, IL-1β, and TNF-α (Luckheeram et al., 2012; Seder and Ahmed, 2003). Among several mechanisms – see for review (Mills et al., 2018; Saidu et al., 2019; Yadav et al., 2019) - DMF acts via the Nrf2, a master regulator of anti-inflammatory and antioxidant signaling (Cuadrado et al., 2019; Grace et al., 2021; Wardyn et al., 2015). Not surprisingly, DMF proved effective in several chronic pain models (Casili et al., 2020; Gao et al., 2022; Li et al., 2020b; Singh et al., 2022) – See additional for review on Nrf2 inducers and chronic pain (Basu et al., 2022; Zhou

et al., 2021). Despite the accumulated knowledge, its potential for pain prevention has not yet been explored.

In the present study, we observed that DMF protected SNI-induced hypersensitivity, with the treated rats exhibiting a significantly higher threshold. Since groups were indifferent regarding thresholds before SNI, such protective effects could not be explained by the discrepancy in basal values. Notably, not only the continuous and early post-surgery treatments but also the pretreatment showed efficacy. This suggests the importance of the basal immune state, which, as demonstrated by others, can subsequently affect the acute immunity response, and potentially predispose individuals to severe diseases (Souquette and Thomas, 2024). Additionally, as DMF reaches its optimal effect against neuropathic pain after 5-7 days of administration (Li et al., 2020b), it is possible that at the time of injury, the drug (DMF-VEH) was at its peak efficacy in regulating the immune response, resulting in protection comparable to other treatments. Indeed, the analgesic effect was obtained even in a pretreatment approach (7 days before SNI) and persisted after the cessation of treatment for at least up to 7 weeks. Chronic pain is frequently associated with comorbidities, including anxiety, depression, and cognitive dysfunction (Bair et al., 2003; Flor et al., 1993; Kremer et al., 2021; Leite-Almeida et al., 2012; McWilliams et al., 2003); the current work is also the pioneering study to examine and show the protection of DMF pretreatment against those behaviors in male SNI rats. Examining cytokines and chemokines serum levels, we confirmed DMF's effects in modulating the immune response. The increase in important anti-inflammatory cytokines (de Oliveira et al., 2011; Hao et al., 2006; Moore et al., 1993; Vanderwall and Milligan, 2019), including IL-10, IL-4, C-GSF, IL-13, along with a reduction of leptin – a pro-inflammatory adipokine (Abella et al., 2017; Paz-Filho et al., 2012) – might explain the anti-allodynic effects of DMF. The treatment also induced IL-6, IFN-γ, and IL-17A production. While these cytokines are pleiotropic (Lee et al., 2017; Miller et al., 2015; Mills, 2022; Mühl and Pfeilschifter, 2003; Scheller et al., 2011), they have been reported as predominantly pro-inflammatory cytokines in peripheral nerve injury models (Kim and Moalem-Taylor, 2011; Lee et al., 2010; Robertson et al., 1997), although IFN-γ has recently gained attention for its protective properties in the neuropathy context (Ferrara et al., 2022). Findings on DMF's effects on these three cytokines remain inconsistent, with not just studies showing suppression (Guo et al., 2021; Zhang et al., 2024) but

also others showing induction that aligns with our results (Carlström et al., 2019; Lima et al., 2024). The cytokine profile observed in the present study, increased levels of both anti- and pro-inflammatory molecules, may reflect an active immune response induced by DMF. This could be because our treatment started before injury, in contrast to others that initiated treatment after allodynia and cytokine elevation had already developed. Cytokines play critical roles in acute inflammation and pain, and they are also widely reported as modulators of synaptic plasticity, an essential mechanism for central sensitization underlying chronic pain (J. L. Jankowsky and Patterson, 1999; Prieto and Cotman, 2017). Changes in these inflammatory markers, especially with a spike in those of anti-inflammatory effects around the injury time, by reducing neuroplasticity, may account for the later protection against chronic allodynia. Furthermore, the reduction in leptin levels observed with DMF-VEH even 7 days after the last DMF dose, along with the decrease seen with DMF-DMF (14 days of treatment), but not with VEH-DMF (only on the 7th day of DMF treatment), suggest that leptin reduction may be a delayed effect of DMF. This could explain the sustained anti-allodynic effects, as increased leptin levels are widely reported to be associated with pain (Kinfe et al., 2019; Nozaki et al., 2018; O'Brien et al., 2014; Younger et al., 2016). Regarding treatment comparison, our results indicated a stronger immune modulation in VEH-DMF compared to DMF-VEH at the examined time. This is expected, given that blood was collected immediately after the final DMF dose in VEH-DMF, whereas in DMF-VEH, it was taken 7 days later. Meanwhile, DMF-DMF with 14 days of treatment, starting from 6 days before SNI, did not produce the most profound response. While future studies are required to clarify this puzzling observation, we hypothesize that extended treatment may have activated self-regulation or compensatory mechanisms in the immune system, restoring the immune profile by the time of assessment (Ilan, 2020; Khoury and Ilan, 2019).

The effects of DMF, which resulted in a group of protected animals, also enable the analysis of early allodynia association with later anxiety-like behaviors. We found that pain hypersensitivity on day 3 can predict anxiety-like behaviors on day 35 after SNI. This is in accordance with evidence found in human studies, which indicates pain is one of the most consistent predictors of mental health problems (Bondesson et al., 2024; Eckhoff et al., 2017). Although hypersensitivity seems to reemerge slightly at around week 7 after SNI, in our opinion, this does not necessarily reflect the

appearance of chronic pain due to specific observations, including the protection against anxiety-, cognitive dysfunctions-like behaviors, and the reduction of the injury marker ATF-3. Regarding potential differences in the effects of pretreatment between male and female rats, these might be because allodynia in male rats depends on microglia involvement, while female rats use adaptive immune cells like T-lymphocytes (Sorge et al., 2015). Besides, the role of estrogen in influencing the immune response and modulating nociception could also be a contributing factor (Chen et al., 2021; Millas and Duarte Barros, 2021). Future studies incorporating detailed molecular analyses are necessary to elucidate the mechanisms and draw more comprehensive conclusions.

To further explore the protective mechanism of DMF, we used trigonelline, an Nrf2 inhibitor, to block its effects. Indeed, in the presence of trigonelline, DMF failed to express its protection against mechanical allodynia, cold allodynia, and maladaptive behaviors. This suggests that Nrf2 activation before injury appears to be associated with the observed DMF effects. Our findings align with previous studies indicating that activation of Nrf2 before injury can prevent later neuropathic pain development (Grace et al., 2016; S M Green-Fulgham et al., 2022). Nrf2 is normally located in the cytoplasm and translocated to the nucleus under stress conditions, where it activates the transcription of antioxidant genes such as *HMOX1* and superoxide dismutase (*SOD*) (Vomund et al., 2017). These downstream effectors regained cellular redox balance against reactive oxygen species, contributing significantly to chronic pain development (Basu et al., 2022). Nrf2 is also involved in the interplay with NF-κB, mediating the production of anti- and pro-inflammatory cytokines, which represents the cross-talk between oxidative stress and inflammation (Wardyn et al., 2015). Therefore, this transcription factor has emerged as a potential therapeutic target for attenuating neuroinflammation and oxidative stress-related conditions like chronic pain (Basu et al., 2022; Ferreira-Chamorro et al., 2018; Kim et al., 2010b; Wang and Wang, 2017; Yang et al., 2018). It is worth mentioning that trigonelline did not completely abolish the DMF's pre-treatment effects. In our opinion, this may suggest that there are other contributing factors aside from Nrf2. However, it cannot be ruled out that the trigonelline effect might have been limited by the dose and treatment scheme in our study (20 mg/kg; once a day), which was lower than that of other studies (300 mg/kg twice a day (Li et al., 2020b)).

ATF-3 is a neuronal injury marker immediately induced following the sciatic nerve cut and maintained the level over 10 weeks after axotomy (Tsujino et al., 2000). While preconditioning exercise could reduce DRG levels of ATF-3 at 14 days after surgery (Grace et al., 2016; S M Green-Fulgham et al., 2022), pretreatment with DMF in our study remarkably brought down ATF-3 even at 49 days following SNI. This finding supports the observed behavioral data, proving that pretreatment protection with an immunomodulator persisted. Furthermore, such an effect was abolished if the activation of Nrf2 before the injury was blocked. Since ATF-3 is also a negative regulator of the inflammatory response, which, by interacting with NF-κB, downregulates pro-inflammatory cytokine production such as IL-6, IL-1β, and TNF-α (Jadhav and Zhang, 2017), there might exist cross-talk between these two stress-response factors. It was reported that Nrf2 is a regulator of ATF-3 expression, and reversely, ATF-3 can mediate the Nrf2/Ho-1 pathway (Bi et al., 2022; Brown et al., 2008; Kha et al., 2019; Kim et al., 2010b; Rao et al., 2015; Wang et al., 2022). However, since the time points of treatment cessation and the resulting molecular changes in our study are distanced (49 days), Nrf2 activation may indirectly, by protecting the rats from stress induced by nerve injury, which in turn reduced ATF-3 expression, rather than directly influence the ATF-3 pathway. Further studies are required to elucidate this mechanism. Other preclinical studies focused on the spinal cord when examining the level of Nrf2 in rodents of pain models treated with DMF (Lee et al., 2021). Data on the supraspinal level, however, is still limited. Since the metabolite of DMF – monomethyl fumarate – can cross the blood-brain barrier, future investigations may examine this transcription factor in the brain.

In conclusion, our findings converge to support that pain susceptibility/resistance can be manipulated prior to or around its onset. The immune landscape during that window might set a course for pain trajectories and chronic pain comorbidities. In terms of DMF mechanisms, Nrf2 and its inflammatory downstream effectors, as well as ATF-3, play significant roles. However, the potential involvement of other factors cannot be dismissed.

3.2. Study 2

3.2.1. Results

3.2.1.1. Differences in pain complaints, as well as anxiety and depression magnitudes, between *NRF2* allele carriers

3.2.1.1.1. Pain complaints and *NRF2* polymorphisms

Mann-Whitney U test, as a non-parametric test, revealed significant differences in sum pain scores at the age of 16 to 17 between the allele groups of rs6721961 ($U(1) = 34052$, $p < 0.01$). The TG/TT genotype is associated with fewer pain complaints than the GG genotype (Table 3) (Figure 1).

Table 3. rs6721961 and sum pain score at age 16-17

Genotypes	Mean pain	SD	N
TG/TT	2.526	2.359	135
GG	3.479	3.466	589
SD: standard deviation; N: sample size			

Regarding rs35652124, the Kruskal-Wallis test showed no significant differences between the genotypes (Table 4).

Table 4. rs35652124 and sum pain score at age 16-17

Genotypes	Mean pain	SD	N
CC	3.507	3.696	75
CT	3.346	3.271	312
TT	3.214	3.258	337
SD: standard deviation; N: sample size			

Sex differences were found in the sum pain score ($U(1) = 74951$, $p < 0.001$), with male adolescents 2.747 ± 2.802, and female adolescents 3.721 ± 3.591. There was no significant association between sex and rs6721961 genotype distributions ($\chi^2 = 0.017$, $p>0.05$). For TG/TT, the ratio of male/female is 57/78, while the number for GG is 255/334.

3.2.1.1.2. Anxiety, depression, and *NRF2* polymorphisms

Mann-Whitney U test showed the TG/TT genotype of rs6721961 is associated with a lower magnitude of anxiety at the age of 14-15 than the GG genotype ($U(1) = 36769.5$, $p < 0.05$) (Table 5) (Figure 1). However, there were no significant differences regarding depression at this age ($U(1) = 39225$, $p > 0.05$) (Table 6).

Table 5. rs6721961 and anxiety at the age of 14-15

Genotypes	Mean anxiety	SD	N
TG/TT	1.156	0.471	135
GG	1.251	0.564	589
SD: standard deviation; N: sample size			

Table 6. rs6721961 and depression at the age of 14-15

Genotypes	Mean depression	SD	N
TG/TT	0.881	0.939	135
GG	0.939	1.022	589
SD: standard deviation; N: sample size			

As regards the SNP rs35652124, no differences in either anxiety or depression were found between alleles.

Additionally, sex differences were found in anxiety (U (1) = 76022, p < 0.001) and depression (U (1) = 77276.5, p < 0.001). For anxiety, male adolescents 1.090 ± 0.328, and female adolescents 1.342 ± 0.648. For depression, male adolescents 0.712 ± 0.822, and female adolescents 1.092 ± 1.100. Sex did not moderate the predictions of pain by either anxiety or depression (p>0.05).

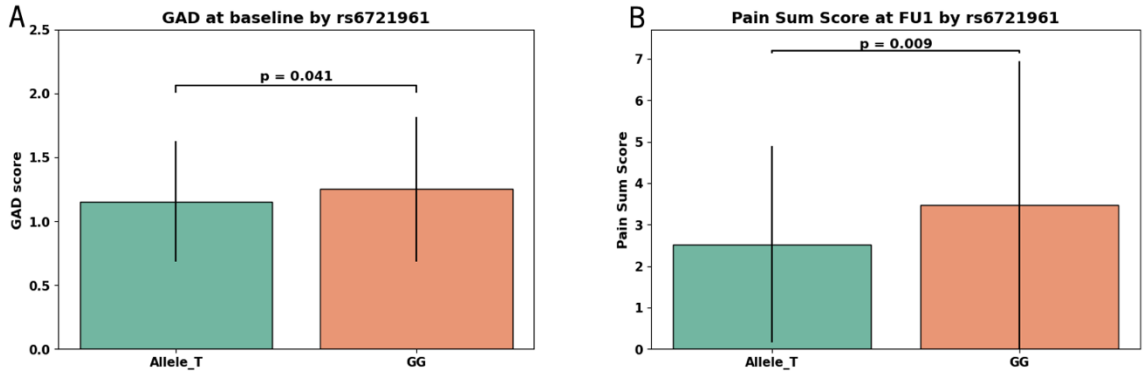

Figure 1. Pain complaints at age 16 and anxiety score at age 14 between rs6721961 *NRF2* variants. The TG/TT allele carriers presented **(A)** a lower anxiety score at age 14 and **(B)** fewer pain complaints at age 16 than those possessing the GG genotype. Mann-Whitney U tests were conducted. Data was shown as mean ± SD. Allele_T indicates TG/TT alleles.

3.2.1.2. Prediction of pain complaint magnitudes at 16 to 17 years by anxiety and depression at the age of 14 to 15 years

Regression analyses showed that pain complaint magnitudes at 16 to 17 years were significantly predicted by anxiety GAD (β = 0.255, 6.357% explained variance, $p<0.0001$), when gender was added as a covariate (β = 0.234, 7.04% explained variance, $p<0.0001$) at the age of 14 to 15 years, independent of rs6721961 and rs35652124.

Depression MDD was also a significant predictor of pain complaint magnitudes. The model outputs (β = 0.273, 7.316% explained variance, $p<0.0001$), and when adding gender as a covariate (β = 0.254, 8.121% explained variance, $p<0.0001$).

3.2.1.3. Prediction of pain complaints by anxiety and depression based on *NRF2* variations

Within carriers of GG of rs6721961, pain complaint magnitudes at 16 to 17 years were significantly predicted by anxiety (β = 0.245, 7.07% explained variance, $p<0.0001$). However, no significant association was found in TG/TT carriers ($p>0.05$) (Figure 2). Meanwhile, the prediction of pain complaints by depression was not affected by rs6721961 variants. Given no differences between alleles of rs35652124 in pain and anxiety, and depression, this SNP was not included in this analysis.

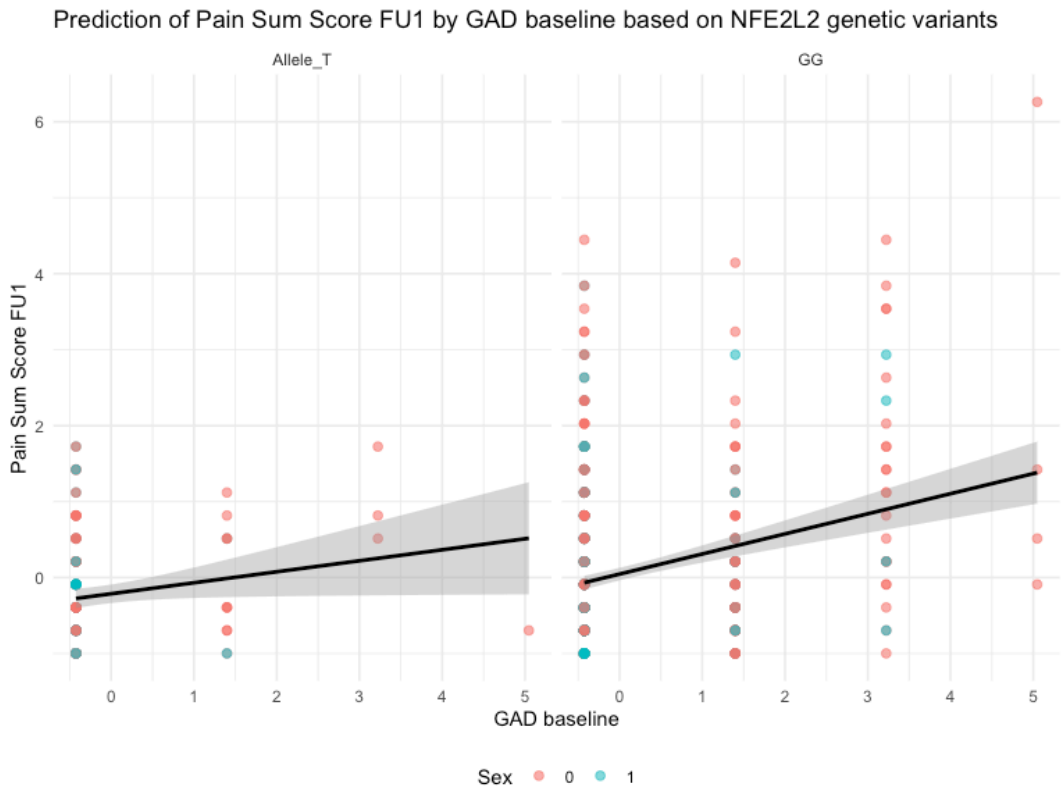

Figure 2. Prediction of pain at age 16 by anxiety GAD at age 14 depends on rs6721961 *NRF2* variants. Within carriers of GG of rs6721961, pain complaint magnitudes were significantly predicted by anxiety (β = 0.245, 7.07% explained variance, p<0.0001). However, no significant association was found in TG/TT carriers (p>0.05). GAD: Generalized Anxiety Disorder. Allele_T indicates TG/TT.

3.2.2. Interim discussion

In this study, we found that anxiety and depression at the age of 14 to 15 were strong predictors of pain complaints 2 years later, and the association was linked to specific SNPs of the transcriptional factor NRF2. Adolescence represents a sensitive developmental period that is vulnerable to developing psychological issues, which subsequently lead to future health problems (Fuhrmann et al., 2015; Steinberg, 2005). Our result aligns with findings from other population-based studies, which reported that adolescents with mental disorders, including anxiety and depression, had a higher risk of pain 2 years later, conducted in Sweden (Bondesson et al., 2024) and the UK (Andreucci et al., 2020). The relationship between early-in-life mental health problems and pain appears to be persistent over time, as it also exists in longer follow-up studies (Larsson et al., 2018; Murray et al., 2024). Additionally, literature shows that not only

is the link between mental health disorders and pain temporal, but it could also be bidirectional (Eckhoff et al., 2017; Noel et al., 2016). It is worth noting that while in most available studies, pain symptoms clinically diagnosed were used as an outcome, we employed sub-clinical symptoms but still found significant associations with anxiety and depression. This helps strengthen the conclusion on the high predictive value of affective symptoms with pain, an implication can be used in the context of chronic postsurgical pain prediction, where presurgical anxiety can be measured.

Even though anxiety and depression have been increasingly shown as determinants for pain symptoms, the underlying mechanisms remain unclear. We found contributions of the SNP rs6721961 *NRF2* variation to the prediction. Carriers of the GG allele showed a higher anxiety score at age 14 and more pain complaints at age 16 than those possessing the TG/TT genotype, while the association between early-in-life anxiety and pain later only exists within the GG carriers. NRF2 is a master regulator of antioxidant and anti-inflammatory pathways, which are both critically important in the defense mechanisms against stress, anxiety, and depression (Basu et al., 2022; Wardyn et al., 2015). Activation of NRF2 has been shown to produce persistent protection against the development of chronic pain and comorbid affect (S M Green-Fulgham et al., 2022; Le et al., 2025). It may be postulated that polymorphisms in *NRF2* change basal expression of *NRF2* or the ability of NRF2 to translocate to the nucleus (Yamaguchi et al., 2019). In adolescence, which is sensitive to changes, this may result in different capabilities of the human body in coping with negative affect, subsequently influencing the development of pain. Our findings may provide new dimensions in the understanding of the SNP rs6721961 *NRF2*. First and foremost, the present study is the first to link *NRF2* polymorphism with pain and anxiety. Second, while the TT allele of rs6721961 has been reported as the risk allele in other works (Marzec et al., 2007; Suzuki et al., 2013), we found that the combination of TG/TT alleles is the protective genotype. This may be because of differences in the mechanisms underlying pathogenesis, as other studies looked at lung disease while we investigated anxiety and pain development. It is worth noting that in conditions where anxiety and pain are more linked, such as Parkinson's disease, the TT allele is not a risk allele (Chen et al., 2013; von Otter et al., 2010). Protein expression analysis also revealed that the TG/TT genotypes are associated with a higher level of NRF2, compared to the GG allele (Yamaguchi et al., 2019). Our results indicate that although

both anxiety and depression were predictors of subsequent pain, the *NRF2* polymorphisms only affected the anxiety-pain relationship. However, whether this may imply a difference in the mechanisms, further works are needed to confirm. Regarding sex differences, we found that female adolescents presented more anxiety and depression, and later more pain complaints, but the association between those symptoms was not affected by sex. Since there was no significant association between sex and rs6721961 genotype distributions, we ruled out the possibility that sex differences reflect genotype differences. The sex differences found in this study align with previous findings (Hankin, 2009; Rutter et al., 2003), reinforcing their importance in mental health and pain research.

Our results revealed that anxiety and depression at age 14 are risk factors for increased pain complaints 2 years later. SNP rs6721961 *NRF2* polymorphisms, which affect anxiety and pain magnitudes, contribute to the prediction of pain by anxiety, but not by depression. Our findings might provide suggestions for susceptibility and resilience factors for pain, anxiety, and mood disorder development, and an important role of the immune system, which is also critical in the context of pain chronicity. Future studies can further elucidate these relationships by investigating the contribution of the neurodevelopmental status in key brain areas such as the ACC, insular cortex, amygdala, hippocampus, and nucleus accumbens, given their already known involvements in both affective and pain symptoms.

4. GENERAL DISCUSSION

4.1. Summary of findings

The heterogeneity in the development of chronic pain and associated comorbidities is an intriguing observation. Evidence indicates that this variability can be attributed to different culprits, including those of psychological, biological, and social natures (Fillingim et al., 2025; Sluka et al., 2023). As one of the major players contributing to mechanisms underlying pain pathogenesis, the immune system, particularly the transcriptional factor Nrf2, was assumed to be critically involved in determining susceptibility and resilience to chronic pain development. In the rat spared nerve injury model, we used dimethyl fumarate – an immunomodulator and Nrf2 inducer – to activate this factor around pain onset, then followed the development of pain and related comorbidities in the animals. What we found is that the preemptive treatment protected against long-term mechanical and cold allodynia, and against associated anxiety, depression, and cognitive dysfunction-like behaviors in SNI rats. Downstream cascade of activating Nrf2 induced by DMF was an increase in serum protein levels of different anti-inflammatory cytokines, including IL-10, G-CSF, IL-4, IL-13, those pleiotropic, such as IL-6, IFN-γ, and IL-17A, and the reduction of pro-inflammatory adipokine leptin. The sustained protection of pre-surgery Nrf2 activation was demonstrated not only on behavioral but also on molecular levels, where we found DMF treatment significantly reduced the neuronal injury marker ATF-3 in SNI rats even at post-surgery day 49.

After the study on animals, we attempted to test our assumption regarding the involvement of Nrf2, the immune system in general, in determining the susceptibility and resilience to pain development in humans. Leveraging data from a longitudinal adolescent cohort, we found that carriers of the GG allele of rs6721961 *NRF2* showed a higher anxiety score at age 14 and more pain complaints at age 16 than those possessing the TG/TT genotype. Pain complaints were predicted by early in life anxiety and depression, regardless of the polymorphisms. Among individuals with anxiety, only carriers of the GG presented a higher risk of developing future pain, which may indicate a susceptibility allele.

4.2. Interpretation of findings

The findings from this set of works have three main interpretations as well as clinical implications.

Firstly, basal immune profile plays an important role in determining susceptibility or resilience to the development of (chronic) pain and associated anxiety and mood disorders. Activating Nrf2 before injury resulted in subsequent changes in immune responses and increased rats' resilience to persistent pain-like behaviors, anxiety, depression, and cognitive dysfunction-like behaviors. The GG allele of rs6721961 *NRF2*, which is linked with lower Nrf2 protein expression (Yamaguchi et al., 2019), showed susceptibility to developing pain and anxiety symptoms in adolescents. Our findings on the role of the immune system as a predisposing risk factor are in agreement with a growing body of literature (Chidambaran et al., 2024; Gandhi et al., 2013; Klyne et al., 2018; Li et al., 2025; Stannus et al., 2013), emphasizing the significance of cytokines such as IL-10, IL-4, IL-13, IL-6, and leptin, which are downstream effectors of the Nrf2 inflammatory pathway.

Secondly, we showed that it is possible to modulate the susceptibility and resilience factors to alter pain trajectories. It took us more than 25 years since Celebrex (Celecoxib) (Clemett and Goa, 2000) to have a new analgesic for acute pain treatment in Journavx (Suzetrigine) (Oliver et al., 2025). Chronic pain management is even more challenging, as demonstrated by suzetrigine's failure in testing trials for chronic pain indications, often requiring a multi-modality approach incorporating exercise, psychotherapy, pain rehabilitation, and pharmacotherapies (Cohen et al., 2021; Finnerup et al., 2015; Shoji Yabuki et al., 2019). Targeted modification of risk factors may offer a great avenue for preventive strategies. Additionally, our findings might imply that early intervention on psychological difficulties such as anxiety and depression in adolescents is necessary in preventing future pain development, especially in individuals carrying the potential susceptibility alleles. However, it is worth mentioning that the effectiveness of psychologically based interventions has been inconsistent – see review (Fisher and de C Williams, 2025).

Finally, this project achieves one step further in the 'bench to bed' process by integrating experimental models and sub-clinical pain studies, bringing us closer to clinical applications. This is an encouraging sign given that translating basic pain

research from rodents to humans appears to be challenging (Burma et al., 2017; Hill, 2000; Mogil, 2009).

In addition to the three points above, given the lack of more SNPs in establishing polygenic scores for pain prediction (van Reij et al., 2020), the newly identified polymorphism associated with pain, rs6721961 *NRF2*, might be a promising candidate for that purpose. Furthermore, the potential interplay between the immune polymorphisms and anxiety in contributing to pain development found in this work validates the biopsychosocial model of pain (Meints and Edwards, 2018) and provides advances in understanding the mechanisms of chronic pain development.

4.3. Limitations and suggestions for future research

Our study on the rat pain model was limited to investigating the effects of activating Nrf2 at the peripheral level; its potential impacts on the central nervous system are, therefore, particularly appealing to examine, given that the treatment showed protection against affective comorbidities, and dimethyl fumarate metabolites can cross the blood-brain barrier (Litjens et al., 2004). The outcome of this will demonstrate the protective effects of DMF pretreatment at different levels and cement the foundation for advancing to clinical trials on humans. Although several cytokines were identified in our study, further research is needed to determine which exert the greatest influence. One approach can be using genetically modified mouse models.

Although our study demonstrates potential translational relevance, its impact could be strengthened through an experimental design that implements a corresponding pharmacological modulation, pretreatment with dimethyl fumarate, in individuals who are planned to have surgery. The fact that the FDA has approved dimethyl fumarate for treating multiple sclerosis is significant for its potential repurposing (Gold et al., 2012). Additionally, examining *NRF2* polymorphisms on a molecular level in participants and linking that with the risk of developing pain could further provide information for evaluating the possibility of clinical translation.

Lastly, pain symptoms in the longitudinal cohort study do not directly reflect chronic pain. However, as the aim of the present project is to identify susceptibility and resilience factors for chronic pain development, investigating young individuals who likely remain unaffected can be considered advantageous for this objective.

5. CONCLUSION

This thesis suggests that the immune system might be critically involved in predisposing the development of chronic pain and associated comorbidities. The transcriptional factor Nrf2 and its downstream cytokines were demonstrated as important contributors in both a preclinical model and in humans. Notably, these factors and the immune response more broadly could be modulated through pharmacological approaches. This is critical for preventive strategies, especially in scenarios where chronic pain is a potential outcome, including surgery, chemotherapy, and professional activities in hazardous environments. The results from this body of work advance current understanding of susceptibility and resilience to chronic pain and associated comorbidities. They might be considered as one piece of the broader puzzle of risk factors, which, perhaps when integrated as biopsychosocial markers, can yield strong predictive values and significant clinical utility (Fillingim et al., 2025).

6. SUMMARY

Susceptibility to develop chronic pain varies substantially across individuals. Evidence suggests that basal immune state is a key determinant of progression. The general aim of this thesis is to identify susceptibility/resilience factors for the development of chronic pain and associated comorbidities. Nrf2, a master regulator of both anti-inflammatory and antioxidant pathways, will be central to the analysis.

In the rat spared nerve injury (SNI) model, we used dimethyl fumarate (DMF) to activate Nrf2 around pain onset. DMF treatment increased rats' resilience to persistent allodynia, associated anxiety, depression, and cognitive dysfunction-like behaviors. The treatment altered immune response, increasing serum protein levels of anti-inflammatory and pleiotropic cytokines, and reducing pro-inflammatory adipokine leptin. The neuronal injury marker ATF-3 was also reduced by DMF, even on day 49 post-SNI. Nrf2 inhibition with trigonelline abolished DMF effects. In humans, we examined the influence of *NRF2* genetic variations in a longitudinal adolescent cohort. Carriers of the GG allele of rs6721961 *NRF2* showed a higher anxiety score at age 14 and more pain complaints at age 16 than TG/TT carriers. Among individuals with anxiety, only carriers of GG presented a higher risk of developing future pain, potentially indicating a susceptibility allele. Meanwhile, pain complaints were predicted by anxiety and depression, regardless of the polymorphisms.

In conclusion, this thesis suggests the involvement of transcriptional factor Nrf2 in determining susceptibility and resilience to chronic pain (and related comorbidities) development in both a preclinical model and in humans. These findings are not only essential for the development of preventive strategies but also critical for our understanding of the mechanisms underlying chronic pain.

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8. APPENDIX

Supplemental material study 1

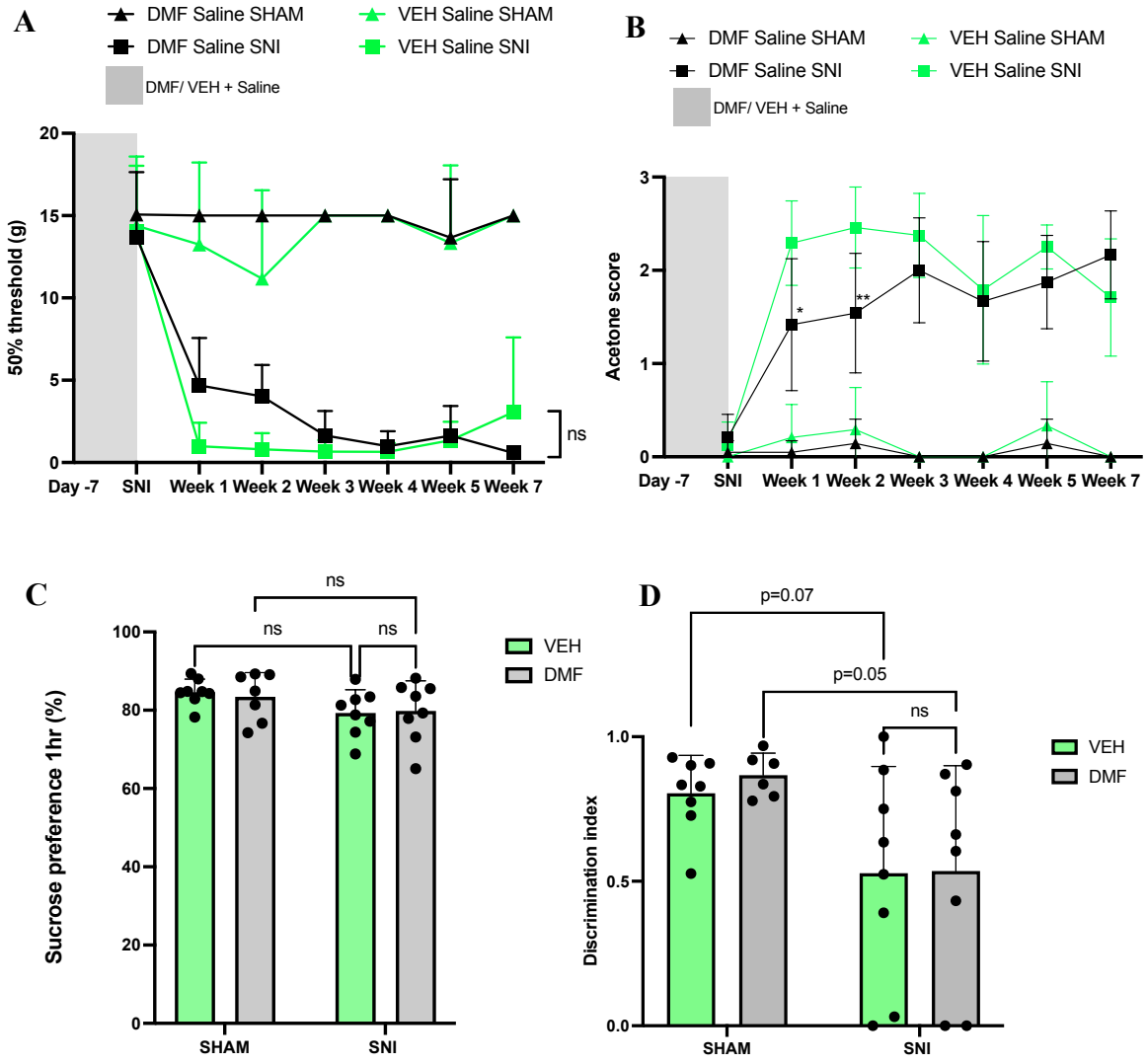


Figure S1. Pretreatment with DMF showed limited effects against allodynia and no effects against relevant comorbidities in female SNI rats. (A, B) Mechanical and cold allodynia were assessed using the von Frey and acetone drop tests. **(C)** Effects of DMF pretreatment on depressive-like behaviors were evaluated using the sucrose preference test. **(D)** Short-term memories were measured by the novel object recognition. Data was analyzed using ANOVA repeated measures and two-way ANOVA with Tukey's multiple comparisons. Data are presented as mean ± SD. n=8 female rats per group. ns: not significant.

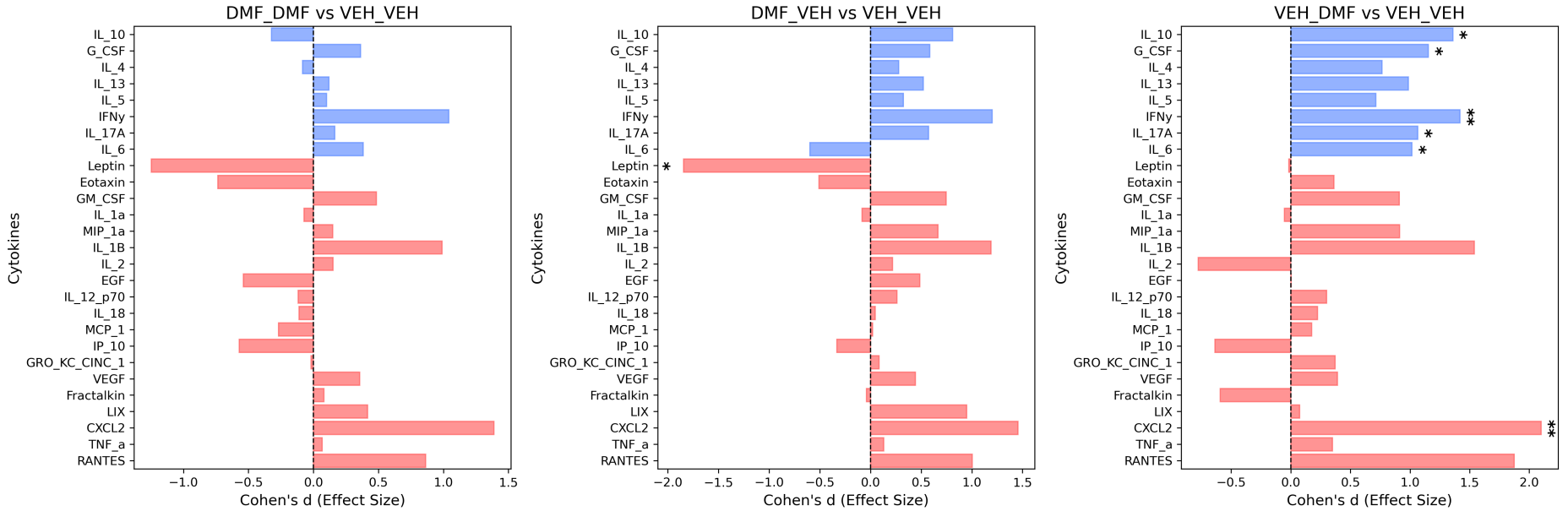


Figure S2. Cytokine/chemokine multiplex assay analyzed the protein levels of 27 cytokines/chemokines. Each panel represents the effect sizes of each DMF treatment compared to the VEH-VEH group, with the effect sizes being calculated using Cohen's *d* statistic. The blue color reflects anti-inflammatory and pleiotropic cytokines, while the red reflects chemokines and pro-inflammatory cytokines. Data was analyzed using one-way ANOVA with Dunnett's multiple comparisons. n=7-9 male rats per group. G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte macrophage colony-stimulating factor; GRO/KC: growth-regulated oncogene/keratinocyte chemoattractant; IFN: interferon; IL: interleukin; MIP: macrophage-inflammatory protein; RANTES: regulated upon activation normal T-cell expressed and secreted; VEGF: vascular endothelial growth factor; TNF: tumor necrosis factor; MCP: monocyte chemoattractant protein; IP-10: interferon-γ-inducible protein 10; LIX: lipopolysaccharide-induced CXC chemokine.

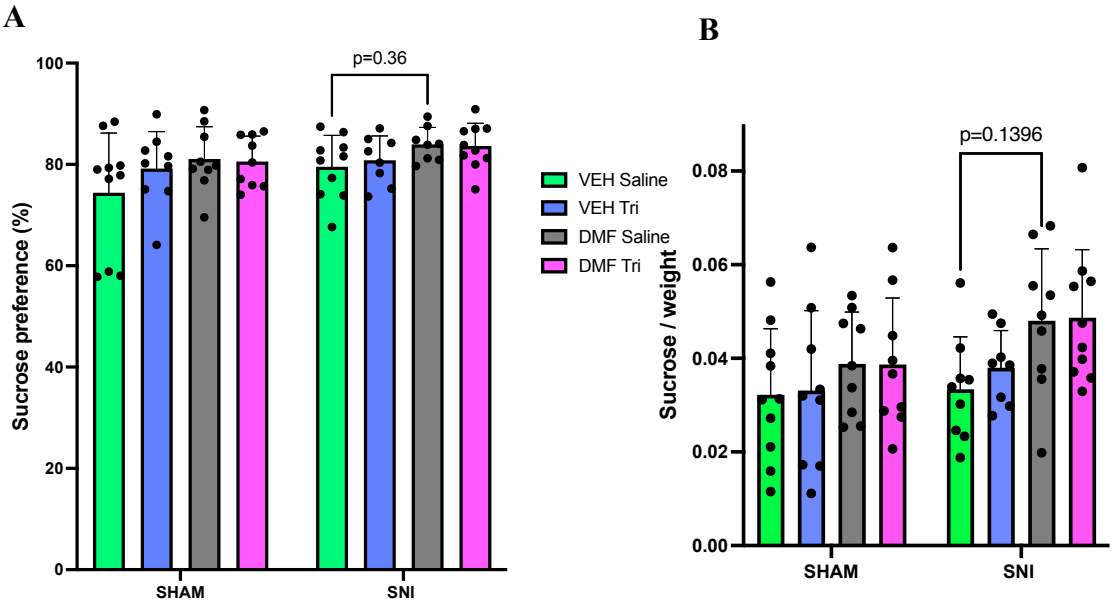
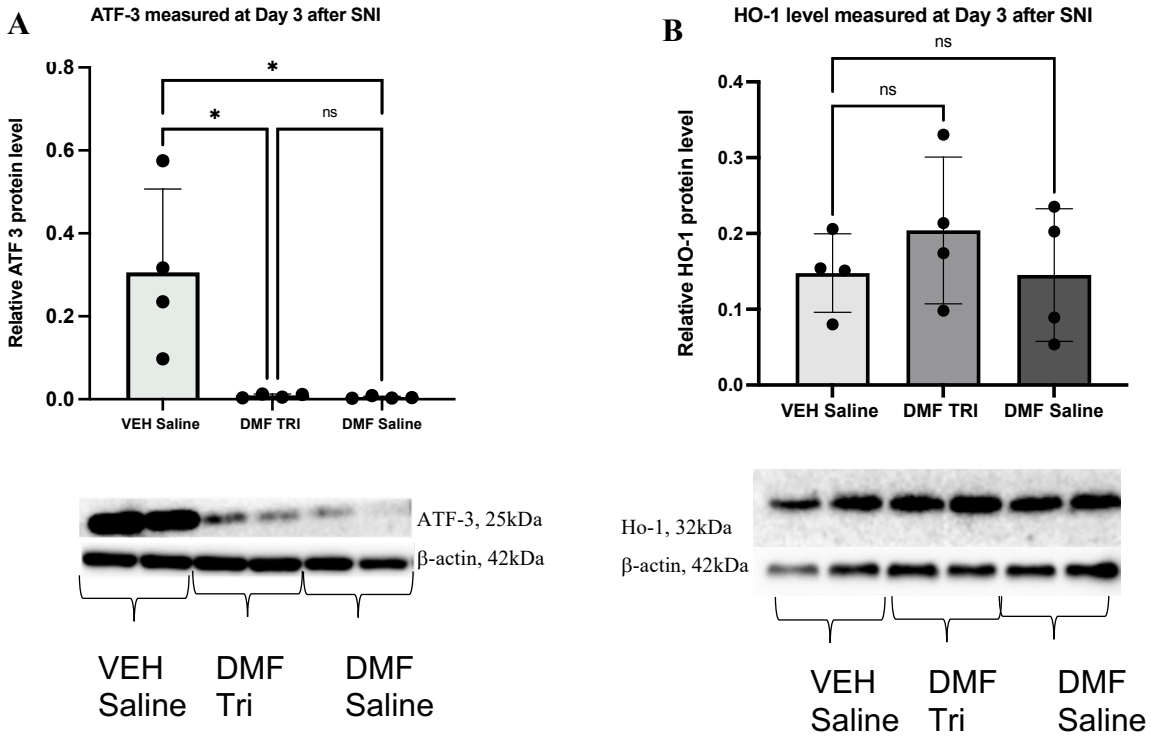


Figure S3. Effects of pretreatment with DMF on depressive-like behaviors in the presence of Nrf2 inhibitor in male rats. The SPT test was assessed at 6 weeks after SNI. The control animal, VEH Saline, expressed no sign of depressive-like behaviors. However, there were trends that DMF-treated rats exhibited less anhedonia and consumed higher sucrose per weight than VEH-treated rats. n=8-10 male rats/group. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001. ns: not significant.



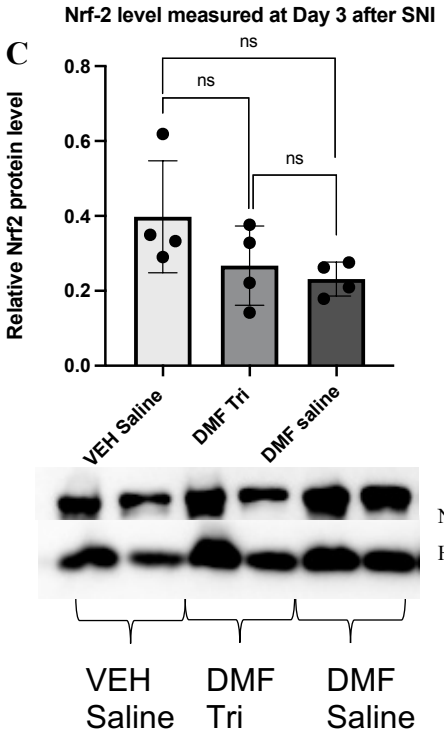
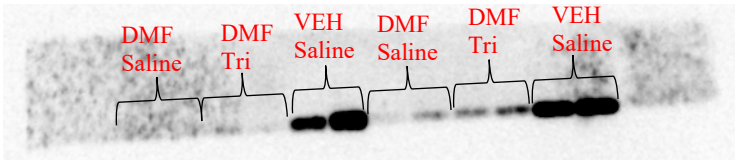
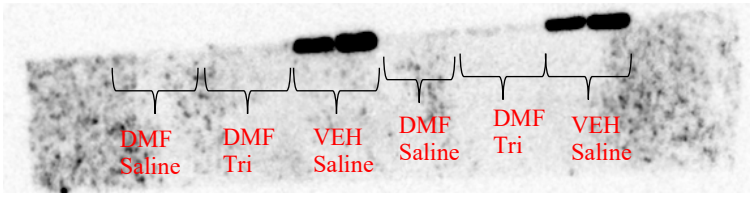
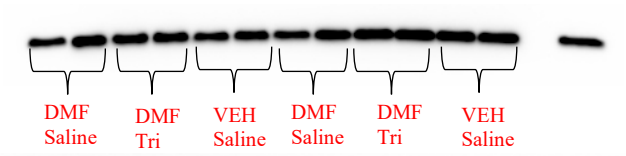
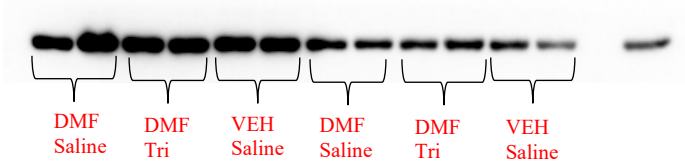
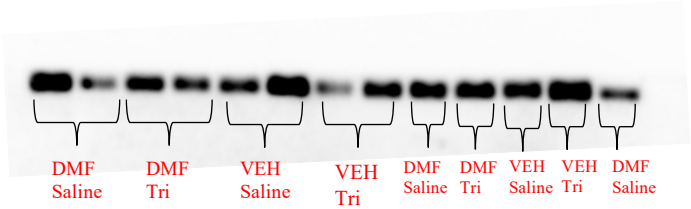
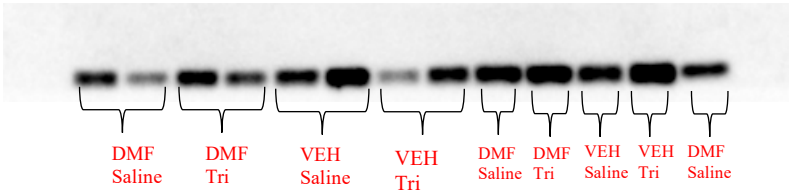
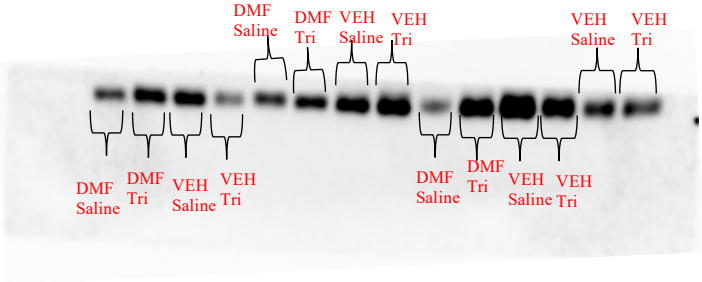
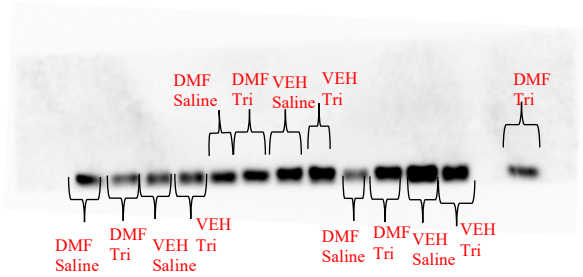
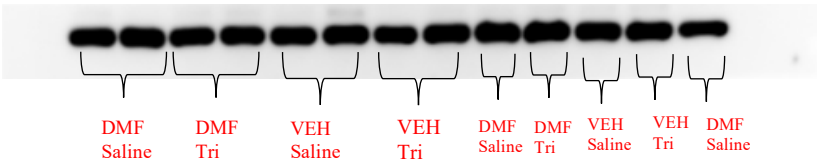
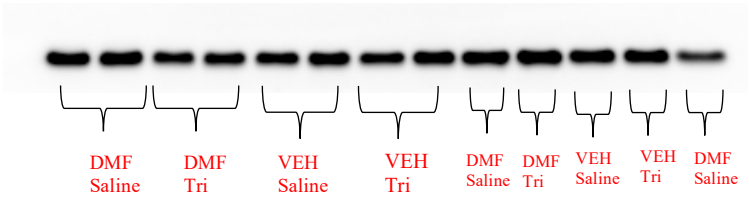
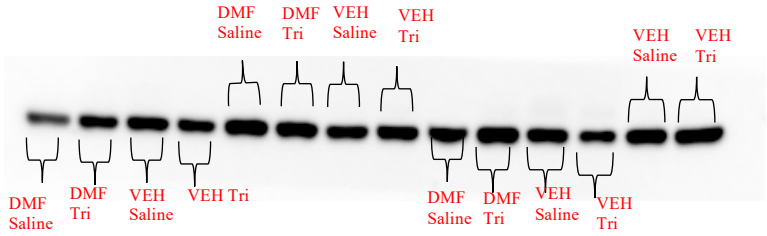
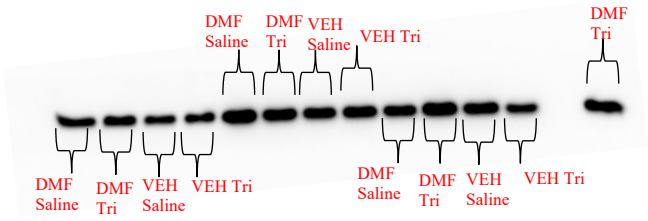
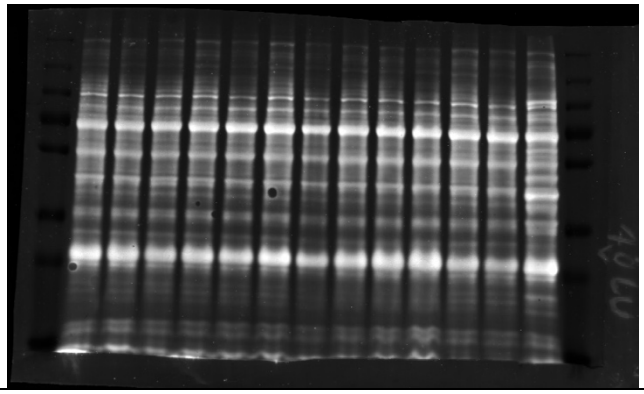
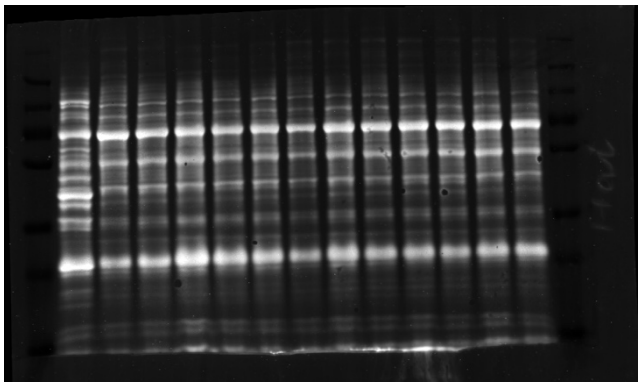
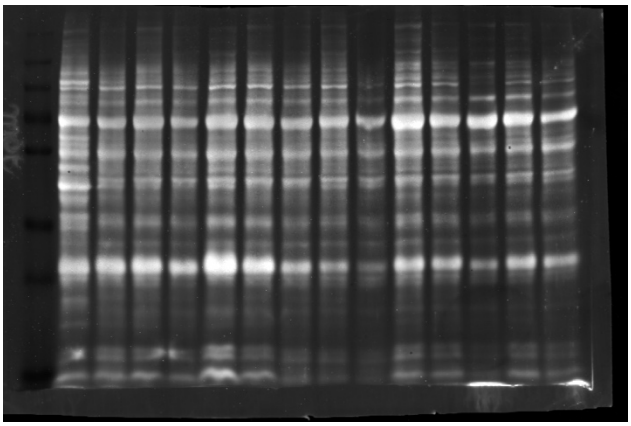
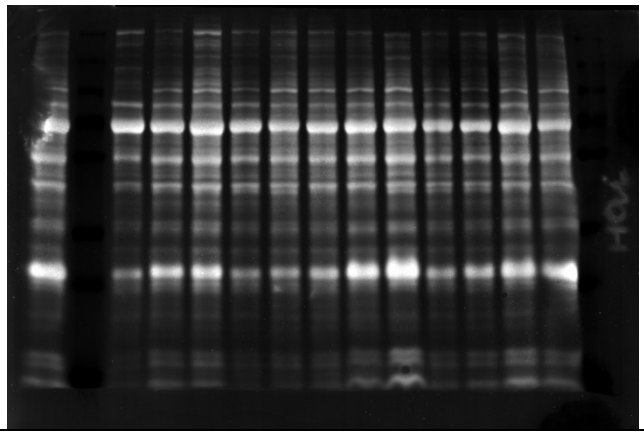
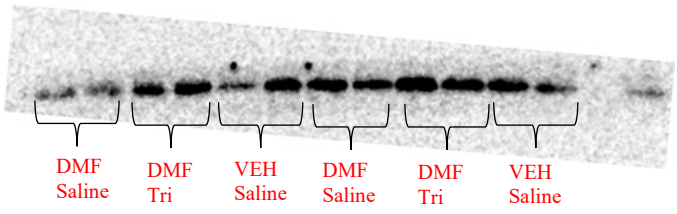
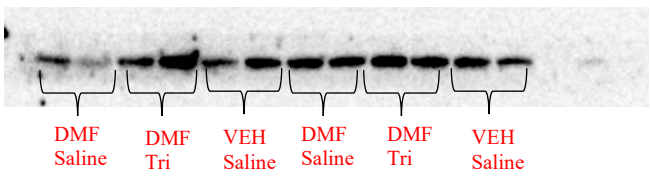


Figure S4. DMF pretreatment significantly reduced ATF-3 protein levels. **(A)** Quantification of ATF-3 protein level, **(B)** Nrf-2 nuclear fraction, and **(C)** Ho-1 protein level at day 3 after SNI/ treatment cessation. All tissue was ipsilateral DRG L4-6. Data was analyzed using one-way ANOVA with Tukey as post hoc test. Data are presented as mean ± SD. *p<0.05. Tri: Trigonelline, Sal: Saline. n = 4-6 samples/group. ns: not significant.

Uncropped Blots

ATF-3 at **day 3**	M1	
	M2	
β-actin at **day 3**	M1	
	M2	
ATF-3 at **day 49**	M1	
	M2	
	M3	

	M4	
β-actin at **day 49**	M1	
	M2	
	M3	
	M4	
Total Stain Q **at day 49**	M1	

	M2  M3  M4 
Ho-1 at **day 3**	M1  M2 

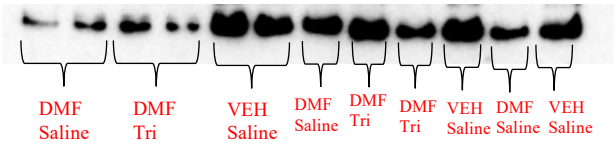
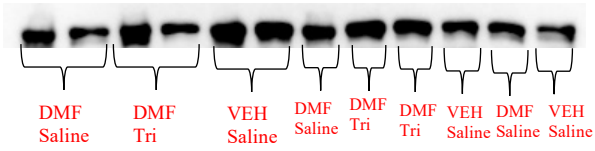
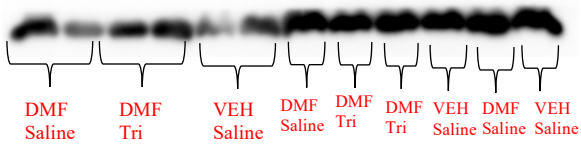
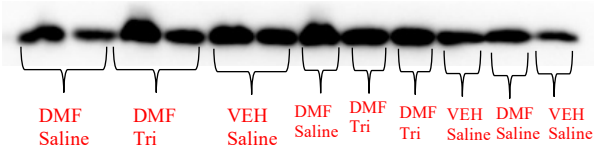
Nrf2 at **day 3**	M1  M2 
Histone H3 at **day 3**	M1  M2 

Table S1. Statistical outputs of behavioral tests

	Three-way ANOVA		Post hoc tests (independent t-test or Mann-Whitney U-test with Bonferroni correction)				
			VEH Saline SHAM vs VEH Saline SNI	**DMF Saline SHAM vs DMF Saline SNI**	**SNI** **DMF Saline vs VEH Saline**	**SNI** **DMF Tri vs VEH Tri**	**DMF Tri SHAM vs DMF Tri SNI**
LDB	Surgery x DMF vs VEH	F (1,60) = 5.81, p < 0.05	t (18) = 4.106, p < 0.01	t (14) = 0.4194, p > 0.05	t (16) = 4.082, p < 0.05	t (14) = 0.9784, p > 0.05	t (15) = 2.824, p = 0.1024
	DMF vs VEH x Trigonelline vs Saline	F (1,60) = 4.980, p < 0.05	63.07 ± 26.19 vs 18.15 ± 22.60	71.23 ± 31.86 vs 65.11 ± 26.22	65.11 ± 26.22 vs 18.15 ± 22.60	30.62 ± 20.18 vs 20.12 ± 22.67	64.34 ± 28.78 vs 30.62 ± 20.18
	Surgery x Trigonelline vs Saline	F (1,60) = 2.403, p > 0.05					
	Surgery x DMF vs VEH x Trigonelline vs Saline	F (1,60) = 0.3281, p > 0.05					
EPM	Surgery x DMF vs VEH	F (1,63) = 3.261, p > 0.05	t (16) = 2.892, p < 0.05	t (16) = 0.8546, p > 0.05	t (15) = 4.933, p < 0.01	U = 22, p > 0.05	t (17) = 1.916, p > 0.05
	DMF vs VEH x Trigonelline vs Saline	F (1,63) = 1.560, p > 0.05	88.97 ± 84.76 vs 1.714 ± 3.294	111.8 ± 92.13 vs 82.44 ± 46.01	82.44 ± 46.01 vs 1.714 ± 3.294	47.75 ± 56.90 vs 8.314 ± 11.12	90.18 ± 35.97 vs 47.75 ± 56.90

	Surgery x Trigonelline vs Saline	F (1,63) = 0.0936, p > 0.05					
	Surgery x DMF vs VEH x Trigonelline vs Saline	F (1,63) = 0.0175, p > 0.05					
NOR	Surgery x DMF vs VEH	F (1,62) = 12.77, p < 0.001	t (18) = 3.443, p < 0.05	t (14) = 0.0281, p > 0.05	t (16) = 3.371, p < 0.05	t (15) = 1.784, p > 0.05	t (16) = 2.915, p = 0.0808
	DMF vs VEH x Trigonelline vs Saline	F (1,62) = 4.142, p < 0.05	0.9014 ± 0.0475 vs 0.5872 ± 0.2846	0.9321 ± 0.0395 vs 0.9328 ± 0.0509	0.9328 ± 0.0509 vs 0.5872 ± 0.2846	0.7680 ± 0.1332 vs 0.6364 ± 0.1706	0.9328 ± 0.0509 vs 0.5872 ± 0.2846
	Surgery x Trigonelline vs Saline	F (1,62) = 0.7413, p > 0.05					
	Surgery x DMF vs VEH x Trigonelline vs Saline	F (1,62) = 1.509, p > 0.05					
SPT			t (18) = 1.217, p > 0.05				
			74.40 ± 11.79 vs 79.53 ± 6.220				

Note: LD: Light-Dark Box; EPM: Elevated Plus Maze; NOR: Novel Object Recognition; SPT: Sucrose Preference Test; DMF: Dimethyl fumarate; VEH: Vehicle; Tri: Trigonelline; SNI: spared nerve injury.

9. CURRICULUM VITAE

PERSONAL INFORMATION

Name and First name:	LE, CONG TUAN ANH
Date of birth:	10/10/1993
Birthplace:	Quang Tri, Vietnam

SCHOOL

2008 – 2011	Gio Linh High School, Quang Tri, Vietnam
Exam and diploma completion	October 1, 2011 - Abitur

UNIVERSITY

9/2011 – 7/2016	Hue University of Medicine and Pharmacy (Viet Nam). Major: Bachelor's in pharmacy, GPA: 8.26/10 (Top 5% of the class of 150)
9/2019 – 12/2020	National University of Ireland, Galway (Ireland) M.Sc. in Neuropharmacology First Class Honors. Highest-mark student
5/2022 – now	Ph.D candidate – MSCA-ITN fellow. European Joint Doctorate: University of Minho (Portugal) and University of Heidelberg (Germany). Website: https://happyejd.com/

WORKING EXPERIENCE

8/2016 – 8/2019	Danang University (Viet Nam) School of Medicine and Pharmacy Department of Pharmacology Role: lecturer and research assistant

ACADEMIC ACHIEVEMENTS

- Marie Skłodowska-Curie Actions PhD fellowship
- Irish Government's Irish Aid IDEAS Scholarship to undertake the MSc in Neuropharmacology at NUI Galway (Ireland) in 2019
- Pharmacology Prize for First Place in MSc. in Neuropharmacology
- Connor Prize for Best Research Project in MSc. in Neuropharmacology
- A scholarship of training at Ludwig Maximilians–Universitat Munchen, Germany
- Graduated in the top 5% of the class of 150 students at the undergraduate level

PUBLICATIONS

- Le, A. C. T., Sousa, Â., Alves, N. D., & Leite-Almeida, H. (2024). Preemptive minocycline decreases allodynia and depressive-like behaviors in a peripheral neuropathy rat model: A preliminary study. *bioRxiv*, 2024.10.23.619782. https://doi.org/10.1101/2024.10.23.619782
- Le, A. C. T *et al.* Pretreatment with dimethyl fumarate prevents chronic pain and its comorbidities via Nrf2 pathway in a rat model of neuropathic pain. *Brain. Behav. Immun.* 128, 725–736. https://doi.org/10.1016/j.bbi.2025.05.003

CONFERENCE PRESENTATIONS

- Speaker at European Pain Federation EFIC 2025. ‘’*Make it HaPpY: Multidisciplinary Databliz on Chronic Pain Comorbidities and Sex Differences by Early Career Researchers’’*
- Speaker at International Symposium on Paediatric Pain ISPP 2025. ‘*’Unraveling the Complexities of Pain and its Comorbidities from Adolescence to Early Adulthood: Insights from the IMAGEN Longitudinal Study’’*
- Poster presentation ‘*Pretreatment with dimethyl fumarate prevented chronic pain and its comorbidities in rat model of neuropathic pain*’ at IASP 2024 World Congress on Pain
- Poster presentation ‘*Susceptibility and resilience factors related to the immune system for chronic pain and its comorbidities: evidence from human and animal studies*’ at European Pain Federation EFIC 2025

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