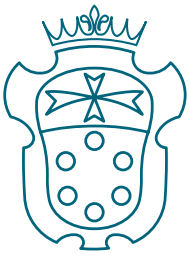

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Doctoral program in Neuroscience

XXXIII cycle

Understanding Hereditary Sensory and Autonomic Neuropathy type IV through a novel knock-in mouse model

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Abstract

The Nerve Growth Factor (NGF) and its high-affinity tyrosine kinase receptor TrkA play a key role in pain sensation. Indeed, a functional NGF-TrkA system is an essential requisite for the generation and maintenance of long-lasting thermal and mechanical hyperalgesia in adult mammals. Mutations in the gene encoding for TrkA are responsible for a rare condition, named Hereditary Sensory and Autonomic Neuropathy type IV (HSAN IV), characterized by the loss of response to noxious stimuli, sweating defects and cognitive impairment. However, to date, there is no available mouse model to properly understand how the NGF-TrkA system can lead to pathological phenotypes that are distinctive of HSAN IV.

Since the diversity of HSAN IV TrkA-related mutations determines variable degrees of clinical phenotype and intellectual disabilities in affected individuals, we have decided to deeply investigate the missense Arg649Trp (R649W) mutation, located in the intracellular tyrosine kinase domain of TrkA receptor and known to induce a diminished kinase activity and reduced phosphorylation after NGF stimulation in transfected cells. First, by *in vitro* biochemical and biophysical analyses, I showed that the pathological R649W mutation leads to kinase-inactive TrkA, reducing the constitutive ubiquitination and also affecting the membrane dynamics and trafficking. Then, after the generation of the knock-in mouse line carrying the HSAN IV TrkA^{R649W} mutation, I demonstrated that TrkA$^{R649W/m}$ mice displayed a lower response to thermal and chemical noxious stimuli, correlating with reduced skin innervation and altered expression of nociceptive markers in Dorsal Root Ganglia (DRGs).

By performing a sweat assay, I also found that the pathological TrkA^{R649W} mutation causes sweating deficits in HSAN IV TrkA$^{R649W/m}$ mice compared to TrkA$^{h/m}$ controls. Moreover, the R649W mutation decreased anxiety-like behavior and compromised cognitive abilities, by impairing spatial-working and social memory. In addition, the results obtained in this thesis uncovered unexplored roles of TrkA in thermoregulation and sociability.

By exploiting suitable control animal models such as HSAN V $NGF^{R100W/m}$ and $TrkA^{+/-}$ mice, I demonstrated that HSAN IV $TrkA^{R649W/m}$ mice mimic the clinical phenotype of HSAN IV patients and they can be considered a suitable experimental platform to explain the clinical aspect of HSAN IV disease, also offering promising new routes for testing future therapies.

TABLE OF CONTENTS

1. Introduction

1. Pain and Nociception: A Historical Perspective

In ancient cultures, it was believed that nobody, except the gods, can live without any pain. For instance, Egyptians assumed that pain was caused by the religious influences of their gods. From an etymological point of view, "pain" comes from the Latin word of "poena" that, in turn, comes from Poinḗ (ποινή), the Greek spirit (daimona) of punishment and penalty for the crime of murder and manslaughter. Indeed, the ancient Greek Hippocrates, father of Western medicine, believed that pain was caused by evil spirits that unbalanced the vital fluids in the body, called four humors: blood, phlegm, yellow and black bile. This mystic sight of pain has been anchored in human beings until the Renaissance when the French philosopher René Descartes suggested a novel concept of pain. In his *Traité de l'Homme (1964)*, Descartes proposed that a movement or touch, initiated at the periphery, propagated to the brain producing a representation of the stimulus. The mechanical view of Descartes described how a noxious stimulus, such as fire, excites the sensory nerve endings in the foot and then is transmitted, as an alarm, to the brain triggering the unpleasant sensation called "pain". The nerves in the skin were considered "tubes" containing a marrow made of fine threads that transmit sensory stimuli (Procacci & Maresca, 1994). The description of the involuntary withdrawal of a foot that makes contact with a noxious stimulus was the beginning of the development of modern physiology (**Figure 1**), influencing the study and the treatment of pain for more than 300 years. Beyond the mechanical description of pain sensation, in his private correspondence, Descartes tackled a more "conscious" concept of pain": *"I once knew a girl who had a serious wound in her hands and had her whole arm amputated because of creeping gangrene. [...] She complained of feeling pain in her fingers, wrist and forearm; and this was obviously due to the condition of the nerves in her arm which formerly led from her brain to those parts of her body [...]"*(Cottingham, John et al., 1985). Descartes observed that this condition may be caused by a type of pain triggered by the stimulation of the

remaining nerves. Then, this sensory information was transmitted to the brain but not always it was interpreted correctly (Finger et al., 2003). Thus, Descartes first hypothesized that some pain sensations, caused by injuries or trauma, are a misinterpretation of sensory information in the brain. Later, the doctor Silas Weir Mitchell, providing assistance to injured soldiers on the battlefields during the American Civil War, noted that amputated soldiers experienced painful complications after surgery. The great mass of American soldiers, who had undergone amputations, were still able to feel pain or itch in their lost limb (Finger et al., 2003; Mitchell et al., 2007). This disorder, predicted by Descartes, was for the first time described with the term "Phantom Limb". In addition to the itchy sensation, a discrete percentage of soldiers with wounds and injuries suffered from a pain described as a "burning", which usually occurs distal to the site of injury and is associated with red or mottled skin areas (Klifto & Dellon, 2021). Even if the understanding of pain sensation was becoming denser, the nature of the sensory nerves in the skin remained still unclear. However, at the end of the 19th century, it was evident to scientists that the skin can be stimulated by different stimuli (Perl, 2007). Silas Weir Mitchell tested the ability of patients to react to mechanical stimuli within a wide range of intensity, from painful stimulus using a pin, to a gentle touch using the tip of a single hair (Louis & York, 2006). Another pioneering work was made by the Austrian-German physiologist Maximilian von Frey that developed an easy, reproducible and currently used method to stimulate the skin using "von Frey filaments". The skin, our largest sensory organ, is a mosaic of sensory spots. By employing thin and sharp fibers, it is possible to produce a painful sensation without touch or a soft pressure sensation without pain (Handwerker, 2014). These findings clarified that sensitive fibers over the skin responded specifically to different types of stimuli. In the meantime, based on physiological experiments, Sir Charles Sherrington suggested "[...] *There is considerable evidence that the skin is provided with a set of nerve endings whose specific office it is to be amenable to stimuli that do the skin injury, stimuli that in continuing to act would injure it still further*'', and, a few years later, called these fibers "nociceptors" (Sherrington CS, 1906). With the discovery of nociceptors, a new term was coined by Sherrington: *nociception* which refers to a physiological process and differs from *pain*

which is instead considered a personal experience that emerges somewhere in the brain (Prescott et al., 2014).

The universality of nociception appears to be one of the most interesting aspects of pain. Indeed, many diverse animals possess nociceptors, for instance, invertebrates, such as Drosophila melanogaster and Caenorhabditis Elegans, and many non-mammalian vertebrates including birds, reptiles, amphibians and fish, and thus process painful stimuli in a similar way (Sneddon, 2017).

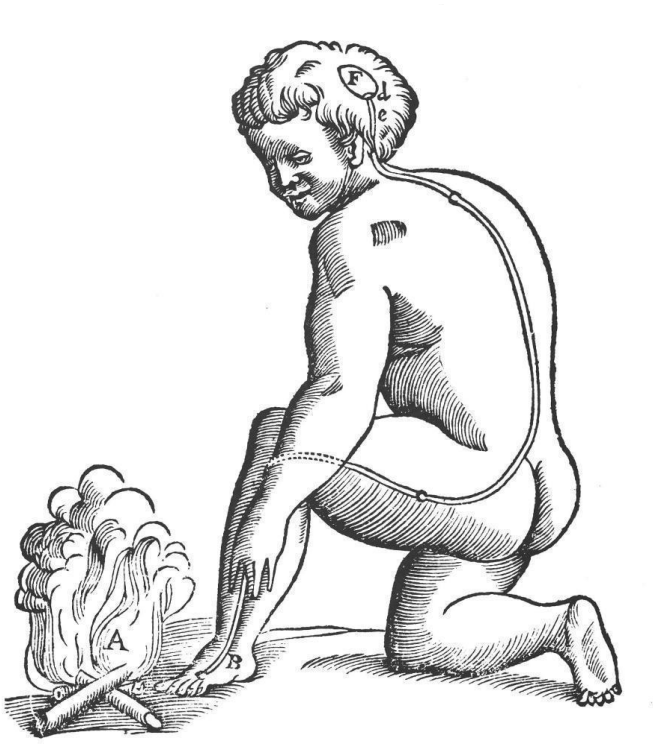


Figure 1. The model of pain perception proposed by Descartes.
Image taken from *Traité de l'Homme, Renè Descartes, 1964.*

2. Theories of pain

Over the centuries, different theories have been postulated to describe mechanisms underlying pain sensation and somatosensory modalities. Specificity, Intensity, Pattern and Gate Control Theories are

the most influential theories postulated about pain, but are sometimes adopted to explain the other sensory perceptions and thus they are not completely specific for pain **(Figure 2)**.

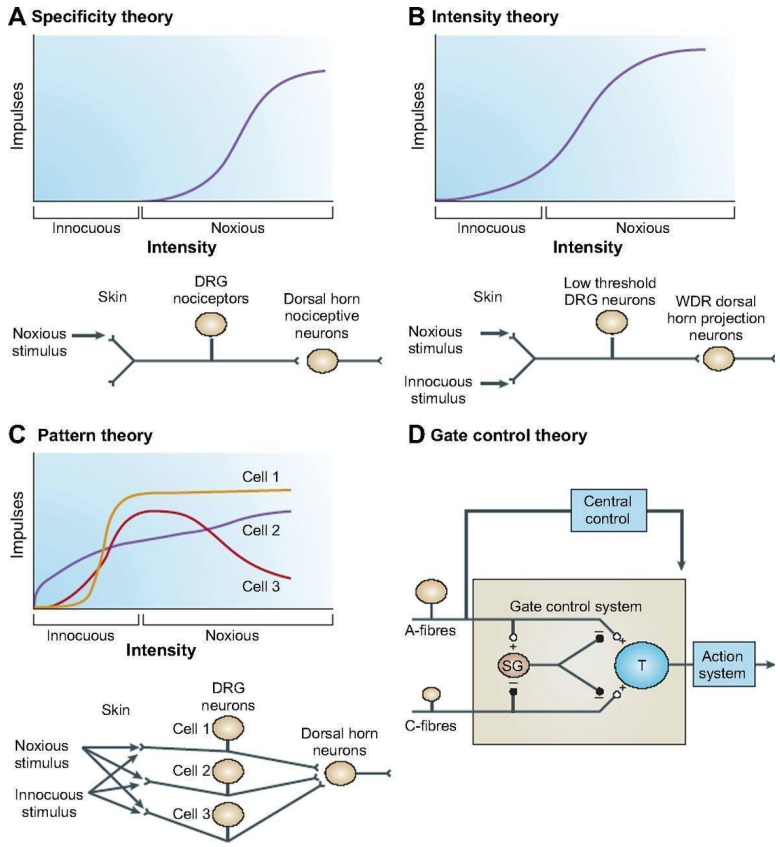


Figure 2. Diagrams of Theories of Pain. Image shows all pain theories: (A) specificity, (B) intensity, (C) pattern and (D) Gate Control Theory. As taken from Perl, E. R. (2007).

1. Specificity Theory

The term "nociception" came from the Latin word *nocere* meaning "hurt" and it is described as the mechanism by which intense thermal, mechanical or chemical stimuli are detected by a subpopulation of peripheral nerve fibers, called nociceptors (Basbaum et al., 2009). Indeed, the existence of specific organs and fibers in the skin that detects and transmit different types of stimuli led scientists to suggest the "Specificity Theory". According to this theory, noxious, mechanical, warm, and cool stimuli are encoded by specialized sense organs and transmitted along different pathways that are integrated into the brain. As suggested by von Frey, each of the four different sensations could be associated with a

morphologically defined receptor type. Through the discovery of cutaneous receptors (Dallenbach, 1939), the model suggested that non-noxious mechanical stimuli are detected by low-threshold mechanoreceptors, whereas the high-threshold noxious stimuli by nociceptors. Thus, painful and mechanical stimuli were encoded by specialized sense organs and then transmitted along distinct pathways to different brain areas (Moayedi & Davis, 2013).

2. Intensity Theory

The Specificity Theory supported by the discoveries of von Frey (von Frey, 1895) and later by Sherrington (Sherrington, 1906), was debated from the beginning. The German physiologist Goldscheider formulated that a weak stimulus applied to the skin induces touch sensations at first, and only when becoming more intense, this sensation becomes pain. The model was described as a summation effect in which repeated weak stimulations converge and summate in the spinal cord leading to the pain sensation (Moayedi & Davis, 2013).

3. Pattern Theory

A completely different theory of pain was postulated by the psychologist Nafe. Ignoring discoveries about specialized sensory nerves in the skin, Nafe suggested that any sensations were the result of a specific pattern of neural firing and that the intensity of the stimulus derived from the spatial and temporal firing of peripheral nerves (Nafe, 1929). Since all cutaneous sensory nerve fibers are similar, with the exception of those that innervate hair cells in the ear, the perception and consequent interpretation of the stimulus depends on the brain. Thus, intense stimulation of any of these nerve fibers can generate the pain sensation (Sinclair, 1955).

4. Gate Control Theory

The most revolutionary theory of pain was developed by Ronald Melzack and Patrick D Wall in 1965 (Melzack & Wall, 1965). A few years before, they had examined the controversial issues about two precedent theories, the specificity and the pattern theories (Melzack & Wall, 1962). The specificity theory proposed the existence of specialized sensory nerves in the skin but did not examine in depth the psychological dimension of pain. Similarly, the pattern theory missed some significant aspects of somatosensation (Melzack & Wall, 1962). The Gate Control Theory (GTO) (**Figure 3**) developed in 1965 was considered a bridge between the precedent findings. During a stimulus, sensory signals produced in primary afferents are precisely transmitted to the spinal cord. Here, in the dorsal horn of the spinal cord, a group of inhibitory neurons located in the substantia gelatinosa (SG) modulates the transmission of information from the sensory afferents to transmission cells (T-cells). This gating mechanism, which modulates the transmission of nerve stimuli from afferent fibers to spinal cord transmission cells, is controlled by. the activity of large- and small-diameter fibers. Large-fiber (A-fiber) activity inhibits (or closes) the gate, whereas small-fiber (C-fiber) activity facilitates (or opens) the gate (Melzack & Wall, 1965). When the C-fiber input exceeds that of the A-fibers, the gate opens, permitting activation of the projection neuron. This theory still has profound implications for the managing of pain and how pain is viewed by clinicians and patients.

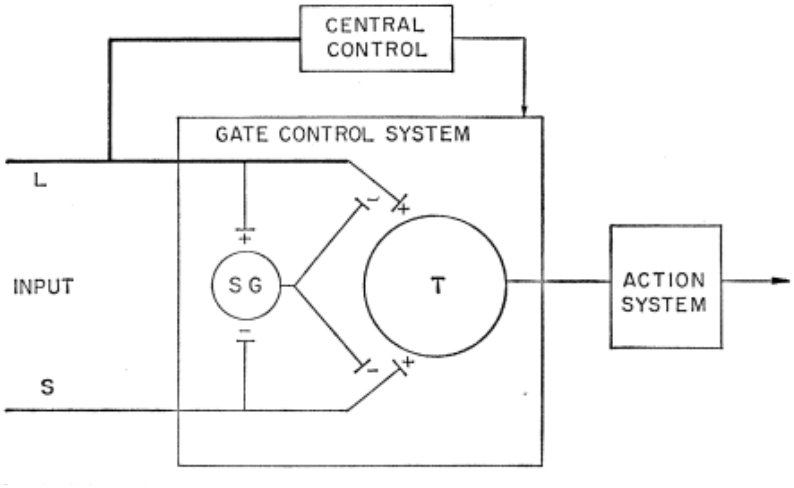


Figure 3. Diagram of the Gate Control Theory mechanism. As taken from Melzack and Wall, 1965

3. The peripheral nervous system and the nociceptive system: anatomy and physiology

Sensory neurons are the principal elements of the Peripheral Nervous System (PNS) and are also known as afferent neurons. They perceive and react to stimuli originating outside and inside of the body using their receptors, such as chemosensors, thermosensors, mechanosensors, nociceptors etc. All these sensors, located in the skin, muscle, joints, and viscera, detect and transmit information about pain, touch, temperature, and body position to the Central Nervous System (CNS). The cell bodies of sensory neurons located within the Dorsal Root Ganglia (DRG) are pseudo-unipolar neurons containing one axon branching that innervate the hairy and the glabrous skin, and a second axon terminating in different laminae of the spinal cord. Based on the level of myelination of the nerves, conduction velocities, adaptation properties, and mechanical threshold, these primary sensory neurons, referred also to as DRG neurons, can be classified into Aβ, Aδ, or C (Brown and Iggo, 1967; Zotterman, 1939). The thickly myelinated Aβ fibers (diameter 6-20 μm) have the faster conduction velocity (16-100 m/s), followed by myelinated Aδ-fibers (diameter 1- 5 μm) with a conduction velocity of 5-30 m/s. Lastly, the small (diameter 0.2- 1.5 μm) and unmyelinated C-fibers have a conduction velocity of 0.4-1.5 m/s (Brown & Iggo, 1967).

Electrophysiological studies based on the mechanical threshold of DRG neurons have shown the existence of low-threshold and high-threshold sensory neurons. The low-mechanical threshold of the majority of Aβ-fibers makes them "light-touch sensitive", whereas many Aδ- and C- fibers with high-threshold are considered nociceptors. However, there are also large subsets of Aδ- (D-hairs) and C-fibers that show a low-mechanical threshold to von-Frey filaments stimulation suggesting their role in detecting innocuous mechanical stimuli (Brown & Iggo, 1967; Burgess et al., 1968).

The sensory inputs detected in the skin and deep visceral organs are processed in the pseudounipolar DRG soma and finally transmitted to the dorsal horn of the spinal cord with a specific distribution

pattern dependent on (1) functional class of sensory fibers and (2) the region of the body that they innervate. The dorsal horn of the spinal cord can be divided into six laminae, each specific for a sensory modality (Perl, 2007). In general, superficial laminae I and II receive nociceptive and thermoreceptive Aδ and C afferents and the vast majority of lamina I projection neurons nociceptive-sensitive express the receptor for neurokinin I (NK1R). Whereas low-threshold mechanoreceptive (LTMR) C-fibers project to the inner portion of lamina II (IIi), Aβ tactile and hair afferents reach the deeper laminae III and part of lamina V that receive also information about the position of limbs (proprioceptors). The complex anatomy of the dorsal horn of the spinal cord also includes projection neurons recognized as an essential element in sensory processing, including pain (**Figura 4**). The axon of projection neurons, located mainly in laminae I and IV–V of the dorsal horn, cross the midline and move rostrally in the contralateral white matter to reach different nuclei in the brainstem and thalamus, where pain and temperature perception are initially processed. Thus, the pain experience appears the result of a series of connections between the periphery, the spinal cord and the higher brain centers.

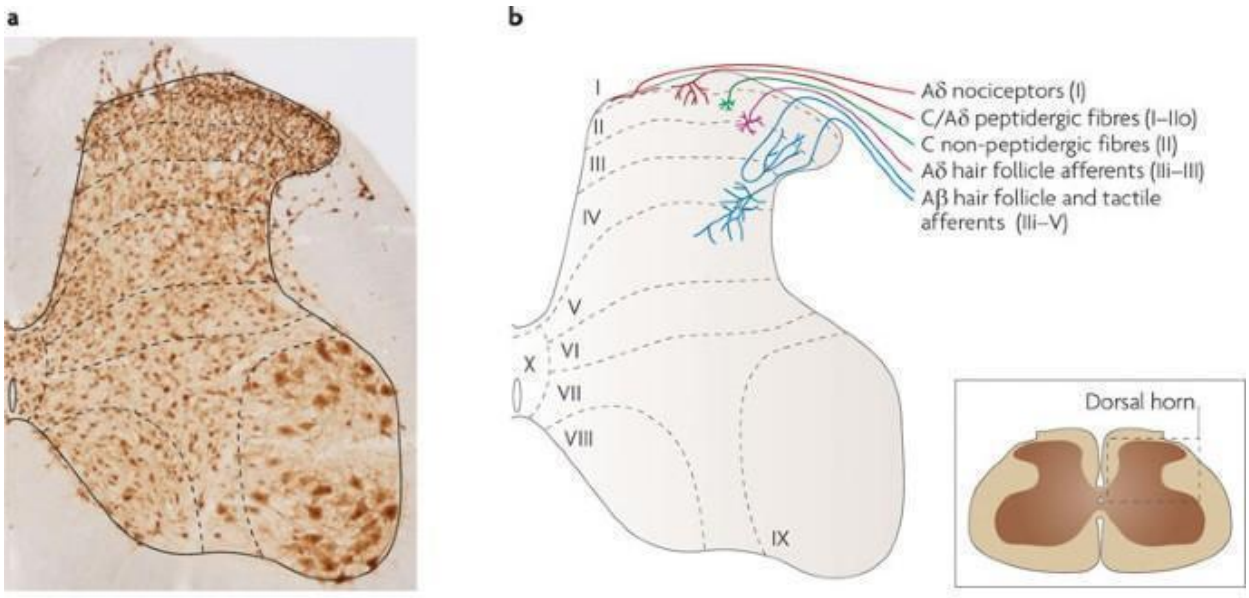


Figure 4. Organization of laminae in the dorsal horn. As adapted from Todd, 2010

4. The development of primary sensory neurons

Sensory neurons are derived from the neural crest cells (NCCs) that, under a defined spatio-temporal control from the neural tube, delaminate and migrate ventrally to generate the dorsal root ganglia (DRG) (Marmigère & Ernfors, 2007). The spatial and temporal signals from the neighborhood tissues, such as the spinal cord, lead to a generation of multiple types of DRG sensory neurons that will specify in the detection of different sensory modalities. Indeed, each neuronal population expresses a unique pattern of molecular markers including ion channels and neurotrophin receptors warranting the responses to unique sets of stimuli (Y. Liu & Ma, 2011; Marmigère & Ernfors, 2007). However, the mechanisms that allow DRG neurons differentiation and specialization during the development remain unclear. The genetic cascade that controls the development and differentiation of sensory neurons is based on the interaction of several genes during the time, such as the two proneural genes, Neurogenin1 and Neurogenin2 (Ngn1/2) (Ma et al., 1999) and the Runt domain transcription factors Runx1 and Runx3 (Chen et al., 2006). However, for complete comprehension of the sensory neuron development, it is necessary to understand the role of neurotrophin receptors, TrkA, TrkB and TrkC, in this process. Indeed, the vertebrate DRGs need neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4 (NT4) and their receptors TrkA, TrkB and TrkC respectively, for controlling the cell survival, the target innervation, functional interactions between cells and for the phenotypic maturation. A combination of factors, including Runx1 and 3, Ngn1 and 2, TrkA and TrkC expression, and Ret receptor (glial-derived growth factor GDNF-receptor) begin in early neurogenesis producing large TrkB+ and TrkC+ neurons (Montelius et al., 2007).

Cells expressing Runx3 and TrkC will become proprioceptors, whereas the loss or the reduction of Runx3 expression will lead to different subpopulation of mechanoreceptive neurons, expressing TrkB+/TrkC+, TrkB alone, Ret alone or Ret+/TrkB+ (Chen et al., 2006).

The expression of the isoform 1 of Runx (Runx1) seems to drive the differentiation of small-diameter TrkA-dependent neurons (Levanon et al., 2002). It has been shown that the mice embryos lacking Runx1 activation also lose TrkA+ trigeminal sensory neurons during neurogenesis (Theriault et al., 2004). Indeed, Runx1 drives not only the diversification of the TrkA+ neurons into different types of nociceptors during the late embryonic and postnatal stages but also the segregation of TrkA+ and Ret+ nociceptors, and the expression of the transient receptor potential (TRP) channel family members (Kramer et al., 2006; Marmigère & Ernfors, 2007) **(Figure 5)**. Moreover, during the embryogenesis, TrkA controls not only the specification of nociceptors, but also of thermoceptors and pruriceptors, becoming a key player in the development of a great variety of sensory neuronal populations (Liu & Ma, 2011).

The late stages of the differentiation of sensory neurons in nociceptors, mechanoreceptors, and proprioceptors, will be described in the following sections.

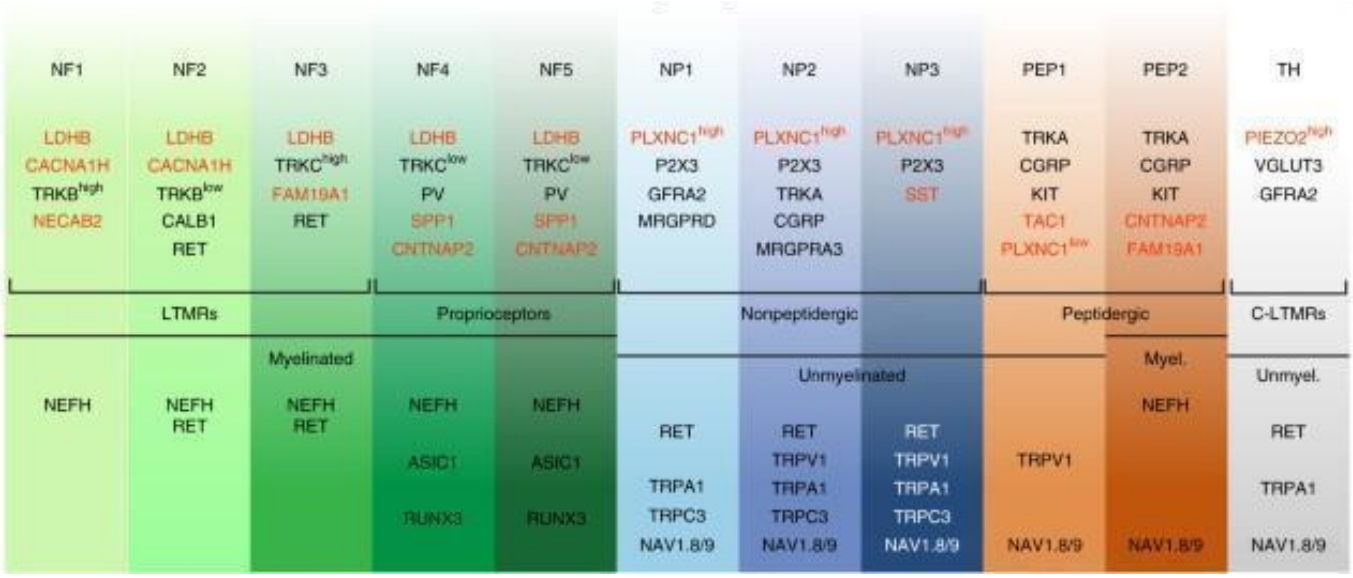


Figure 5. Classification of DRG sensory neurons based on unbiased full RNA transcriptome analyses. As adapted from Usoskin et al., 2015

1. Nociceptors

Nociceptors, the first drivers of noxious stimuli, are high-threshold sensory neurons activated by intense mechanical, thermal, or chemical stimuli and classified as Aδ- and C- nociceptors (Woolf & Ma, 2007). Another classification is based on the molecular phenotype of these neurons. One group expresses the GDNF-receptor, Ret, and comprises around 60% of total DRG neurons. The other group is smaller and expresses the high-affinity NGF-receptor, TrkA (Molliver et al., 1997; Priestley et al., 2002).

Contrary to large diameter neurons, at late embryonic stages, the differentiation of small sensory neurons is dependent on the expression of Runx1. Indeed, neurons that maintain Runx1 expression become TrkA– /Ret+ /Runx1+ cells and are called *non-peptidergic* nociceptors, by their ability to bind to the lectin *Griffonia simplicifolia* IB4. On the other hand, the downregulation of Runx1 drives embryonic sensory neurons to acquire a *peptidergic* phenotype (TrkA+ /Ret–/Runx1–) expressing the neuropeptide calcitonin gene-related peptide (CGRP) (Luo et al., 2007; Patel et al., 2000). Interestingly, the Runx1 deficiency in DRG of mice (Runx1-/-) causes a switch of non-peptidergic neurons to peptidergic phenotype affecting also in vivo the thermal sensation (Chen et al., 2006).

During the early embryonic events of the development the majority of cells are TrkA+ and are responsive to NGF (White et al., 1996). However, around embryonic day 16 (E16), some sensory neurons switch from NGF- to GDNF-dependence, downregulating TrkA expression (Molliver et al., 1997). The selective downregulation of TrkA in Ret+/Ib4+ non-peptidergic nociceptors persists also during postnatal (PN) life, up to PN21 (D. L. H. Bennett et al., 1996). The central axonal projections of mature non-peptidergic Ret+/Ib4+ and peptidergic TrkA+/CGRP+ nociceptors reach different portions of the dorsal horn of the spinal cord. TrkA+/CGRP+ peptidergic central innervation terminates in lamina I and outer lamina II (IIo), while the Ret+/Ib4+ neurons end in inner lamina II (IIi) (Luo et al., 2007). Both "nociceptive" laminae in the spinal cord are characterized by the presence of excitatory interneurons that express the isoform gamma of protein kinase C (PKCγ), which has

been implicated in injury-induced persistent pain (Malmberg et al., 1997), and a great number of nociceptive-sensitive lamina I projection neurons respond to noxious stimuli and express the receptor for neurokinin I (NK1R) (Peirs et al., 2015). An important role for the development and differentiation of nociceptors, is played by the low-affinity NGF-receptor $p75^{NTR}$, deeply discussed below, that is expressed and required for survival of a subpopulation of RET+ nonpeptidergic nociceptors, indeed its absence causes the loss of 20% of adult mouse DRG neurons (Chen et al., 2017) **(Figure 6)**.

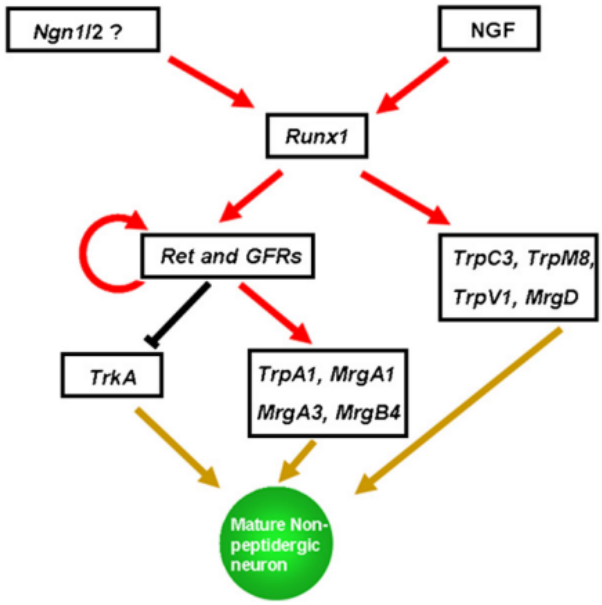


Figure 6. Critical role of NGF on the development of sensory neurons destined to become non-peptidergic RET+ neurons. As adapted from Luo et al., 2007

2. Mechanoreceptors

The ability to detect mechanical forces is crucial for all living organisms to interact with the physical world. This capacity is known as mechanosensation, which is described as the conversion of mechanical stimulus into electrical currents and represents the basis of physiological processes such as the sense of touch, balance, proprioception and hearing. In mammals, mechano-sensitive afferents that detect a wide range of mechanical forces with specific threshold sensitivities are classified in

low-threshold mechanoreceptors (LTMRs) and high-threshold mechanoreceptors (HTMRs). Both LTMRs and HTMRs afferents are extensively distributed in the skin, tendons, muscles, joints and viscera.

LTMRs are classified in Aβ, Aδ, or C fibers, based on different properties, such as action potential, level of myelination and conduction velocities. Aδ- and Aβ- LTMRs are lightly and heavily myelinated, exhibiting intermediate and rapid conduction velocities, respectively, whereas the latter are unmyelinated with the slowest conduction velocity. A further classification is based on the cutaneous end organs they innervate. Indeed, Aδ LTMR fibers innervate hairs in the skin and detect hair movement, Aβ-fibers terminate on Merkel cells, Pacinian corpuscles and hair follicles detecting texture, vibration, and light pressure and C-fibers terminate as free nerve endings and are associated with 'tickling' sensations (Iggo & Andres, 1982). The kinetic characteristics of LTMRs currents determine another classification in rapidly adapting (RA) (~3-6ms) or slowly adapting (SA) (~200-300ms). Both RA-LTMRs and SA-LTMRs can be further classified into type I and type II that are common to most, if not all, vertebrate animal models (Zimmerman et al., 2014). The sensory structures in mammals glabrous skins responsible for discriminative touch (texture and shape), such as Merkel cell-neurite complexes, Ruffini corpuscles, Meissner and Pacinian's corpuscles, are innervated by SA and RA LTMRs (Abraira & Ginty, 2013). The response to a complex touch, as usually happens in vertebrates, results from the combined action of SA and RA LTMRs. Indeed, in case of an accident, indentation on skin, like a jab, would activate SA and RA LTMRs that are both mainly made up of Aβ-fibers, and so called "indentation detectors" and "velocity detectors", respectively (Bai et al., 2015) (**Figure 7).**

HTMRs represent a broad group of mechano-nociceptive sensory neurons that are activated by noxious mechanical stimuli. This category includes Aδ- and C- fibers that innervate both glabrous and hairy skin. The mechano-nociceptors Aδ-HTMRs are responsible for the detection of mechanical

pain, noxious both heat and cold pain. On the other hand, C-HTMRs respond specifically to mechanical but not thermal stimuli (Abraira & Ginty, 2013).

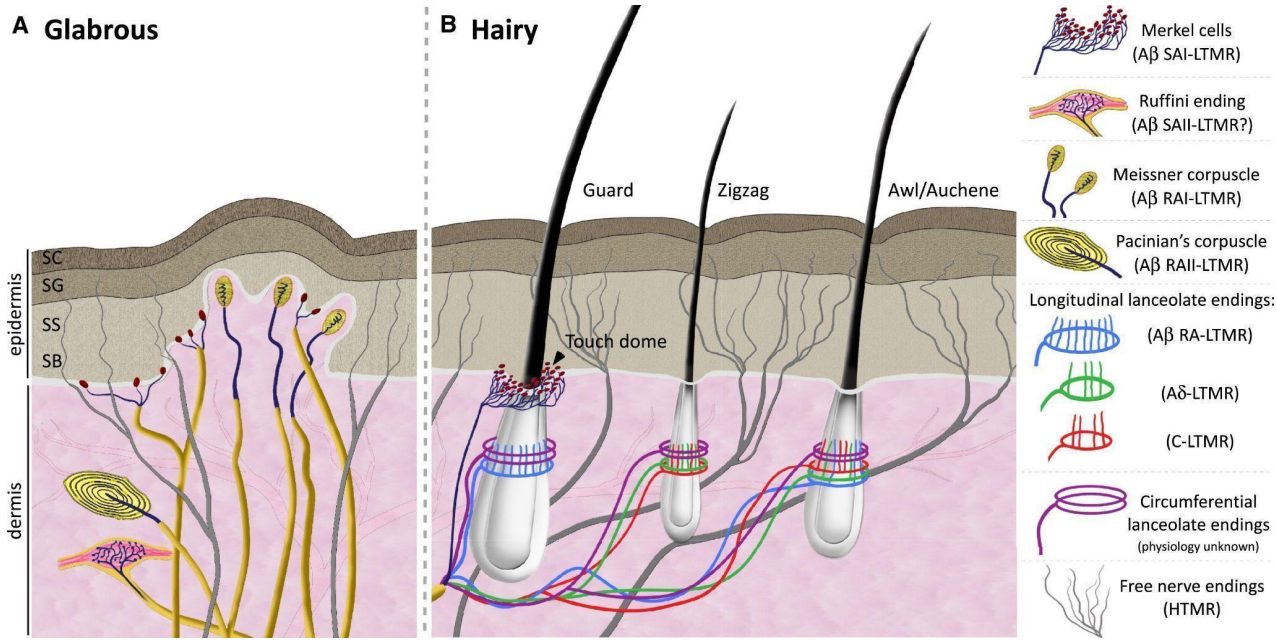


Figure 7. Glabrous and Hairy skin innervation. Meissner corpuscle, Merkel cells and nerve endings mainly innervate glabrous skin, while hair follicles are innervated by different subtypes of longitudinal lanceolate endings. As taken from Abraira & Ginty, 2013.

3. Proprioceptors

The "sense of self" in mammals is detected by specialized sensory neurons located in muscles, joints and tendons, and called proprioceptors. Information about joint angle, muscle length, tension etc are necessary to sense the position and movement of the limb in the space and the sense of balance (Proske, 2005). Muscle spindle and Golgi tendon organs, innervated by different fibers that detect and then transduce information from the periphery to the CNS, are the most characterized proprioceptors. Muscle spindles notice changes in muscle length and the velocity of contraction, whereas Golgi tendon organs provide information about changes in muscle tension (Hillier et al., 2015). Because primary sensory neurons, cell bodies of proprioceptors are located in DRG and their projections reach the dorsal horn of the spinal cord in lamina V. They are large neurons (13-20 μm) with myelinated axons and a conduction velocity of 80-120 m/s. Recently, using deep single cell

RNAseq coupled with virus and genetic tracings, researchers have identified three main types of proprioceptive sensory neurons (PNs), type Ia, Ib and II, that segregate into eight distinct subgroups (Wu et al., 2021). These discoveries contribute to filling the gap in the scientific knowledge about the understanding of the molecular diversity within proprioceptors and to develop new strategies to study and modulate the sense of self.

5. Sensory transductors

1. TRP channels

The superfamily of Transient Receptor Potential (TRP) channels was discovered for the first time in *Drosophila melanogaster,* in which a spontaneous mutation made blind the animal during prolonged intense light (Cosens & Manning, 1969). Electrophysiological studies highlighted that, instead of a sustained current of wild-type channels, the mutant Drosophila photoreceptors displayed a "transient" current, and so it was named TRP (Cosens & Manning, 1969). Expressed in a large number of tissues, from nerves to epithelial cells, the 28 types of TRPs identified so far in mammals were initially classified in 6 subfamilies: TRPC (classical or canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin-like), TRPPP (polycysteine), TRPML (mucolipin). However, more recent studies have discovered other three subfamilies, such as TRPN (no mechanoreceptor potential C), TRPVL (vanilloid-like) and TRPML (mucolipin) widely expressed across vertebrates (Himmel & Cox, 2020) **(Figure 8).** Of the nine sub-families, only TRPV1, TRPA1 and TRPM8 seem to be associated with pain sensation. Indeed, these members of the TRP channel family are activated by different plant-derived agents that produce irritation or pain, such as capsaicin, menthol, the cooling agent from mint, as well as isothiocyanate and so on. Molecular, functional and histological analyses have revealed that sensory neurons express specific patterns of these three channels (explained in detail below).

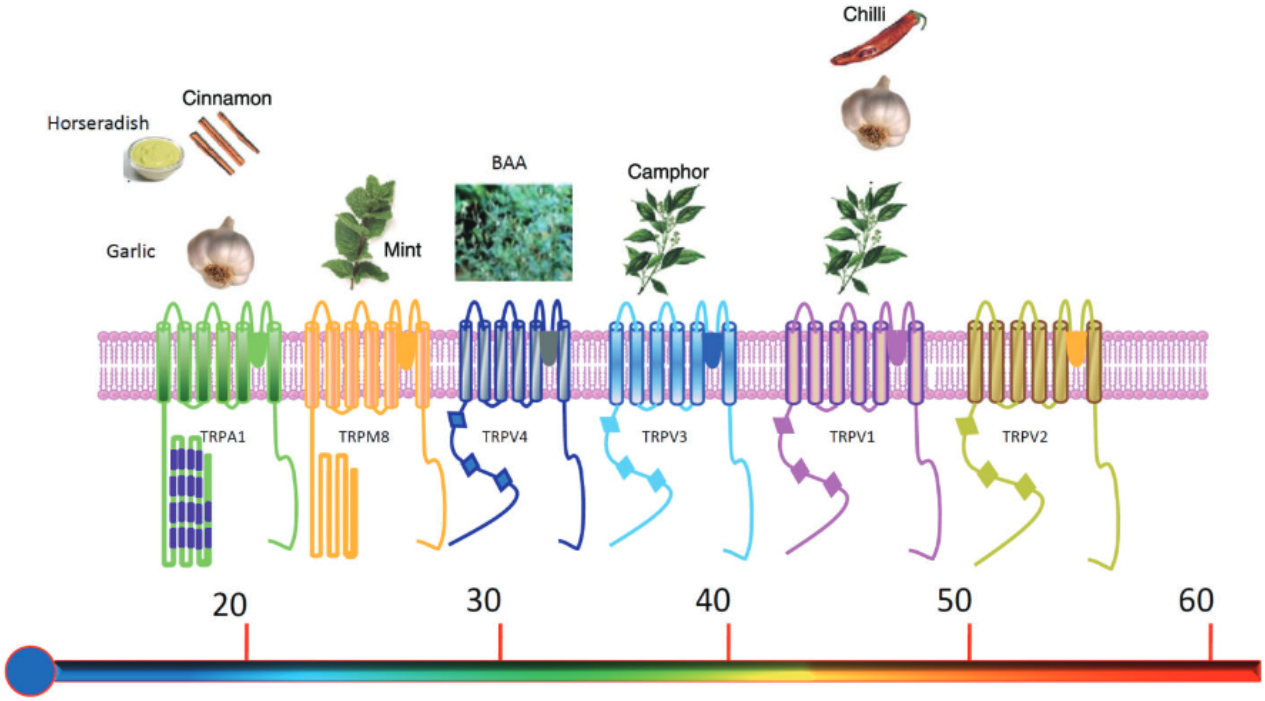


Figure 8. Schematic description of the six mammalian Transient Receptor Potential (TRP) channels. As taken from Vay et al., 2011

TRPV1. The Transient Receptor Potential Vanilloid 1, TRPV1, is the prototype and the most studied receptor within the TRP family. It can be activated by high temperature (>43°C) and capsaicin, its main natural agonist (Caterina et al., 1997). Moreover, due to its sensitivity to low pH (<5.9) that usually occurs during an injury, TRPV1 is a key mediator during inflammation, contributing to pain state (Marrone et al., 2017). The TRPV1 transcript and protein were found to be abundantly expressed in sensory neurons, especially in small-diameter unmyelinated C-fibers (Caterina et al., 1997). These C-fibers labeled by TRPV1 also express different neuropeptides such as substance P (SP), TrkA, and CGRP and constitute approximately 30–50% of all DRG somatosensory neurons, contributing to key events during inflammation (Kobayashi et al., 2005; Tominaga & Tominaga, 2005). TRPV1 is indeed considered one of the leading molecular markers for defining the nociceptor subpopulation involved in the response to noxious stimuli, inflammation, and thermal hyperalgesia (Julius, 2013). In addition to its role in inflammatory pain attenuation, (Caterina et al. 2000; Davis et al. 2000), TRPV1

transduction is involved in other processes such as, itch- and warm-temperature-coding neurons (Bautista et al. 2014; Yarmolinskyet al. 2016), thus suggesting a broader role in the somatosensory system.

TRPA1. Originally called ANKTM1, or Wasabi receptor, the Transient Receptor Potential Ankyrin 1 (TPRA1) is the only member of the ankyrin subfamily found in mammals and it is abundantly expressed in nociceptors. The function of TRPA1 is to detect noxious cold, less than 17°C, and chemical irritants that elicit acute and inflammatory pain (Story et al., 2003). A key role of TRPA1 is also to contribute to cold hypersensitivity caused by an injury in which reactive oxygen species and proinflammatory mediators are released and activate the channel (Camino et al., 2010; Obata et al., 2005). Among TRPA1 activators, irritants such as mustard oil and garlic that contain isothiocyanate or thiosulfinate compounds, respectively, activate sensory nerve fibers that express TRPA1, causing acute pain and inflammation (Bandell et al., 2004; Bautista et al., 2005, 2006). This activation leads to a peripheral release of CGRP and SP neuropeptides and other transmitters that trigger robust thermal and mechanical hypersensitivity (Bautista et al., 2006). After inflammation, the increased expression of TRPA1 in DRGs, especially in TrkA+ neurons, seems to be dependent on NGF signaling (Obata et al., 2005), and interestingly, in trigeminal sensory neurons, NGF contributes to the upregulation of TRPA1 activity leading to the development of hyperalgesia in the orofacial region (Diogenes et al., 2007).

TRPM8. Transient Receptor Potential Melastatin 8 (TRPM8), is a cold-sensitive ion channel, activated also by natural and synthetic cooling agents, such as menthol, and for this reason called the "menthol receptor". ∼15% of somatosensory neurons, mostly small-diameter unmyelinated C-fibers, express TRPM8 and a small subset of these are polymodal, expressing also TRPV1 (TRPM8+/TRPV1+) (Julius, 2013; Kobayashi et al., 2005). Indeed, these menthol-sensitive neurons also respond to capsaicin, highlighting that TRPM8-expressing sensory neurons derive from the population of TRPV1-expressing neurons (Hjerling-Leffler et al., 2007)). The involvement of

TRPM8 in cool sensation is evident in TRPM8-deficient mice that show profound diminished responses to cold and fail to distinguish warm from cool (Bautista et al., 2007).

2. ASICs

A large number of painful stimuli, such as inflammation, causes a decrease in the extracellular pH that is promptly detected by pH-sensitive receptors expressed on nociceptive fibers. These receptors are called Acid-Sensing Ion Channels (ASICs). ASICs are a family of proton-gated ion channels composed of 7 isoforms, mostly expressed in mechanosensory and nociceptive neurons (Lingueglia et al., 1997; Moshourab et al., 2013). Evidence of the importance of ASICs channels in sensory neurons came from analysis of somatosensation in mice lacking the subunits 2 or 3 (ASIC2 or ASIC3) showing an opposite phenotype. Indeed, ASIC2-null mice showed a reduced mechanical sensitivity (Price et al., 2000), whereas ASIC3-null mice exhibit an altered response to mechanical stimuli and a reduced sensitivity to acid and noxious heat (Price et al., 2001). The differences among the various isoforms of ASIC channels seem to be dependent on the different sensitivity to pro-inflammatory mediators, including NGF, that determines the increased level of ASIC mRNA during inflammation. NGF injections in rat DRG neurons lead to upregulation of ASIC3, promoting the acid-mediated NGF-sensitization (Mamet et al., 2002).

3. Voltage-gated sodium channels (VGSCs or Navs)

Since the first discoveries of Hodgkin and Huxley in studying the squid giant axon (Hodgkin & Huxley, 1952), the voltage-dependent activation of the sodium current (due to movement of Na+ algon the axon) is fundamental for the generation and propagation of the action potential.

This important task is mediated by Voltage-gated sodium channels (VGSC or Navs) that are, nowadays, of special interest also in pain sensation due to their involvement in the genesis and

propagation of sensory stimuli. Navs are widely expressed throughout the body and mainly three different Nav-subtypes are expressed in adult mammalian sensory neurons: the tetrodotoxin-sensitive (TTX-S) subtypes Nav1.7; and the two TTX-resistant (TTX-R) subtypes Nav1.8 and Nav1.9 (Goodwin & McMahon, 2021). All these three channels have been deeply investigated because of their direct link with abnormal sensitivity to pain (Niels Eijkelkamp et al., 2012).

Nav1.7. Nav1.7 encoded by SCN9A gene, has been of particular interest because it is related to a large number of human heritable pain disorders. Indeed, gain-of-function mutations are responsible of debilitating chronic pain conditions, such as inherited erythromelalgia and paroxysmal extreme pain disorder, causing episodes of burning pain sensations in affected individuals (Dib-Hajj et al., 2005; Fertleman et al., 2006). In contrast, loss-of-function mutations cause congenital pain conditions characterized by insensitivity to a wide range of noxious stimuli that normally elicit pain (Cox et al., 2006).

Nav1.8. Nav1.8, encoded by SCN10A gene, is widely expressed in C-fibers and it is linked to painful states (Goodwin & McMahon, 2021). The expression of Nav1.8 in nociceptors seems to be regulated by NGF-TrkA signaling (Dib-Hajj et al., 1998) and, surprisingly, the electrophysiological properties of some Aβ- rapidly adapting fibers Nav1.8+ are influenced by TrkA (Fang et al., 2005). Some mutations causing a gain of Nav1.8 function have been identified in individuals diagnosed with painful peripheral neuropathies (Faber et al., 2012) as well as loss-of-function Nav1.8 mutations that are associated with a reduced pain sensitivity (Duan et al., 2016). Moreover, Nav1.8 hyperexcitability leads to an increase in calcium influx into nociceptors through mitochondrial proteins contributing to neuropathic pain conditions and nerve degeneration that usually occurr in diabetic patients (George et al., 2022).

Nav1.9. Nav1.9, encoded by the gene SCN11A, has been found at all neuronal compartments in DRG, in the neuronal somata, along the nerve fibers and in central terminals within the spinal cord (Bennett et al., 2019). Predominantly expressed in C-fibers, Nav1.9 has been also detected in

myelinated nociceptive Aδ and Aβ fibers (Fang et al., 2002). The involvement of this Nav in pain sensation, it is evident in SCN11A knockout mice that exhibit a robust painless phenotype confirming Nav1.9 is an important player in generating hyperalgesia in inflammatory pain states and in the response to cold in chronic pain conditions (Priest et al., 2005).

4. PIEZO2

Until 2010, the mechanisms underlying the response to mechanical stimuli through the activation of ion channels expressed by sensory neurons, have remained elusive. Indeed, the discovery of Piezo proteins, Piezo1 and Piezo2, opened new findings about mechanosensation (Coste et al., 2010). Piezo1 is expressed in endothelial cells and it is fundamental in the development of blood vessels in mice (Ranade et al., 2014). On the other hands, Piezo2 has been detected in around 20% of DRG sensory neurons and it is activated by innocuous mechanical stimuli (Ranade et al., 2014; Woo et al., 2014). In addition to touch, Piezo2 is also associated with mechanical pain (N. Eijkelkamp et al., 2013; Murthy et al., 2018)The loss of Piezo2 in adult sensory neurons and Merkel cells caused a profound loss of touch sensation in mice, without affecting the response to thermal stimuli (Ranade et al., 2014), whereas the conditional knock-out of Piezo2 showed profound defects in proprioceptive behavior related to limb position and fine movement (Woo et al., 2015). Interestingly, the Piezo2 knockdown in DRGs mediated by intrathecal injections of Piezo2 antisense oligodeoxynucleotides leads to reduced activation of Piezo2+ fibers that innervate bones. Since these fibers are also TrkA+ and the loss Piezo2 prevents the NGF-induced sensitization of bone afferent neurons, Nencini and colleagues reported that Piezo2 is involved in the response to noxious mechanical stimuli and interactions with NGF are essential for its function (Nencini et al., 2021).

6. Central pain processing: spinal, cortical and subcortical organization

1. Dorsal horn: ascending and descending pain pathways

Pseudounipolar primary sensory neurons located in the dorsal root ganglion (DRG) have distal nerve projections that reach the skin and deep tissues and apical central projections in the dorsal horn of the spinal cord. As aforementioned, the dorsal horns are organized into distinct laminae relying on pain, itch, touch and temperature. Indeed, the sensory modalities detected in the periphery are processed in the different laminae of dorsal horn of the spinal cord through neuronal and non-neuronal circuits of excitatory and inhibitory neurons and microglia (Chen et al., 2018). Neurons within the dorsal horns can be divided into projection neurons that reach the brain regions, and interneurons whose axons make contact with other spinal neurons. Pain- and itch-related projection neurons, distributed in the superficial laminae, are a heterogeneous excitatory neuronal population fundamental to stimulus transmission (Wercberger & Basbaum, 2019). In addition to these cells, also interneurons play a key role in sensation. By using histological procedures, it has been shown that spinal interneurons belong in inhibitory cells, which use GABA and/or glycine and excitatory interneurons, which use Glutamate (Todd, 2017). Interestingly, the intra-laminae connections in the dorsal horns mediated by interneurons seem to be responsible for pain sensation observed under pathological conditions, such as mechanical allodynia, discussed below (Cheng et al., 2017; Takazawa & MacDermott, 2010). Recently, to have a comprehensive classification of the molecular profile of dorsal horn neurons, Haring and colleagues performed RNA-sequencing of 1,545 single neurons from the dorsal spinal cord, identifying 15 inhibitory and 15 excitatory molecular subtypes of neurons, then confirmed in situ in the spinal cord (Häring et al., 2018). The anatomical and molecular complexity of the dorsal horn of the spinal cord results in a precise organization of projection neurons within laminae. In fact, from lamina I and V, neurons send projections up to the brain via ascending spinothalamic and dorsal column–medial lemniscal tract, in which axons decussate at the spinal level and then ascend on the contralateral side towards the thalamus, and finally terminate in the parabrachial nucleus and

periaqueductal gray (PAG) nucleus. On the other hand, descending tracts that originate from several brain regions such as hypothalamus, amygdala, hippocampus, prefrontal cortex, synapse within the rostral ventromedial medulla (RVM), and locus coeruleus (LC) building up the descending projections that reach the dorsal horn laminae (Harding et al., 2020) **(Figure 7)**.

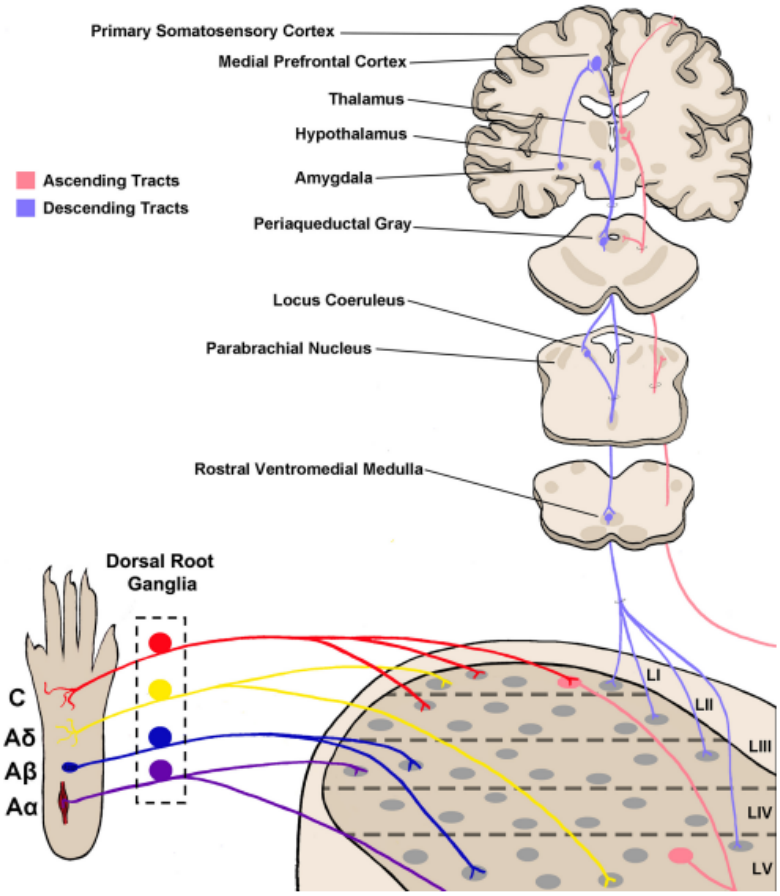


Figure 7. Spinal circuits of somatosensation, focusing on ascending and descending tracts. As taken from Harding et al., 2020

2. Brain matrix of pain

The advancement made about pain brought to expand the definition of pain itself. Indeed, the International Association for the Study of Pain (IASP) defines pain as "An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage", referring to the "subjective" nature of this sensation (IASP, 1978). As commonly thought in the past century, painful information was integrated and limited to subcortical brain regions, not extending beyond the thalamus. The importance of the cortex as a multidimensional integration site turned up with Melzack and Casey that defined pain experience as a result of the interaction of three dimensions: sensory, affective and cognitive (Melzack & Casey, 1968). Nowadays it is known that noxious stimuli detected in the periphery reach and activate both subcortical and cortical brain regions, resulting in an orchestrated activation in time and space that can lastly generate the "pain sensation" (Larrea & Peyron, 2013).

The coordinated network of several brain areas activated by different painful stimuli was defined as the "pain matrix", a slightly modified version of the "neuromatrix" proposed by Melzack (Melzack, 1990). By using Positron-Emission Tomography (PET) and functional Magnetic Resonance Imaging (fMRI) that evaluate the human brain activity during a pain state, it has been possible to identify the main central components of this matrix, such as primary and secondary somatosensory cortices, insular cortex (IC), anterior cingulate cortex (ACC) and prefrontal cortex, amygdala, nucleus accumbens (NAc), thalamus and periaqueductal gray (PAG) (Apkarian et al., 2011; Larrea & Peyron, 2013); Baliki and Apkarian, 2015) **(Figure 8)**. Through these non-invasive functional neuroimaging techniques, investigating cortical and subcortical structures involved in pain sensation allowed to find that there is a strong connection between the intensity of painful stimuli and the magnitude of the neural responses in the Pain Matrix, and consequently, factors modulating pain also affects the neural activation of the pain matrix (Iannetti & Mouraux, 2010). The pain matrix represents a novel view of

pain perception that cannot be referred exclusively to as nociception but also includes consciousness. Indeed, if nociception refers to a peripheral noxious stimulus independent of conscious perception, pain means a more complex sensation based on a cortical and subcortical representation (Apkarian & Baliki, 2015). This new concept is constantly confirmed by studies in which pathological conditions, such as chronic pain, lead to a re-modulation of brain connectivity. Recently, researchers have shown that peripheral nerve injury modulates functional connectivity of NAc and causes a sustained decrease in the expression of dopaminergic receptors (Pei-Ching et al., 2014). Another key brain area involved in the top-down response to pain is the PAG which conveys and regulates the processing of nociceptive inputs from the spinal cord, modulating the brainstem-spinal cord communication (Eippert & Tracey, 2014).

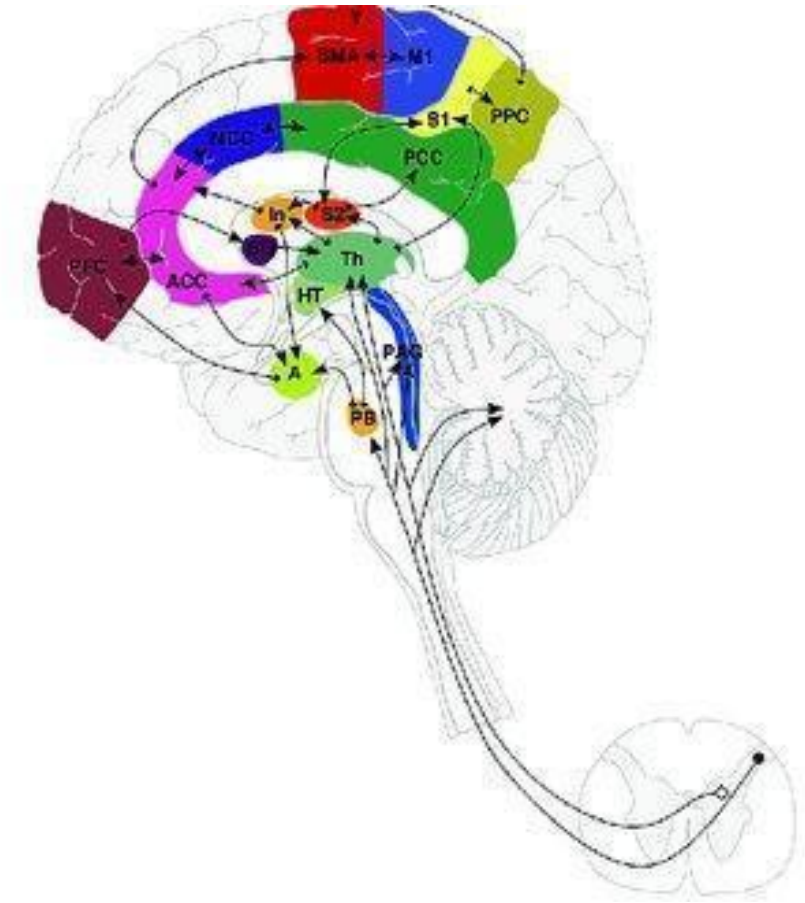


Figure 8. Cortical and sub-cortical brain areas involved in the elaboration of pain sensation.

As adapted from Apkarian et al., 2005

3. Neuroimaging of human pain

The recent progress of neuroimaging techniques applied to humans allowed us to understand how pain is encoded in the brain. Since pain is a complex combination of sensory, emotional, cognitive, and motor responses, fMRI, PET and Electroencephalography (EEG) techniques offer a potential breakthrough in these efforts. Using the intracranial EEG approach, nociceptive responses of 27 patients were recorded by 300 intracortical electrodes, highlighting around 14 sensory, associative, limbic and cortical brain areas involved (Bastuji et al., 2016). Interestingly, based on this data, researchers try to build a spatio-temporal picture of the brain activity during pain state, discovering three main sets of areas that are active in different moments. Indeed, the noxious stimulus was found to immediately activate sensory cortices (posterior insula and parietal operculum), motor regions and amygdala, suggesting a simultaneous activation of these areas. A few milliseconds later, Bastuji and colleagues registered the activation of cortical structures, which may lead to the transition from unconscious to conscious pain perception, and lastly, brain areas involved in memory encoding, such as the hippocampus **(Figure 9)**.

Noteworthy, there is no evidence that the activation of a set of brain areas is specific to pain perception because the same brain areas are observed to be active also during touch or visual stimuli. A similar pattern of brian areas active during pain state was found also in individuals with congenital insensitivity to pain. As mentioned above, loss-of-function mutations in Nav1.7 encoded by *SCN9A* are associated with pain insensitivity (Cox et al., 2006). Brain activity recorded by fMRI of Nav1.7-null patients, referred to as “pain-free” during the administration of noxious mechanical stimuli, resulted in a similar level of sensation to healthy control individuals, revealing normal activation of brain regions involved in the pain-matrix (Salomons et al., 2016).

In order to improve and fill the knowledge gap about pain processing, recently it has been highlighted the individual variability in brain representations of pain based on the activation of several brain regions. Indeed, contrary to somato-motor cortices that show more stable pain representations across

individuals, ventro-medial and ventro-lateral prefrontal cortices have a larger variability across people tested suggesting that individual differences in pain elaboration may be helpful in the development of personalized treatment for pain conditions (Kohoutová et al., 2022).

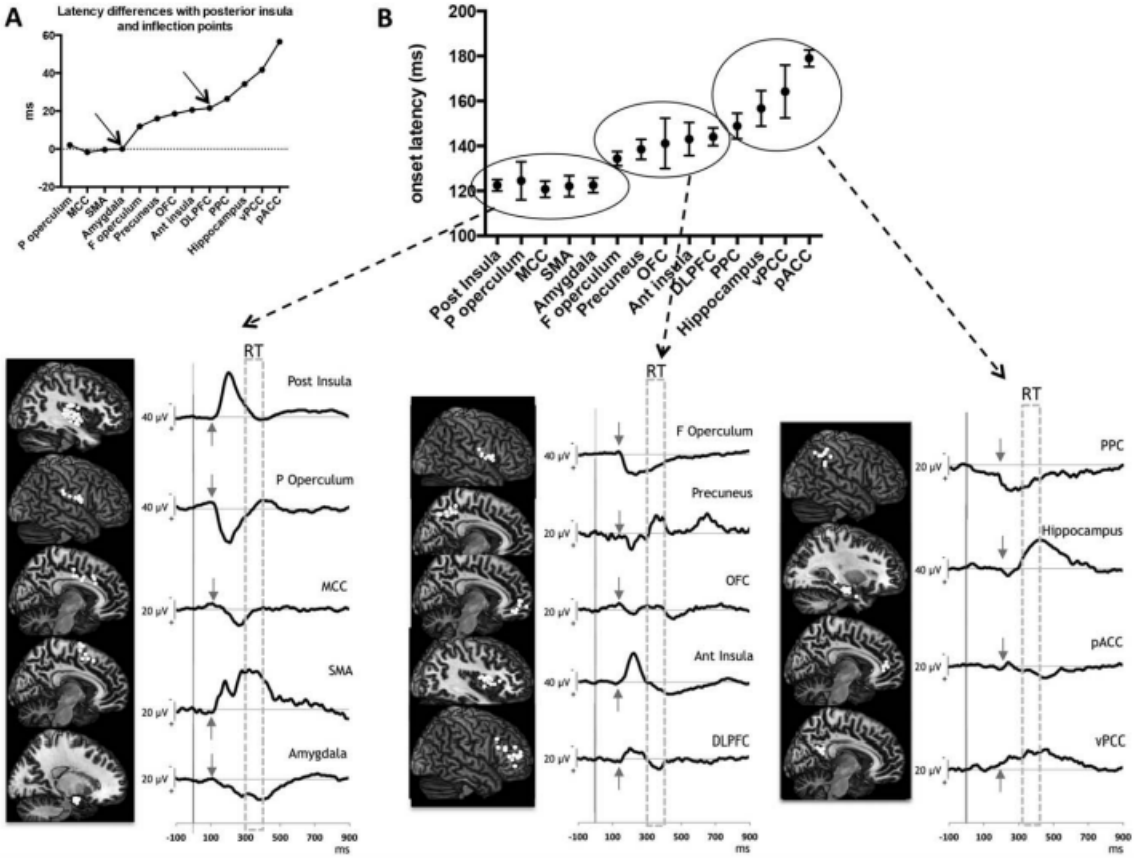


Figure 9. Electroencephalogram recording of brain areas involved in human pain perception. Onset latencies of the nociceptive responses are different across areas.

As adapted from Bastuji et al., 2016

7. Neuropathic pain

The ability to experience pain has a fundamental and protective role across all vertebrate species. Avoiding potential or actual tissue damage elicits a complex pattern of behavioral responses making *pain* in fact an evolutionary necessity. On the other hand, chronic pain conditions do not have any biological advantages and are responsible for suffering and distress (Woolf & Mannion, 1999).

Based on clinical considerations and findings obtained in animal models, the current definition of Neuropathic Pain (NP) is "*Pain caused by a lesion or disease of the somatosensory nervous system*" (Loeser & Treede, 2008), in other words, "pain due to a stimulus that normally does not cause pain".

Neuropathic pain thereby develops as a result of damage or disease affecting the somatosensory nervous system either in the periphery or centrally. Peripherally, lesions damage peripheral fibers (Aβ, Aδ and C fibers) inducing a dramatic fall in pain threshold and amplifying both the duration and the amplitude of the response. Whereas in the central nervous system, spinal neurons are the main players. NP can be classified as spontaneous or evoked. The former occurs when an injured fiber discharges spontaneously, the latter is caused by a non-noxious stimulus able to generate a painful sensation, such as the touch of a feather. This evoked condition is usually referred to as Dynamic Mechanical Allodynia (DMA) or touch-evoked allodynia which is a type of allodynia caused by a stimulus that normally, is not capable of activating nociceptors (Laird & Cervero, 1996). This persistent and weakening condition can appear as an enhanced response to a noxious stimulus, called hyperalgesia, or as a pain sensation caused by an innocuous stimulus, allodynia. The former usually happens in the site of the injury or in an adjacent area and it is believed to be caused by the sensitization of free nerve endings after a noxious stimulus or by changes in sensory processing (Treede & Baron, 2008). The latter refers to pain that is clearly caused by the excitation of low-threshold sensory nerve fibers. Both hyperalgesia and allodynia are thought to be mediated mainly

by peripheral sensitization of C-fibers that thus show a reduced activation threshold resulting in an increased response to a stimulus (Woolf & Mannion, 1999). Peripheral sensitization is usually followed by central sensitization which occurs in the spinal cord driven by nociceptor activation after nerve injury. Together these mechanisms are responsible for neuropathic pain conditions.

1. Primary hyperalgesia

The phenomenon of hyperalgesia, caused by different damages, is a neural mechanism in which peripheral nerve fibers show enhanced responsiveness to noxious stimuli (Treede et al., 1992).

Tissue damage, for instance, a heat damage, leads to the peripheral sensitization of nociceptive fibers in the skin and to the central sensitization in the spinal cord. This neural adaptation is named "primary hyperalgesia" and it is generally associated with the release of inflammatory mediators. The "inflammatory soup" is constituted by peptides, such as CGRP and substance P, cytokines, chemokines and the nerve growth factor NGF, mainly released by non-neuronal cells, including mast cells, platelets, endothelial cells, macrophages and fibroblasts, that directly act modifying the thermal/mechanical thresholds and activation properties of peripheral C- and Aδ- nerve endings (Basbaum et al., 2009; Woolf & Ma, 2007). The most common condition is heat hyperalgesia that usually causes a burning sensation. Studies on mice lacking TRPV1, the heat and capsaicin receptor, have shown the importance of this ion channel in the onset and maintenance of heat hyperalgesia, indeed TRPV1 knockout mice failed to develop inflammatory hyperalgesia (Caterina et al., 2000). The inflammatory soup released during damage includes also Bradykinin which is involved in mediating a variety of inflammatory responses such as vasodilation and (Dray & Perkins, 1993). The key role of bradykinin in hyperalgesia is highlighted by the over-expression of its receptor B1 during inflammation which leads to amplified responsiveness of the inflamed tissue (rat hindpaw) to bradykinin resulting in a significant reduced mechanical threshold (Fox et al., 2003), and the selective blocking of B1 bradykinin-receptor by antagonist administration reduces mechanical hyperalgesia in

animals (Schuelert et al., 2015). Another pro-algesic agent is the neurotrophin NGF which activates TrkA receptors promoting peripheral sensitization of nociceptors during inflammation (Lewin et al., 1993). Interestingly, nociceptors sensitization mediated by both bradykinin and NGF modulates the activation of other pain mediators like TRPV1 possibly via PLC activation (Chuang et al., 2001). The biological role of neurotrophin, especially NGF, will be deeply discussed in the following sections.

2. Mechanical allodynia

As mentioned above, mechanical allodynia, also referred to as Dynamic mechanical allodynia (DMA) or touch-evoked allodynia, is a pathological condition in which pain sensation is caused by a non-noxious stimulus (Treede et al., 2008). Since this condition usually occurs in an area surrounding the site of injury, it is also called secondary mechanical hyperalgesia which is defined as *"increased pain sensitivity outside of the area of injury or damage"* (Treede et al., 1992).

In the past years, with developments in genetic labeling techniques, many works have shown that the selective blocking of A-fibers abolished brush-evoked allodynia, whereas Aδ- and C- nociceptors were not required for the induction of mechanical allodynia in a nerve injury animal model (Abrahamsen et al., 2008). This view has been supported by the discovery of several populations of peripheral sensory neurons. LTMRs Aβ- and D Aδ- nerve fibers expressing TrkB (BDNF high-affinity receptor) have been suggested implicated in mechanical allodynia. Indeed, the ablation of TrkB-expressing sensory neurons erased the sensitivity to light touch, under physiological conditions, and mechanical allodynia after nerve injury (SNI model) in mice (Dhandapani et al., 2018).

Usually associated only with the control of pain, Delta and Mu opioid receptors, DOR and MOR respectively, are reported to be also involved in mechanical allodynia. However, since both DOR and MOR are expressed in subpopulation of myelinated and unmyelinated CGRP+ neurons, their involvement in the understanding of neuropathic conditions is limited (Bardoni et al., 2014; Ceredig

et al., 2020). Toll-like receptor 5 (TLR5) has been identified as a new marker of Aβ-fibers and the targeted silencing of A-myelinated TLR5+ fibers suppresses mechanical allodynia-induced in mice (Xu et al., 2015). Piezo2, a proper marker of mechanotransduction, mediates RA-currents in sensory neurons and its permanent blockade recovers neuropathic pain conditions in mice (N. Eijkelkamp et al., 2013).

Noteworthy, the onset and maintenance of mechanical allodynia state are also due to spinal central neurons. Indeed, after a peripheral injury, it has been shown dramatic neuronal plasticity at the spinal cord level resulting in a shift of LTMR Aβ-fibers onto 'nociceptive' fibers. The injury can cause a disinhibition-mechanism of dorsal horn spinal circuits guiding mechanosensitive neurons towards pain projecting neurons in lamina I through a polysynaptic circuit composed of Somatostatin (SOM)+ and SOM+ /Protein Kinase Cγ (PKCγ)+ excitatory interneurons (Duan et al., 2018). The Vesicular Glutamate Transporter isoform 3 (VGLUT3), required for acute mechanical pain and chronic mechanical pain (Seal et al., 2009) and expressed by neurons located in lamina III, a region important for touch because the LTMR inputs, is fundamental as "critical entry point" in spinal pain circuit participating to persistent mechanical pain (Peirs et al., 2015).

There are several possible theories proposed for the development and maintenance of allodynia including alteration in gene expression in DRGs and spinal cord neurons, sprouting of myelinated fibers within dorsal horn laminae from lamina III/IV to lamina I, and a disinhibition of inhibitory interneurons in the dorsal horn (Lolignier et al., 2015). Another interesting mechanism proposed by Campbell and colleagues is related to a human study in which the mechanical allodynia injury-induced was erased by the selective blockade of Aβ-fibers while the selective blockade of the C- and Aδ-fibers did not affect the condition, suggesting a cross-talk mechanism at the site of injury that leads to a cross-activation of fibers (Campbell et al., 1988). Examples reported in this section clearly indicate that mechanical pain condition is the result of both peripheral and central sensitization, and the latter can be perfectly explained by the gate control theory.

3. Spinal mechanisms of hyperalgesia and allodynia: role of glial cells

The importance of the immune system in the onset and maintenance of pain hypersensitivity has been reported in many models of peripheral neuropathies (PNI). For instance, mechanical allodynia observed in patients affected by chemotherapy-induced PNI is caused by nociceptors that produce and release CCL2 chemokine that regulates local macrophage activation in DRG via Toll-like receptor TLR-signaling, resulting thereby in a neuropathic pain state (Zhang et al., 2013). Moreover, the activation of the chemokine CXCL12/CXCR4 signaling in nociceptors is fundamental in the pathogenesis of mechanical allodynia conditions in painful diabetic neuropathy (Jayaraj et al., 2018). Among immune cells, a fundamental contribution to the understanding of pain sensation has been given by new findings on microglia. Microglia is located only in the central nervous system and for this reason, it has never been detected in DRGs. As a point of interest, microglia has been abundantly investigated in the last decade as a pain-modulator in brainstem regions and, especially, in the spinal cord, in which it was observed a robust microglia proliferation few days after injury (Chen et al., 2018). The inflammation injury-induced causes a great release of cytokines and chemokines that can rapidly activate immune cells, including microglia. In particular, the activation of C-fiber elicits spinal microglial activation, but the activation of large A-fibers is central for the maintenance of microglial activation, indeed under physiological conditions, only stimulating microglia themselves can lead to pain hypersensitivity. Indeed, injections of fractalkine, also named CX3C ligand-1 (CX3CL1) are able to produce mechanical allodynia in a dose-dependent manner. On the other hand, fractalkine antagonists reduce the hypersensitivity caused by nerve injuries (Clark & Malcangio, 2014). Recent findings have suggested that spinal microglia exist in “surveillance mode”, switching to a “reactive state” in case of injury, leading to an enhancement of the excitability of spinal nociceptive circuits (Salter & Stevens, 2017). However, peripheral nerve injuries can promote the microglia shift to a reactive state causing a long-term condition of pain hypersensitivity through the

persistent expression of some markers, such as P2X purinoceptor 4 (P2X4), which interacts with BDNF-TrkB pathway, or through the activation of the neuronal K-Cl cotransporter 2 (KCC2) pathway that modulates the GABA receptor activation, compromising the neuronal excitability (Chen et al., 2018; Salter & Stevens, 2017). Interacting with excitatory and inhibitory neurons, microglia participate in the spinal cord synaptic plasticity after nerve injury, the phenomenon already referred to as central sensitization (Carniglia et al., 2017). Its role in the modulation of spinal pain circuitry has been recently reported to be linked to the extracellular matrix, especially perineuronal nets, that are degraded by microglia after peripheral nerve injuries contributing to enhancing the activity of projection neurons and inducing robust pain-related behaviors (Tansley et al., 2022).

8. Modulation of pain: anti-pain therapies

Nowadays, understanding the mechanisms underlying neuropathic pain (NP) and developing appropriate treatments is still challenging. NP causes a heterogeneous set of symptoms, including sleep disorders, anxiety, psychosis, strongly affecting the quality of life of patients. It is reported that, in western societies, one in five individuals is affected by NP, and the failure of pharmacological therapies has a high economic impact on the individual and society (Dahlhamer et al., 2018).

Peripheral neuropathies (PNI), which include those caused by metabolic dysfunctions, diabetes, chemotherapeutic agents, infectious diseases and inherited neuropathies and channelopathies, make the assessment and diagnosis of the condition difficult. Recently, a meta-data analysis of all NP drug treatments published and unpublished since 1966, finds that available treatments for NP are still insufficient and with a modest efficacy (Finnerup et al., 2016). Among several drugs administered for the treatment of NP, the Special Interest Group on Neuropathic Pain (NeuPSIG) has suggested as first-line drugs gabapentinoids, tricyclic antidepressant (TCAs), selective serotonin–norepinephrine reuptake inhibitors (SNRI) and nonsteroidal anti-inflammatory drugs (NSAIDs) whose analgesic effects results, sometimes, not completely valid for the treatment of NP.

Based on their ability to bind voltage-dependent channels reducing Ca^{2+} influx into the cells, the antiepileptic drugs gabapentinoids are known for their analgesic effects acting on neurotransmitter release or neuronal firing. Both gabapentin and pregabalin approved by FDA are used for the treatment of diabetic neuropathies, sciatic nerve injury and also for phantom limb conditions.

The efficacy of antidepressant drugs TCAs and SNRI, especially amitriptyline and duloxetine respectively, is evident in a variety of painful neuropathies, by their capacity to inhibit the reuptake of serotonin and norepinephrine at the synaptic level.

Used as second- and third-line drugs for NP, the opioids need to be administered cautiously due to their long list of contraindications including a history of substance abuse and suicide risk. Widely used for pain management, opioid-agonists block the transmission of nociceptive chronic stimuli by binding μ-opioid receptors. By their ability to inhibit the serotonin and noradrenaline reuptake, opioid-agonists, such as tramadol, block pain transmission at different levels of both ascending and descending pain pathways, acting on multiple brain areas (PAG, ACC, amygdala, etc) whose inhibition is usually associated with a decrease of pain sensation (Martínez-Navarro et al., 2019; Ossipov et al., 2010). Although the analgesic effect and the clinical efficacy in the treatment of chronic pain conditions, opioids are responsible for an abuse epidemic called the "opioid overdose crisis" with dramatic consequences representing a tremendous issue in the United States (NIDA, 2021).

To avoid abuse consequences and behavioral side effects, scientists have proposed the inhibition of the neurotrophin NGF as a potential therapeutic candidate in the treatment of musculoskeletal conditions such as osteoarthritis (OA) and chronic low back pain (CLBP). Indeed, in 2008, among several neuropathic conditions, OA and CLBP affected more than 100 million individuals in the USA resulting in the most frequent and invalidating NP conditions (Wise et al., 2021). NGF-inhibition via anti-NGF monoclonal antibodies that bind inactivating NGF, are today tested for their efficacy in the reduction of pain (Lane & Corr, 2017).

Among several anti-NGF clinical candidates as a therapy for chronic pain, one of the most prominent is Tanezumab, a humanized monoclonal antibody that neutralizes NGF directly blocking the interaction with TrkA and $p75^{NTR}$ (Abdiche et al., 2008). Tanezumab administration during clinical trials in mild-OA patients demonstrated both the efficacy and safety of the treatment, suggesting that anti-NGF treatment significantly reduces chronic pain caused by OA (F. F. Hefti et al., 2006; Lane et al., 2010). However, preclinical and clinical studies with tanezumab have reported side effects such as increased cartilage and joint damages, slowed down the treatment and culminating in the loss of

FDA approval for tanezumab in OA (LaBranche et al., 2017; Wise et al., 2021; Lilly and Pfizer 2022). The systemic administration of anti-NGF-based therapies has been investigated also in rescuing the thermal hyperalgesia observed in animal models of chronic constriction injury (CCI). Indeed, the local injections of anti-NGF was able to prevent the sensory hypersensitivity and the sprouting of nociceptive fibers into rodent skin (Ro et al., 1996). In other studies, the administration of both rodent and human anti-NGF aD11 monoclonal antibodies has been reported to lead to a significant and long lasting analgesic effect in the NP animal model induced by CCI (Covaceuszach et al., 2012).

In addition to anti-NGF antibodies, another strategy to treat pain could be preventing the activation of TrkA via anti-TrkA antibodies. Indeed, neutralizing TrkA using the anti-TrkA monoclonal antibody MNAC13 (Cattaneo et al., 1999) has been shown to induce analgesia in both inflammatory and neuropathic pain models. After sciatic nerve ligation mice developed robust mechanical allodynia that was reverted by repeated i.p. injections of MNAC13 (Ugolini et al., 2007). Moreover, in animal models of inflammatory pain, the administration of NGF-neutralizing molecule trkA-IgG in parallel with a pro-inflammatory compound, such as carrageenan, prevented the sensitization of the nociceptors erasing behavioral pain-related response in animals (Koltzenburg, 1999), and the trkA-IgG-infusion alone produced a sustained thermal and chemical hypoalgesia (McMahon, 1996).

Even if some side effects observed during anti-NGF or anti-TrkA treatments have been observed and have not yet been clearly explained, the analgesic efficacy of these therapies for chronic pain conditions can be considered unique.

9. NGF and the "Neurotrophic hypothesis"

The term "Nerve Growth Factor" (NGF) was introduced for the first time in the middle of the 1950s by the eminent scientists Cohen, Levi-Montalcini and Hamburger to describe a protein isolated from

mouse sarcoma with the ability to promote growth and differentiation of sensory and sympathetic neurons (Cohen, Levi-Montalcini and Hamburger, 1954). Mouse sarcoma transplantation experiments in chick embryos helped to discover that the exogenous tissue was able to promote a "hyper-innervation" of the chick internal organs, possibly by the release of a diffusible molecule later described as NGF (Levi-Montalcini & Angeletti, 1968). NGF, the "*gift from a malignant tissue"* as it was defined by Levi-Montalcini, was purified by mouse salivary glands extract (Cohen, 1960) and injections in different tissues helped to clarify the biological properties of this molecule. Interestingly, it was found that the injections of anti-NGF molecules in newborn rodents had a dramatic effect on the development causing a profound loss of the sympathetic system, suggesting for the first time the importance of NGF for in vivo development (Levi-Montalcini, 1987; Levi-Montalcini & Angeletti, 1968).

NGF is the prototype of the neurotrophin family and by its discovery other three molecules with neurotrophic potentials have been described, the Brain-Derived Neurotrophic Factor (BDNF), neurotrophin -3 and -4 (NT-3 and NT-4) that are linked to the survival and function of several neuronal populations in cortical and subcortical areas of the brain (Barde et al., 1982; Huang & Reichardt, 2001; Rosenthal et al., 1990). All neurotrophins are initially synthesized as a precursor or "pro-neurotrophin" (30-35 kDa) and after translation, these pro-forms interact making non-covalent dimers that will be then cleaved in the Golgi or in secretory vesicles with the ultimate goal to produce mature active neurotrophins (about 12 kDa). Immediately evident to scientists since their discoveries, the main role of neurotrophins is promoting the health and well-being of the nervous system, supporting the growth, differentiation and survival of neurons, and the NGF discovery has been the seeds to the development of the "Neurotrophic Hypothesis" (Davies, 1996).

The original view of the neurotrophic hypothesis suggested that a single neurotrophic factor, released from a target tissue, was sufficient to promote the survival of a specific neuronal population. Later, *in vitro* experiments demonstrated that a combination of multiple neurotrophins can cooperate in

regulating the survival and differentiation of neurons. Indeed, during the development of sensory neurons, BDNF, NT-3, NT-4 and NGF act sequentially to promote survival. For instance, during early stages (embryo day 11), BDNF, NT-3 and NT-4, but not NGF, appear necessary for axonal growth and their absence causes neuronal death. After a few days, these same neurons lose the responsiveness to BDNF, NT3 and NT4 and start to need NGF for their survival (Lallemend & Ernfors, 2012; Snider & Wright, 1996). However, the importance of the neurotrophins in many fundamental neuronal processes came to light also by their receptor. As previously mentioned, NGF binds most specifically to TrkA, BDNF and NT-4 to TrkB, and NT-3 to TrkC. Each neurotrophin also binds to the low-affinity neurotrophin receptor $p75^{NTR}$, a member of the tumor necrosis factor receptor superfamily. Indeed, haploinsufficiency of neurotrophins or their receptor is linked to several impairments and lethality in animal models (Crowley et al., 1994; de Carlos et al., 2006; Ernfors et al., 1994; Perez-Pinera et al., 2008; Smeyne et al., 1994).

In addition to the role on neuronal cell growth and survival, neurotrophins are also involved in axonal guidance, dendritic growth, synaptic plasticity influencing spines structures and neurotransmitter release, and long-term potentiation (LTP). The widespread action of neurotrophins is related both to cortical and subcortical regions during the development and in adulthood, like the high expression of BDNF and TrkB in the neocortex and hippocampus (Cellerino et al., 1998). Neurotrophin' signaling, especially BDNF- and NGF-mediated, is fundamental for the development and maintenance of cortical circuitry. For instance, during the formation of ocular dominance columns, the density of thalamic innervation to the visual cortical layer IV depends on the expression of BDNF, and a decrease can cause a delay in visual system development dark rearing-related (Pizzorusso et al., 2000). Intracerebroventricular administration of NGF in monocularly deprived rats during the critical period attenuated the expected ocular dominance (OD) shift (Domenici et al., 1993), and, on the contrary, infusion of anti-NGF antibodies to visually normal rat pups caused anatomical and functional visual alterations (Berardi et al., 1993; Caleo et al., 1999). Interestingly, a key NGF-target population is represented by basal forebrain and striatal cholinergic neurons, in which NGF regulates

the activity of these neurons, increasing the level of choline acetyltransferase (ChAT) and possibly accelerating synapse formation (Johnston et al., 1987; Mobley et al., 1986). The connection between NGF and basal forebrain cholinergic neurons has attracted many interests due to the importance of these cells in cognitive processes and their involvement in neurodegenerative conditions, such as Alzheimer's disease (Capsoni et al., 2002; Cattaneo & Calissano, 2012; Latina et al., 2017). It has been shown that NGF and BDNF administration have a protective effects on basal forebrain cholinergic neurons in adult rats with partial fimbrial transections (Hefti et al., 1993), as well as the intranasal NGF administration in rescuing synaptic plasticity and memory deficits in a mouse model of Alzheimer's disease (Capsoni et al., 2017).

All together these and many other observations helped to explain the necessity of neurotrophins during both development and adulthood and their active participation in several brain mechanisms, from molecular to behavioral aspects.

10. NGF and its receptors: TrkA and $p75^{NTR}$

As mentioned above, NGF binds TrkA, members of the Trk (tropomyosin-related kinase) receptor tyrosine kinase family and $p75^{NTR}$, member of the tumor necrosis factor receptor superfamily. By the description of the three-dimensional structures of both NGF and TrkA, it has been possible to obtain detailed information about the interactions of the ligand and the receptor (Ultsch et al., 1999). Based on human TrkA sequence nomenclature, cytoplasmic TrkA domains contain 10 evolutionarily conserved tyrosines, of which three (Y670, Y674, and Y675) are responsible of the tyrosine kinase activity and recruiting adaptor proteins after phosphorylation, such as Grb2 and SH2B. The binding of NGF to TrkA induces conformational changes of the extracellular domain promoting the dimerization which not only stabilizes the receptor but also facilitates the autophosphorylation (Ullrich & Schlessingert, 1990), possibly via a re-organization of the extracellular juxtamembrane

region and the consequent rotation of the transmembrane domain of TrkA (Franco et al., 2020). TrkA autophosphorylation initiates a cascade of events that lead to the activation of phosphatidylinositol 3-kinase (PI-3K), mitogen-activated protein kinase (MAPK), and phospholipase C-γ (PLC-γ), with the aim to modulate TrkA trafficking and degradation (Kaplan et al., 1991; Kaplan & Miller, 2000; Soltoff et al., 1992). After the binding, the NGF-TrkA complex is internalized and retrogradely transported to cell bodies along the axon over extremely long distances to support cell survival (Ginty & Segal, 2002). The ubiquitination mechanism has been considered fundamental in controlling the turnover, degradation and thus, the signaling TrkA-mediated. Among four different E3 Ub-ligases, TrkA ubiquitination is mainly mediated by Nedd4-2 that binds to the PPXY TrkA-specific motif (not found in TrkB or TrkC receptors) (Arévalo et al., 2006) and the depletion of Nedd4-2 in TrkA-positive sensory neurons impairs the TrkA trafficking, degradation and consequently the signaling (Yu et al., 2011). In addition to the ubiquitination mechanism, TrkA-interactors have also a key role in signaling transduction. Several pieces of evidence have shown that the interactions between TrkA and $p75^{NTR}$ are necessary for NGF-triggered signaling (Chao, 2003) because $p75^{NTR}$ seems to delay TrkA internalization through reducing TrkA ubiquitination and promote a prolonged NGF-TrkA signaling (Makkerh et al., 2005). $p75^{NTR}$, which can bind all neurotrophins with a similar affinity but different dissociation rates, is involved in several processes related to neuronal and non-neuronal cells, such as Schwann cells contributing to the regeneration of peripheral nerves (Hempstead, 2002; Johnson et al., 1988). A broad overlap between TrkA and $p75^{NTR}$ has been observed in neurons, especially in DRGs and sympathetic neurons (Franco et al., 2021a; White et al., 1996) and in PC12 indicating that NGF-binding requires the coexpression of both receptors because $p75^{NTR}$ proximity can increase the affinity of TrkA for NGF (Hempstead et al., 1991). Indeed, the DRGs and sympathetic neurons collected from $p75^{NTR}$ -deficient mice show a reduced sensitivity to NGF application, supporting the necessity of $p75^{NTR}$ in neuronal development (Lee et al., 1994). $p75^{NTR}$ seems to be able to modulate the response of TrkA to lower concentrations NGF and TrkA-$p75^{NTR}$ interaction could mediate their reciprocal activation (Franco et al., 2021).

Neurotrophins, including NGF, are synthesized as precursors or pro-proteins and they become biologically active proteins after proteolytic cleavage. The uncleaved NGF precursor, known as proNGF, binds both to $p75^{NTR}$ with high affinity promoting cell death and with a minimal binding affinity to TrkA to promote cell survival. proNGF is the most predominant form of NGF in rodent and human brains, and it can be secreted as a precursor or remain unprocessed (Fahnestock et al., 2004). The different binding affinity for $p75^{NTR}$ and TrkA indicates distinct functions of proNGF and mature NGF in mediating apoptosis or promoting survival, respectively (Hempstead, 2002; Lee et al., 2001). Interestingly, after proNGF binding, $p75^{NTR}$ steers apoptosis through contacting other interactors such as Sortilin, a type I membrane receptor expressed in neuronal tissues, and the neurite outgrowth inhibitor reticulon 4 (NOGO). $p75^{NTR}$ can form a heteromeric receptor with Sortilin activating the pro-apoptotic cascade induced by the proNGF binding (Nykjaer et al., 2004). The activation of the complex made by proNGF-$p75^{NTR}$-Sortilin is responsible for the apoptosis of mouse DRG after nerve injury (Arnett et al., 2007). On the other hand, the matureNGF-$p75^{NTR}$ binding has been observed to stimulate the neurite outgrowth of ciliary neurons isolated from chicken and spinal and sensory nerves of $p75^{NTR}$-deficient mice, by reducing the activation of Rho, a small GTPase that regulates the state of actin polymerization (Yamashita et al., 1999). Despite the strong effort to improve the understanding of the mechanisms underlying the biological actions mediated by $p75^{NTR}$ or TrkA, nowadays, it is still unclear how neurotrophins regulate biological processes at the cellular level, consequently how this can affect cognitive processes **(Figure 10)**.

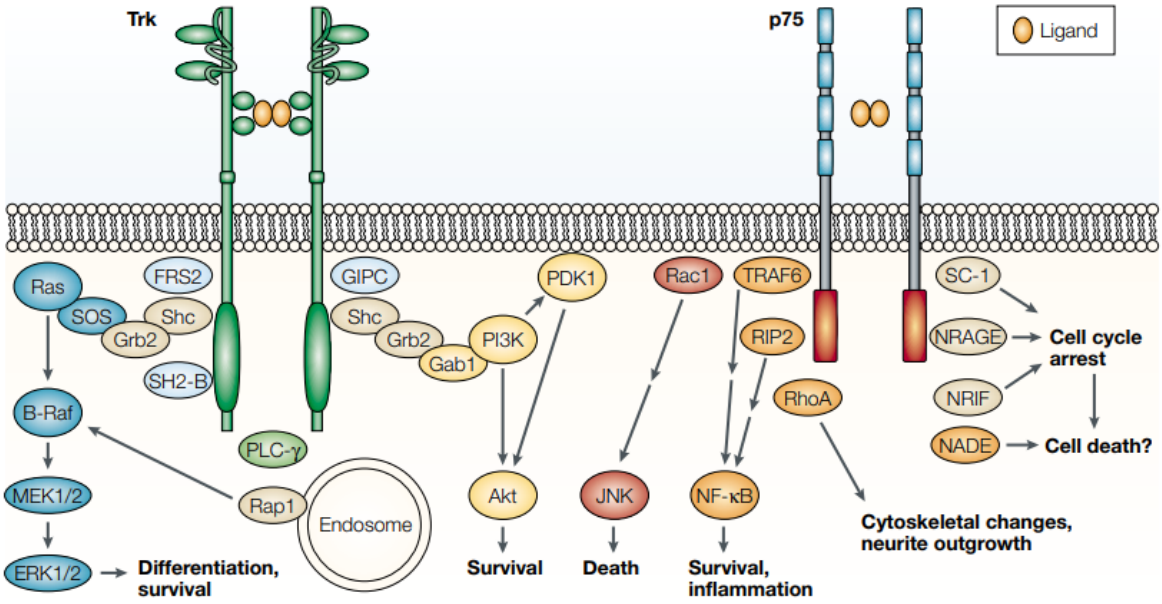


Figure 10: TrkA and p75^{NTR} signaling mediated by NGF binding.

As taken from Chao, 2003.

1. Retrograde and anterograde: a bi-directional signaling

The neuronal survival, including transcriptional control and other cellular processes, neurotrophins-induced requires a retrograde transport from nerve terminals to cell bodies. The first evidence of this retrograde transport came from *in vivo* studies in which radiolabeled neurotrophins, such as NGF (^{125}I-NGF), were injected at the terminal part of the axon and later found accumulated in the cell bodies (Hendry et al., 1974). Nowadays, scientists agree on the ability of neurotrophins to transmit retrograde signals from distal axons to neuronal soma. For instance, basal forebrain cholinergic neurons that project to the cortex and hippocampus receive NGF by retrograde transport from the target area (cortex and hippocampus). In distal axons, the NGF-TrkA complex is internalized into signaling endosomes and retrogradely transport along microtubules to cell bodies by the activation of proteins such as PI3K, Akt, ERK and Cre element binding protein (CREB), to control changes in gene expression and metabolic activity required for neuronal survival and differentiation. Sometimes the length of the axon may be more than a thousand times the width of the cell body and this long-

distance communication between the nerve terminals and the cell body is obviously based on a retrograde transport that has been explained by two models: the endosome hypothesis and the wave propagation model.

The "signaling endosome hypothesis" is based on the interaction of the NGF-TrkA complex with a variety of interactors, such as signaling effectors Ras and ERK and the motor protein dynein. The endosome containing the NGF-TrkA complex and many attached proteins are retrogradely trafficked along microtubules to reach the cell body (Howe & Mobley, 2004). By using three-chamber apparatus, it has been shown that, in parallel to NGF application to nerve terminals, the injection of anti-NGF antibodies to neutralize the neurotrophin contained in endosomes, led to sympathetic cell death without affecting NGF-induced TrkA activation (Ye et al., 2003). This and a more recent study (Ye et al., 2018) strongly support the idea that the NGF-TrkA complex needs to be retrogradely transported within endosomes to support survival, maturation, and synapse formation and maintenance. The retrograde transport of the NGF-TrkA system is considered also the key mechanism adopted by sympathetic neurons for axonal growth and maintenance (Kuruvilla et al., 2004). The second hypothesis, known as "the wave propagation model" or "Domino Model" proposes that NGF binding to TrkA on distal axons induces a wave of TrkA phosphorylation. The retrograde transport of NGF is thus not required for NGF-TrkA signaling, because the wave of "*ligand-independent phosphorylation*" of TrkA is sufficient to be propagated to cell bodies promoting neuronal processes (Ginty & Segal, 2002; MacInnis & Campenot, 2002).

Nowadays, the "signaling endosome hypothesis" is the more accepted theory to explain the retrograde NGF-TrkA transport.

As already mentioned, the outcomes of the NGF-TrkA retrograde signals are mainly related to survival, axonal growth and maintenance. In sympathetic neurons, the NGF-TrkA complex, retrogradely transported to neuronal soma, regulates the expression of anti-apoptotic factors and metabolism-related proteins via activation of transcription factors CREB and MEF2D. This

mechanism is reported also to promote the over-expression of its own TrkA receptor prolonging TrkA-mediated signals (Deppmann et al., 2008) **(Figure 11)**.

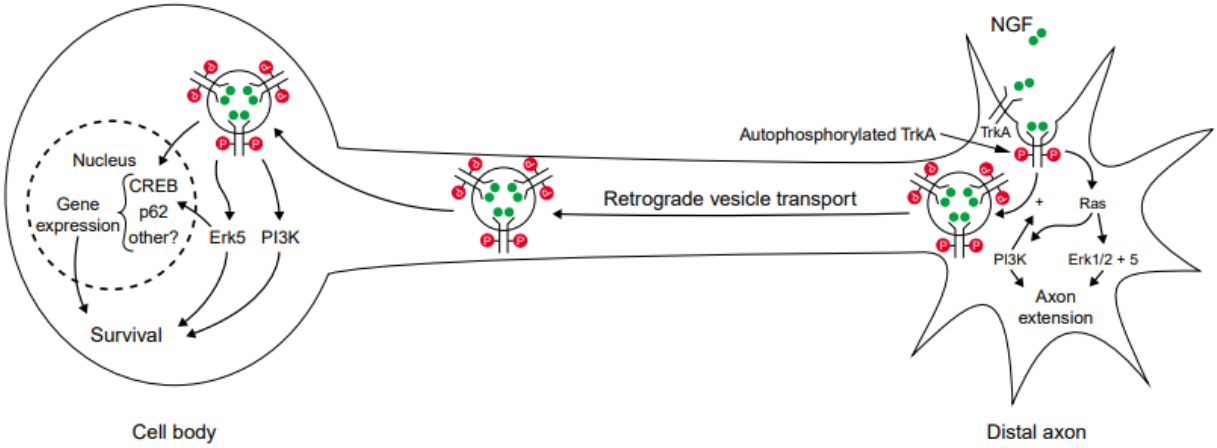


Figure 11. Signaling endosome hypothesis of the retrograde NGF-TrkA complex transport. As taken from Ginty & Segal, 2002.

The internalization mechanism of the NGF-TrkA complex can occur via clathrin-dependent or clathrin-independent manner. In the former, endosomes are covered by clathrin making clathrin-coated vesicles that activate Ras-MAP kinase pathway, including phosphorylated Erk1/2 (Howe et al., 2001). In the latter, clathrin-independent mechanism happens via macropinocytosis, which involves the formation of multivesicular bodies composed of multiple NGF-TrkA-containing vesicles, and it is modulated by Pincher, an NGF upregulated GTPase involved in the TrkA internalization and whose inhibition causes neuronal death (Shao et al., 2002). To reach the cell body, internalized endosomes containing the NGF-TrkA complex recruit several proteins including RAS-related C3 botulinum toxin substrate 1 (RAC1) and cofilin that promote actin rearrangement. Thus, by interacting with other effector proteins such as Rab5, Rab7 and motor proteins (dynein), endosomes are transported along axonal microtubules to the cell bodies. At the soma, TrkA receptor can be exocytosed onto the soma surface and it can interact with naïve TrkA receptors promoting

their anterograde transport to axons, forward trafficking called “transcytosis”. The somatic NGF-TrkA complexes activate transcriptional programs in order to promote neuronal growth and survival (Harrington & Ginty, 2013; Scott-Solomon & Kuruvilla, 2018) **(Figure 12).**

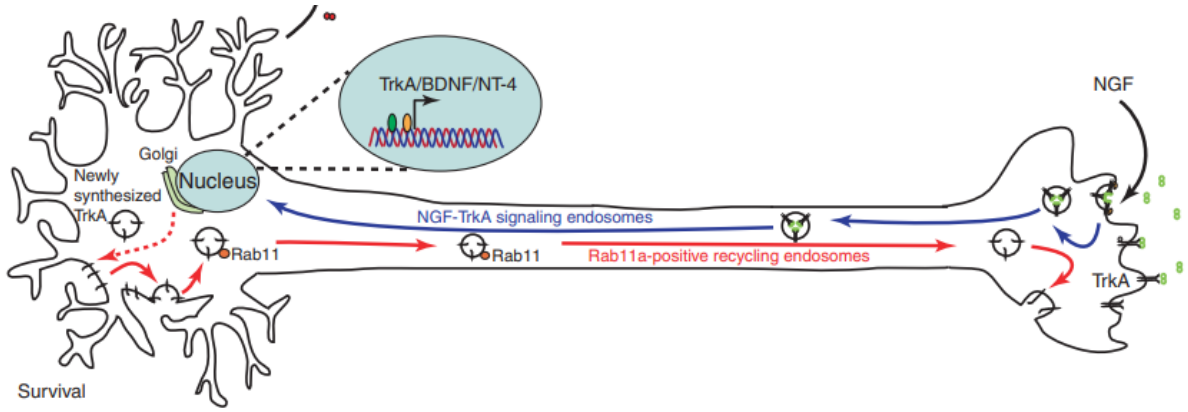


Figure 12. Anterograde trasport of the NGF-TrkA system.

As taken from Scott-Solomon & Kuruvilla, 2018

The anterograde transcytosis, which can be considered a feedback mechanism that allows neurons to gain access to limited amounts of NGF, is still debated. Strongly supported by findings from Kuruvilla’s group, the anterograde transport of TrkA receptor is an essential process to balance the newly synthesized and somatic recycled receptors that must be transported to axons, and it can happen especially in developing sympathetic neurons (Ascaño et al., 2009; Scott-Solomon & Kuruvilla, 2018). Interestingly, a crucial role in the anterograde transport of Trk-receptors is played by Sortilin, mainly located in intracellular compartments, such as trans-Golgi network (TGN) and endosomes (Willnow et al., 2008). After a physical interaction with Trks to form heterodimers, Sortilin supports the anterograde Trk transport in peripheral axons, and, indeed, the lack of Sortilin strongly reduces neurite outgrowth in DRG cultures (Vaegter et al., 2011). The anterograde transport is reported to be linked also to an ATP-dependent motor protein: the kinesin superfamily protein 1A (KIF1A). The loss of KIF1A seems to impair the TrkA transport to axons, resulting in a decreased expression of

TrkA in DRGs and in a diminished response to noxious stimuli of Kif1a-deficient mice, finally suggesting that the KIF1A-mediated NGF/TrkA/PI3K signaling pathway may be responsible for sensory modalities in the peripheral nervous system (Y. Tanaka et al., 2016).

As mentioned previously, the survival/death balance of several neuronal populations depends on the different functions of proNGF and matureNGF. However, it is still unclear whether both forms are internalized and retro- or antero-gradely transported at the same rate (Fahnestock et al., 2004). Interestingly, by using a biophysical approach to label neurotrophins (single-particle tracking strategy) Cattaneo's group has shown that, unlike NGF that is possibly transported in both directions, vesicles containing proNGF can move exclusively from the axon terminal to the cell soma of neurons, via retrograde transport. Moreover, they found that proNGF and NGF vesicles hold a different number of molecules per vesicle (1 or 2 proNGF and between 2 and 8 NGF dimers) and the pro-apoptotic signaling proNGF-mediated emerges in absence of NGF-mediated trophic signaling (De Nadai et al., 2016).

The importance of NGF-TrkA trafficking has been observed also in sensory neurons, in which the retrograde transport of the NGF-TrkA complex has been shown to promote TRPV1 transcription in PC12 cells and to increase the TRPV1 expression on the cell surface of cultured DRGs neurons (Zhang et al., 2005). Moreover, the premature degradation of the retrograde NGF-TrkA transport is reported to potentially contribute to a common inherited peripheral neuropathy called Charcot-Marie-Tooth type 2B which is characterized by severe axonal degeneration (Zhang et al., 2013).

The NGF-TrkA transport mediates several cellular processes, including longer-term changes in gene expression or membrane protein localization, both of which can directly affect sensory neurons, especially nociceptors.

11. Role of NGF-TrkA in development and adulthood

The relevance of correct NGF-TrkA signaling in sensory and sympathetic neurons during development is evident in animal models with the loss of both NGF and TrkA expression. NGF- and TrkA-null mice show a severe loss of sympathetic and sensory neurons, especially nociceptive peptidergic DRGs, resulting in premature death (Crowley et al., 1994; Smeyne et al., 1994). The loss of TrkA leads to extensive cell death of sympathetic neurons of TrkA-mutant mice that start during embryonic life E17.5 and progressively continue after birth (Fagan et al., 1996). Moreover, in TrkA-null mice, it was reported that the striatal and basal forebrain cholinergic neurons were smaller than those of wild-type controls, without affecting the number and density of cholinergic neuron in the target area (hippocampus) (Fagan et al., 1997). Markedly in contrast with the robust decrease observed in the peripheral nervous system, authors found that the absence of TrkA did not compromise the survival of striatal and basal forebrain cholinergic neurons in the central nervous system in 4–6 week mice (Fagan et al., 1997). However, even if the central nervous system did not appear immediately affected by TrkA loss, TrkA-null mice show a short lifespan that precludes testing several aspects of the NGF-TrkA pathway. On the contrary, loss of one allele of NGF, resulting in heterozygous NGF-mutant mice, led to a reduced expression of NGF in the hippocampus (area of NGF secretion), a decrease in cholinergic innervation to the hippocampus, leading to a severe cognitive impairment that was rescued by NGF-infusion into the lateral ventricle in heterozygous NGF knockout mice (Chen et al., 1997). The peripheral nervous system is the most affected by the loss of both NGF and TrkA. For instance, peripheral cutaneous nerves fail to reach the hindlimb in the absence of NGF-TrkA signaling, causing a loss of skin innervation in mice (Patel et al., 2000). It is also observed that the deprivation of NGF by using anti-NGF antibodies in pregnant rats leads to the depletion of around 70% of sensory neurons, mostly Substance P-containing neurons, in newborn rats (Mantyh et al., 2011). Similarly, the expression of other DRG markers such as CGRP, is directly affected by alteration in NGF-TrkA pathway (Patel et al., 2000).

On the other hand, little is known about the NGF-TrkA function during adulthood. Especially for sympathetic neurons, it has been observed that, even in maturity, sympathetic cells remain dependent on NGF for survival and maintenance of dendrites, and indeed, the prolonged administration of NGF-antiserum reduces the number, size and dendritic length of sympathetic neurons (Ruit et al., 1990). For instance, in adult rats, NGF can still promote extensive sprouting of sympathetic neurons into DRGs (Jones et al., 1999). This mechanism could be explained by the ability of satellite cells, glial cells that surround DRGs, to stimulate the synthesis of p75 after nerve injury promoting sympathetic sprouting (Zhou et al., 1996).

One of the most important features of NGF-TrkA signaling found in adults is mediating hyperalgesia. Already discussed in previous sections, NGF acts as a mediator of inflammatory pain in the adult mammals directly acting on the sensitization of nociceptors in vivo (Lewin et al., 1993; McMahon, 1996). *In vitro* DRG cultures treated with NGF show a rapid activation of TrkA and rapid sensitization to noxious stimuli by increasing the expression of TRPV1 (X. Zhang et al., 2005). Interestingly, the TrkA hypofunctionality observed in naked mole rats does not cause deficits in C-fibers innervation in pups but leads to a decrease in adult animals, suggesting a peculiar role of TrkA during the life of these rodents (Omerbašić et al., 2016).

In humans, the level of NGF starts to decrease during puberty possibly reflecting the maturation of the nervous system, and increases after inflammatory conditions (Barker et al., 2020), confirming the dual effect of the NGF-TrkA system in young and adult mammals.

12. NGF-TrkA signaling in pain

Over the years, the physiological role of the NGF-TrkA system in developing neurons has been well established, especially by exploring the effects of NGF or TrkA deprivation *in vivo*. Indeed, sensory and sympathetic neuronal survival during the development is NGF-dependent, but, as mentioned in the previous section, NGF-dependence decreases across time, and NGF expression is low in adults. Changes in the NGF-TrkA signaling during the transition from development to adulthood are related to the switch from MEK/PI3K to JAK pathways that modulate the development and regeneration of axons, respectively (Markus et al., 2002). It has been proposed a critical period for the NGF-TrkA system, among post-natal day 4 and 10 (P4-P10), during that DRGs became sensitive to noxious stimuli, particularly to noxious heat and capsaicin, acquiring the molecular features that determine the peripheral sensitization (Zhu et al., 2004). Scarcely found in normal conditions in rodents and humans, NGF levels increase during inflammation states and its main sources are keratinocytes, mast cells, macrophages and, more recently, bone marrow-derived mast cells (Denk et al., 2017). This increase is clear in experimental models of inflammatory pain caused by Complete Freund's adjuvant injection (Woolf et al., 1994) or caused by autoimmune conditions, such as arthritis (Shelton et al., 2005). Injections of NGF into healthy skin are reported to cause localized pain associated with inflammatory hyperalgesia, both in humans and rodents (Dyck et al., 1997; Lewin et al., 1993). NGF application, via TrkA, leads to a rapid sensitization of sensory neurons to noxious heat and chemical stimuli (capsaicin) by promoting the release of other pro-inflammatory mediators and then recruiting several proteins involved in the retrograde transport of the NGF-TrkA complex. The NGF-TrkA-activated cascade increases both the phosphorylation and trafficking of membrane receptors and ion channels, especially TRPV1. The retrograde transport to the cell body, deeply discussed in previous sections, aims to increase the synthesis of peptides, including SP, CGRP and neurotrophins (BDNF), receptors, ion channels and other pain modulators (Barker et al., 2020; Basbaum et al., 2009). In addition to the transcriptional regulation of pain-related genes, other NGF-TrkA-target genes are

those involved in the axonal growth, a mechanism called "nociceptors sprouting or hyperinnervation", observed in conditions like bone cancer pain (Mantyh et al., 2011). TRPV1 sensitization plays a key role in NGF-TrkA pain-induced. Indeed, TRPV1-deficient mice (full-knockout mice) do not develop the thermal hyperalgesia usually NGF-associated (Caterina et al., 2000) and the "thermal and chemical-insensitive" naked mole rat, deeply investigated by Lewin's group, show a reduced TRPV1 sensitization NGF-induced (Omerbasic et al., 2016). Moreover, also the bradykinin-mediated thermal hypersensitivity requires in vivo the expression of TRPV1, and thus is abolished in TRPV1 knockout mice (Chuang et al., 2001).

Interestingly, as discussed in the section *"Modulation of pain: anti-pain therapies*", targeting the NGF-TrkA complex may be a potential therapy in order to treat several types of chronic pain conditions. For instance, the NGF-TrkA system is physiologically involved in bone metabolisms, ossification and sensitivity, and in pathological conditions, such as osteoarthritis or rheumatoid arthritis (Nencini et al., 2017; Tomlinson et al., 2017). Animal studies had shown the presence of NGF in endothelial cells in unfractured bones that strongly increased after a painful fracture or bone damage, suggesting NGF role in mediating pain sensation after injuries (Grills & Schuijers, 1998). In NP joint conditions, such as OA, the higher level of NGF activates pain signals via TrkA promoting the overexpression of other pain-mediators, substance P and CGRP, and triggering peripheral sensitization (Wise et al., 2021). The involvement of NGF-TrkA pathways in musculoskeletal chronic conditions has been observed in patients affected by two rare genetic conditions named Hereditary Sensory and Autonomic Neuropathy type IV and V (HSAN IV and HSAN V), caused by mutation in gene encoding for TrkA and NGF, respectively, that cause pain insensitivity deeply discussed in following sections. In addition to the loss of pain sensation, HSAN IV and V patients develop bone and joint complications that degenerate in neurogenic arthropathies called Charcot joints (Indo et al., 1996; Minde et al., 2004). However, the mechanism by which the NGF-TrkA system influences these features in such patients is still unclear.

13. NGF-TrkA signaling in itch

Contrary to the extensive information about pain and its related conditions, little is known about itch sensation and a possible involvement of the NGF-TrkA signaling axis. Itch is defined as *"an unpleasant sensation that evokes a desire to scratch and consists of sensory, emotional, and motivational components"* (Ikoma et al., 2006).

Based on the "intensity theory" (discussed above), itch has been initially linked to a weak activation of nociceptors (von Frey). Nowadays, scientists have found several subpopulations of sensory neurons that specifically respond to histamine application, suggesting distinct sets of neurons that mediate itch and pain (McMahon & Koltzenburg, 1992). The most common itch manifestations, pickle and tickle, usually well recognized by humans, provoke similar stereotypical behavioral responses in many mammals, however developing appropriate animal models of itch is still challenging (Dong & Dong, 2018). Itch agents, such as capsaicin and histamine, activate neuronal responses both in the PNS and in CNS eliciting scratching. During inflammation, these pruritogens activate pruriceptors or itch fibers and sensitize nociceptors promoting a great release of mediators, including NGF. The neurotrophin, highly expressed in injured and inflamed tissues, triggers pain signalings via TrkA and promotes the sprouting of nerve fibers in the skin. This mechanism is reported to contribute to itch sensation in skin diseases, such as atopic dermatitis (AD). Indeed, high levels of neurotrophins NGF and BDNF enhance nerve sprouting, resulting in hyper-innervation of the inflamed skin that can lead to chronic invalidating conditions such as hyperkinesis and alloknesis that, similarly to hyperalgesia and allodynia, depend on a lower threshold for nerve fibers activation and induction of itch mediated by non-pruritic stimuli, respectively (Legat, 2021). The increased nerves' density in the skin seems to be responsible for intense itching sensations in patients with atopic dermatitis and it is usually linked to a great release of pro-itchy molecules, including NGF. Plasma analysis of patients with AD has shown high levels of NGF compared to controls, suggesting the involvement of NGF and substance P in the pathogenesis of allergic diseases and a strong correlation between the levels of plasma NGF and the severity of symptoms (Toyoda et al., 2002). In

a mouse model of AD, NC/NgaTnd mice that spontaneously develop atopic dermatitis-like skin lesions, was observed NGF abundance in the skin lesions, especially in keratinocytes, fibroblasts and mast cells of affected skins, and an increased density of fibers PGP9.5-positive in NC/NgaTnd mice, suggesting that an over-production of NGF can contribute to development of itch (Tanaka & Matsuda, 2005). Interestingly, administration of anti-NGF antibodies has been observed to inhibit the development and proliferation of skin lesions and to block the epidermal hyper-innervation also ameliorating the scratching behavior in mice (Takano et al., 2005). NGF is released mainly from keratinocytes and mast cells, and high immunoreactivity to NGF and TrkA is also found in human samples of other skin-related diseases, such as psoriasis, suggesting the role of the NGF-TrkA in the development of itchy symptoms.

Keratinocytes, located in the skin's intercellular spaces, make contact with sensory nerves, including pruriceptive fibers, which convey itch sensation to the upper laminae in the dorsal horns of the spinal cord. Like nociceptors, pruriceptive fibers are usually classified as unmyelinated C- and thinly-myelinated Aδ- fibers that transmit both pain and itch to laminae I/II, as well as lamina V (Dong & Dong, 2018). The itch signal is transferred via the lateral spinothalamic tract, which crosses to the contralateral side, to the thalamus, usually recognized as the relay station for spinal stimuli, and then is transmitted to multiple brain regions involved also in the elaboration of scratching behavior like somatosensory and motor cortices, ACC and PFC associated with hedonic aspects of scratching, and also PAG common to pain-mediated activation (Chen & Sun, 2020).

Pruriceptive sensory fibers express multiple itch-transmitting receptors, some of which belong to the large families of G-protein-coupled receptors (GPCRs) referred to as Mas-related GPCRs (MRGPRs). Many studies have shown that the ablation of MRGPRs lead to profound scratching deficits in chloroquine-induced itch mice, without affecting the response to pain stimuli (Liu et al., 2009; Sun & Chen, 2007). In addition to endogenous pro-itch molecules, a great number of FDA-approved drugs, such as neuromuscular blocking drugs, can elicit allergic reactions by activation of Mrgprb2, the orthologue of the human MRGPRX2 expressed on mast cells and sensory fibers

(McNeil et al., 2015). Interestingly, a subset of pruriceptors expressing MrgprA3 are non-peptidergic primary sensory neurons and co-express histamine receptors H1 and H4 resulting in sensitivity to both chloroquine and histamine (Liu et al., 2009).

The itch sensation elicited by histamine, the best-studied pruritogen, is thought to be dependent also on the activation of interleukin-31 receptor A (IL-31RA) and the downstream activation of TRPV1. Indeed, the photoablation of IL-31RA-positive fibers in the skin of a mouse model of hereditary skin disorder, resulted in the long-term rescue of the scratching behavior evoked by the injection of IL-31 (Nocchi et al., 2019). Other cytokines, such as IL-23 and IL-17A that boost itch signaling via a direct modulation of both histaminergic and non-histaminergic sensory fibers, are reported also to modulate TRPV1 in a not-well understood way (Pavlenko et al., 2020).

Pain and itch, fundamental for the survival of all living beings and for protecting the body from potentially harmful agents, show an extensive overlap in neuronal subpopulations and central circuits mediating the elaboration of both these sensations. Many observations have suggested a crucial involvement of the NGF-TrkA signaling pathway in the development and maintenance of itch, and targeting the NGF-TrkA complex can be useful for developing novel therapeutic approaches for the treatment of diseases associated with pain and itch.

14. NGF-TrkA signaling in thermoregulation

The maintenance of the body's homeostasis is one of the essential functions accomplished by the nervous system. The regulation of the body temperature requires a great number of activities with the aim to produce an optimal thermal environment necessary for survival. Major players of this function are neurons located in the hypothalamus (Morrison, 2016). Changes in neuronal temperature can cause severe alterations of molecular properties, including altered enzyme efficiency, and reduced membrane fluidity that can affect cellular functions, such as energy availability and membrane ion flux. Cold and warm activate a variety of brain responses in order to maintain the body temperature. These brain actions include both autonomic effectors such as thermogenesis, vasodilation, and sweating, as well as behavioral strategies for controlling and maintaining local thermal environment and body temperature that include warmth- or cold-seeking, nesting, or more complex behaviors adopted by humans, such as putting on clothing (Morrison & Nakamura, 2011). To protect the thermal homeostasis of the brain, and, consequently, the body, is necessary a synergistic action of both peripheral and central nervous systems. For example, in rats it has been observed that cool environments activate sympathetic thermogenesis in brown adipose tissue (BAT), metabolic and cardiovascular mechanisms, such as expired CO2, and arterial pressure and/or heart rate. Interestingly, all these events were inhibited by blocking GABAA receptor into hypothalamic areas, including the preoptic area (POA) the dorsomedial hypothalamic nucleus (DMH), and into the rostral raphe pallidus nucleus (rRPa) (Nakamura & Morrison, 2007). Environmental temperature changes are first detected by thermoreceptors in primary sensory afferents in the skin and transmitted through the spinal projecting neurons to both the thalamus and the pontine lateral parabrachial nucleus (LPB) via spino-thalamo-cortical pathways. Thermal informations are finally transferred to POA and DMH in a feedforward mechanism. The POA hypothalamic region is indeed considered a key integration site for thermoregulation because it receives sensory inputs from peripheral thermoreceptors and then

it provides efferent command signals to peripheral effectors through the sympathetic and somatic motor systems **(Figure 13)** (Tan & Knight, 2018).

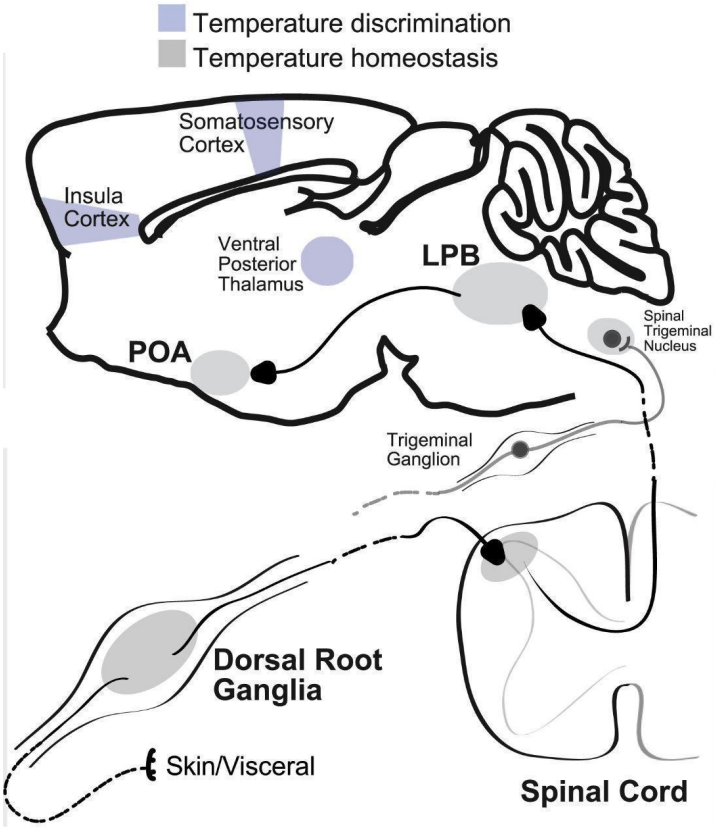


Figure 13. Thermoregulation ascending pathway. Warm and cold are transmitted from the periphery to central brain regions. Adapted from Tan & Knight, 2018.

As mentioned in previous sections, peripheral sensory neurons are able to respond to stimuli of distinct modalities including touch, pain, itch, and temperature. Especially for nociceptors, different elettrophysiological neuronal properties determine the DRG's sensitivity to heat or cold and, for instance, thermoreceptors are considered unmyelinated C-fibers (Ma, 2010). The main peripheral mediators of thermal stimuli belong to the family of TRP ion channels (previous paragraph), and among them, TRPM8 and TRPV1 are considered fundamental thermo-detectors. Indeed, the selective ablation diphtheria toxin-mediated of TRPM8- or TRPV1-expressing DRG neurons leads to a reduction of mild cooling- and heating-evoked neuronal activities in the spinal cord, respectively,

suggesting that TRPM8+ neurons are activated by mild cooling stimuli, whereas TRPV1+ neurons drive spinal responses to heat and strong cold stimuli (Ran et al., 2016). These thermal informations are first transmitted by glutamatergic projecting spinal neurons to LPB, and then to neurons in the POA, whose molecular identity is still debated. For years, traditional models for the thermoregulatory circuit have considered POA neurons, also referred to as warm sensitive neurons (WSN), GABAergic that tonically inhibit central and peripheral structures involved in thermogenesis (Morrison & Nakamura, 2011). However, more recent findings have shown that the chemogenetic or optogenetic activation of glutamatergic VgluT2+ but not GABAergic Vgat+ neurons in POA led to changes in body temperature in mice (Song et al., 2016). The glutamatergic profile of WMS has been supported by results from single-cell RNA-sequencing of ~1 million cells in POA and from discoveries of Machado and colleagues (Machado et al., 2020; Moffitt et al., 2018). Contrary to previous considerations (Nakamura & Morrison, 2011), glutamatergic POA neurons express the EP3 receptor which binds to prostaglandin-2 (PGE2) to stimulate fever via connection with raphe pallidus (RPA) to control BAT thermogenesis, shivering and sweating (Machado et al., 2020). Pyrogenic molecules or bacterial lipids are able to stimulate POA endothelial cells to produce PGE2, that are thought to inhibit the activity of POA neurons causing an uncontrolled increase of body temperature, thereby producing hyperthermia (Nakamura & Morrison, 2011; Tan & Knight, 2018). Against fever, water evaporation is one of thermoregulatory strategies adopted by mammals to dissipate heat and control the body temperature. This process, called sweating, is due to the spread of sweat glands in the skin that are innervated by sympathetic nerves. HSAN IV, caused by mutations in the gene encoding for TrkA, is a congenital insensitivity to pain syndrome characterized also by the inability to sweat or anhidrosis, often associated with recurrent episodes of fever which can be fatal (Indo et al., 1996; Loewenthal et al., 2005; Rosemberg et al., 1994). Interestingly, HSAN IV patients' skin biopsies indicate normal sweat glands, but patients are affected by robust anhidrosis and pharmacological stimuli, such as pilocarpine both systemic and local injections, fail to evoke sweating (Swanson et al., 1963, 1965; Indo, 2018; Itoh et al., 1986). These observations suggest that the affected NGF-TrkA

signaling system can act not only on peripheral sensory and sympathetic nerves but also on central neurons.

A great amount of information about NGF and TrkA in the central nervous system is related to the NGF-target brain regions, as cholinergic neurons of the basal forebrain and hippocampus and cortex, both innervated by cholinergic neurons. Indeed, few studies have investigated the presence of NGF and TrkA in not target-brain regions, such as the hypothalamus. It has been observed in rodents and afterward in humans that NGF expthe ression in hypothalamus is up-regulated by stress conditions, such as intermale fighting, suggesting a possible involvement of hypothalamic NGF in the maintenance of physiologic homeostasis (Alleva et al., 1996). NGF and TrkA mRNA have been detected in rat hypothalamus during the neonatal period, proposing the existence of NGF-sensitive neurons in the hypothalamus itself and/or elsewhere in the brain (Ojeda et al., 1991).

Nowadays, no other findings have been reported about the role of NGF and TrkA in hypothalamus, but this does not rule out that the NGF-TrkA system can impact on thermoregulation, possibly via intra- and inter-hypothalamic connections. Indeed, TrkA mRNA has been found in the paraventricular nucleus of the thalamus (PVT or PVA) (Allen Brain Atlas) that acts as a relay station for the integration of arousal-related signals and in mediating defensive responses (Kirouac, 2021) and modulates core temperature and energy balance during chronic stress (Bhatnagar & Dallman, 1999).

15. Peripheral neuropathies: HSANs

"The great object of life is sensation:

to feel that we exist, even though in pain"

Lord George Gordon Noel Byron, 1863

The ability to perceive a wide variety of different stimuli, especially painful ones, is an evolutionary advantage of all animals because it allows avoiding injuries and damage. However, the universality and necessity of nociception are questioned when some species do not show any nociceptive behavior in response to painful stimulation, such as the naked mole-rats (Smith & Lewin, 2009). Indeed, naked mole-rats do not respond to capsaicin, the active component of chili peppers, nor the sting of acid and their pain insensitivity is linked to mutations in genes encoding for TrkA and the voltage-gated sodium channel, Nav1.7 (Smith et al., 2020).

In humans, an excellent example of pain insensitivity is represented by a heterogeneous group of very rare hereditary neuropathies, named Hereditary Sensory and Autonomic Neuropathies (HSANs). The first HSANs' report dates 1852 and describes chronic ulcerations of the feets of three brothers (Nelaton, 1852). Decades later, HSANs have been classified into four types based on the mode of inheritance, natural history, including onset and symptoms, and type of sensory neurons affected (Dyck et al., 1983) with an estimated incidence of 1 in 25,000 persons (Axelrod & Gold-von Simson, 2007). Nowadays, by novel genetic and diagnosis techniques, eight phenotypically diverse HSAN types have been recognized, with variable involvement of the sensory fibers, both unmyelinated and myelinated peripheral nerve fibers resulting in distinct phenotypes (Schwartzlow & Kazamel, 2019). HSANs are generally caused by mutations in different genes. For instance, missense mutations in SPTLC1 and SPTLC2 (serine palmitoyltransferase ling chain subunit 1 and 2) genes are found in

individuals affected by HSAN type I (Nicholson et al., 1996; Rotthier et al., 2010). Mutations in the endosomal protein RAB7 cause Charcot-Marie-Tooth disease type 2B (Verhoeven et al., 2003), whereas those in WNK1, KIF1A, FAM134B, and SCN9A genes are linked to HSAN type II (Schwartzlow & Kazamel, 2019). HSAN type III, also known as Familial Dysautonomia or Riley-Day syndrome, is caused by mutations in the IKBKAP gene (inhibitor of Kappa-light polypeptide gene enhancer in B cells, kinase complex associated protein) (Slaugenhaupt et al., 2001). Mutations in the genes encoding for TrkA or NGF are responsible for HSAN IV and V, respectively, both characterized by loss of pain and temperature sensation that usually fall into self-mutilations, and frequent bone and joint fractures (Einarsdottir et al., 2004; Indo, 1996). Mutations in genes encoding for DST (dystonin), SCN11A (Nav1.9) and PRDM12 (PRDI-BF1 and RIZ homology domain-containing protein 12) are found to be the main cause of HSAN VI, VII and VIII respectively (Chen et al., 2015; Edvardson et al., 2012; Leipold et al., 2013). All of these eight types generally cause a prominent loss of sensation leading to ulcerations in feet and hands that can degenerate in more severe complications, such as infections or amputations of toes and fingers. Moreover, in patients affected by some HSAN types, such as type IV, VII and VIII, sweating abnormalities, fever, blood pressure fluctuations and mental impairments are usually evident (Schwartzlow & Kazamel, 2019).

The involvement of the NGF-TrkA system in HSAN IV and V disorders has been deeply investigated in the current thesis. Indeed, even though both diseases affect neurotrophic signaling, HSAN IV and V clinical features do not fully overlap, suggesting distinct roles of the TrkA and NGF in these painless syndromes.

In addition to pain and thermal insensitivity, HSAN IV is also accompanied by anhidrosis and mental retardations, profoundly discussed in the following section. On the other hand, HSAN V, also known as Norrbottnian congenital insensitivity to pain, has been first described in three patients belonging to a large multi-generational family located in the Norrbotten region of Sweden and it is associated with the point mutation 661 C>T in the exon 3 of the *NGFB* gene that results in a substitution of tryptophan (W) for arginine (R) at position 221 in the proNGF polypeptide finally corresponding to

residue R100W in mature NGF (Einarsdottir et al., 2004; Testa et al., 2021). Contrary to HSAN IV, patients affected by HSAN V do not present any significant cognitive impairments (De Andrade et al., 2008). Among several HSAN V clinical features, lower legs and feet fractures that appear during childhood in homozygous subjects, and not in heterozygous, can explain the reduced pain sensation at the forearms and legs and the normal sensitivity in the trunk (Capsoni, 2014; Einarsdottir et al., 2004; Minde et al., 2004; Minde et al., 2006). Interestingly, also the cornea of HSAN V patients was found affected by NGF mutations resulting in a decreased number of C-fibers (Perini et al., 2016).

As well as humans, the HSAN V mouse model, recently published by our group, shows impaired thermal and chemical pain sensation and not-affected cognitive abilities, suggesting that the R100W mutation may differentially affect the NGF action in the central and peripheral nervous system (Testa et al., 2019). The phenotype can be explained by the reduced secretion of the mutant NGF^{R100W} protein and by its reduced capability to trigger NGF-mediated pain signaling (Testa et al., 2019b). These and other findings (Sung et al., 2018) have confirmed the necessity of the NGF-TrkA signaling system in pain sensation contributing to greater knowledge about HSAN V disease. However, to obtain a more complete clarification about painless syndromes, it is necessary to develop a mouse model for HSAN IV.

1. HSAN IV

HSAN IV (OMIM# 256800), also referred to as Congenital Insensitivity to Pain with Anhidrosis (CIPA), is an autosomal recessive disorder manifesting at birth or early childhood characterized by insensitivity to pain, self-mutilatory behaviors, anhidrosis (inability to sweat) and variable degree of mental retardation usually described as emotional lability, social deficits and hyperactivity (Indo, 2001; Levy-Erez et al., 2010).

NTRK1, encoding for TrkA receptor, has been the first gene to be identified to cause CIPA (Indo, 1996). More than 100 different mutations in the human *NTRK1* have been detected in HSAN IV patients from various countries *(HGMD 2021.2*). The mutations, including missense, nonsense, frameshift and splicing, are located in the extracellular and/or intracellular domains and correlate to a wide phenotypic severity of affected individuals (Indo, 2001). All mutations in *NTRK1* (or *TRKA*) gene, located on chromosome 1 (1q21-q22), are reported to lead to the impairment of the NGF-TrkA signaling and consequent alterations in NGF-dependent systems in both central and peripheral nervous, thus resulting in the most representative features of HSAN IV: pain insensitivity, anhidrosis and cognitive impairment. HSAN IV results in an extremely dangerous and, in many cases, lethal disease causing the survival of patients until 25-30 years of age. The reported odds of HSAN IV newborns are about 1 in 125 million with a few hundred of recognized cases worldwide (Daneshjou et al., 2012).

The first report of an HSAN IV patient, dated back to the early 1900s, describes the inability of the patient "*to recall any pain except headache and his memory is good"* (Dearborn, 1932). Reported and published cases of HSAN IV have allowed to list all symptoms and collect dozens of pieces of information about the biology of this disease.

Based on previous knowledge about the key role of NGF-TrkA in the development, differentiation and survival of sensory and sympathetic neurons (Levi-Montalcini, 1987), TrkA mutations are considered directly responsible for alterations during HSAN IV embryogenesis. Insensitivity to pain and noxious temperature is caused by the loss of small myelinated and unmyelinated fibers in the PNS that convey both stimuli (Rosemberg et al., 1994; Swanson, 1963). The lack of pain sensation could explain and justify the self-mutilations observed in patients that are missing "self-protective behaviors" (Indo, 2018). As well as in HSAN V patients, nerve and skin biopsies of HSAN IV individuals have shown a reduction of myelinated Aδ- and unmyelinated C-fibers (Indo, 2018; Einarsdottir et al., 2004) that appear less pronounced in heterozygous HSAN V patients justifying the milder phenotype (Minde et al., 2009).

Touch, vibration and position senses are found not affected by *NTRK1* mutations, possibly even because Aβ- LTMRs sensory fibers are unimpaired (Indo, 2002).

The inability to sweat, or anhidrosis, alters the homeostasis of the body temperature and can lead to a higher susceptibility to fever, frequently observed in HSAN IV patients. Recurrent febrile episodes, linked to anhidrosis, are one of the most specific and recognizable clinical signs that begin during infancy or early childhood and can cause hyperthermia or hypothermia (Indo, 2008; Loewenthal et al., 2005). The severity of mental retardation varies across HSAN IV patients ranging from mild to moderate (Indo, 2001, 2018; Li et al., 2019). The cognitive delay is usually described as hyperactivity, symptoms of severe attention-deficit–hyperactivity disorder (ADHD) and social-interaction deficits (Levy-Erez et al., 2010).

Collected genetic analyses of patients have suggested that mutations located in TrkA intracellular domains are linked to a more severe phenotype (Altassan et al., 2017; Indo, 2001; Liu et al., 2018; Shaikh et al., 2017). For instance, point mutations in *NTRK1* gene associated with HSAN IV can strongly reduce the NGF-induced phosphorylation and the kinase activity of the receptor. In vitro studies have shown that missense mutations lead to defects in NGF-mediated autophosphorylation of TrkA receptors. Indeed, the missense mutation R643W found in a Spanish boy was linked to a severe

HSAN IV phenotype (Greco et al., 2000; Mardy et al., 1999; Miranda et al., 2002). Recently it has been found in an 8 years old girl that the missense mutation Arg643Gln was the cause of the anhidrosis, pain insensitivity and mental retardation, ADHD-like (Altassan et al., 2017).

In addition to pain insensitivity, sweating and cognitive defects, HSAN IV is also associated with skeletal disorders such as short stature, tooth loss, and delayed fracture healing (Bonkowsky et al., 2003; Toscano et al., 2000). Clinicians have also reported orthopedic deformities, mainly pronounced at the elbow, knee, and ankle joints in HSAN IV patients that can lead to severe bone complications, such as neuropathic arthropathy or Charcot Joints (Minde et al., 2006; Rotthier et al., 2009; Szoke et al., 1994). These orthopedic deficits can possibly be related to the premature degradation of the retrograde NGF-TrkA transport, already observed in the Charcot-Marie-Tooth type 2B neuropathy (Zhang et al., 2013) and that can be negatively affected by TrkA mutations.

2. Aim

Since the first publications in the ‘90s, big efforts have been put forward in understanding the NGF-TrkA signaling involvement in pain sensation. However, less is known about the role of this pathway in genetic pain-related disorders.

The necessity of a fully functional NGF-TrkA system is evident in congenital forms of insensitivity to pain named Hereditary Sensory and Autonomic Neuropathies (HSANs). Mutations in the TrkA receptor causing HSAN type IV seems to lead to a more pathological phenotype in patients than mutations in the NGF gene, resulting in the loss of pain sensation, anhidrosis and cognitive impairment. However, despite decades of novel insights about nociception and pain from a large number of transgenic mouse models, HSAN IV disease is still missing a suitable mouse model to study this disease.

In this study I aim:

1) to generate and characterize the first animal model of HSAN IV, the knock-in mouse carrying the human TrkA-R649W mutation

2) to elucidate the contribution of the R649W mutation in somatosensation and cognition, behaviorally and histologically

3) to verify the possible novel involvement of TrkA in sweating and thermoregulation

3. Materials and Methods

1. Plasmids for TrkA^{WT} and TrkA^{R649W} expression in cultured cells

Human TrkA^{WT} cDNA sequence (isoform II) in pReceiver-M03 (OmicsLink, ImaGenes, Berlin) was used as a template (Marchetti et al., 2014) and then subcloned in pcDNA3.1 plasmid (Invitrogen). The mutation R649W was obtained starting from wild type sequence in pCDNA3.1, using the QuikChange Site-Directed mutagenesis kit (Agilent) and a pair of specific primers (Forward: CAT TTT GTG CAC TGG GAC CTG GCC ACA CGC; Reverse: GCG TGT GGC CAG GTC CCA GTG CAC AAA ATG). pCDNA3.1-human TrkA^{WT} and pCDNA3.1-human TrkA^{R649W} plasmids were transfected in Hek293 cells to perform western blot and ubiquitination assay described below.

The cloning to obtain S6-tagged human TrkA cDNA sequence in an "all-in-one" third generation Tet-on lentiviral pTRE vector has been described previously (Marchetti et al., 20214; Amodeo et al., 2020a). This construct was used to generate S6-tagged human TrkA^{R649W} mutant, using QuikChange Site-Directed mutagenesis kit (Agilent) and the same pair of primers reported in the paragraph above. The mutant clone (S6-tagged TrkA^{R649W}) was checked by DNA sequencing and used for the transduction of immortalized and primary cells.

2. Cell culture and dorsal root ganglion neuron primary cultures

SK-N-BE (2) (ATCC® CRL-2271™) and SH-SY5Y (ECACC 94030304) cell lines were grown in DMEM/F-12 medium supplemented with 10% Fetal Bovine Serum, 1% Penicillin-Streptomycin, 1% L-Glutamine and 25 mM HEPES. HEK293T/17 cells (ATCC® CRL-11268™) were grown in DMEM High-Glucose (4.5 g/L) medium supplemented with 10% Fetal Bovine Serum, 1% Penicillin-Streptomycin, 1% L-Glutamine, 1% Sodium Pyruvate. Dorsal root ganglion (DRG) neurons were prepared from neonatal (P3-P4) wild type mice, by following the protocol described in (Amodeo et al., 2020b; Testa et al., 2019b), and plated onto coverslips coated with 30 µg/ml poly-D-lysine

(Sigma-Aldrich) and 2 μg/ml laminin (Thermofisher). Dissected neurons were maintained on coverslips in Primary Neuron Basal medium (PNBM, Lonza) supplemented with 1% L-glutamine (Lonza), 0.1% Gentamicin Sulfate/Amphotericin-B (Lonza), 2% NSF-1 (Lonza), and 50 ng/ml of mouse NGF. Twenty-four hrs after seeding, 2.5 μM cytosine β-d-arabinoside (AraC, Sigma) was added to inhibit glia proliferation. Neuron culture medium was changed every 3–4 days, removing about 1/3 of the volume and substituting it with warm, fresh Neuron Growth Medium.

3. Western Blot

Hek293 cells were transfected with pCDNA3.1-human $TrkA^{WT}$ and pCDNA3.1-human $TrkA^{R649W}$ plasmids following the manufacturer instructions for Invitrogen Lipofectamine 2000 (Thermo Fisher Scientific). Forty-eight hours transfection, Hek293 cells were stimulated with NGF^{WT} (100 ng/ml) for 30 minutes or maintained in basal conditions and then lysed in RIPA buffer. Equal amounts of cell extracts were resolved by SDS-PAGE (10%), transferred on nitrocellulose membranes and probed overnight with primary antibodies: anti-TRKA (1:1,000, Cell Signaling #2505), anti-phospho-TRKA (1:1000, Cell Signaling #9141), anti-Tubulin (1:10000, Sigma #T6074). The primary antibody was detected using an appropriate secondary antibody. To analyze the state of ubiquitination of TrkA, samples were subjected to western blot using an anti-Ubiquitin (1:500, Santa Cruz P4D1) antibody that detects both poly- and mono-ubiquitinated proteins. The signal was revealed with ECL solutions (BioRad) and acquired using a ChemiDoc system (BioRad). The optical density was quantified using the ImageJ software (NIH).

4. Viral transduction of immortalized and primary cells

Lentiviral particles containing S6-tagged $TrkA^{WT}$ or S6-tagged $TrkA^{R649W}$ were produced and concentrated following the procedure described in Gobbo, Bonsignore et al., 2018. One day before transduction, about 0.8×10^5 of SK-N-BE neuroblastoma cells (ATCC) were seeded in a 30mm-

diameter culture dish and incubated at 37 °C, 5% CO_2. On the day of transduction, after the removal of all culture medium and two washes with PBS (supplemented with 1 mM $CaCl_2$, 0.5 mM $MgCl_2$), 0.36 mL of non-supplemented DMEM/F12 medium containing 35 µL of concentrated viral stock (1–2.5 × 10^7 infection-forming units per ml) and 4 µg/ml polybrene (Sigma-Aldrich) was added to the cultures. Cells were incubated at 37 °C, 5% CO_2 for 1 h. Then, the infection medium was replaced with a complete neuroblastoma cell medium to allow the transgene integration for at least 48 hrs. Cells were then split and seeded in Willco glass-bottom dishes for cell imaging.

For the transduction of neurons, the day after their plating 1 mL of warm PNBM supplemented with 4 µg/mL polybrene was prepared and mixed with 70 µL of concentrated viral stock. Then, the solution was vortexed for a few seconds to mix the viral particles. The medium was carefully removed from neuron cultures, and the solution containing the virus was added to the cultures and incubated at 37 °C in a 5% CO_2 humidified chamber for 2 hrs. Then, the infection medium was removed and fresh PNBM supplemented with 50 ng/ml of mouse NGF and 2.5 µM Ara-C was added. Cells were maintained at 37 °C under 5% CO_2 humidified atmosphere for 48 hrs. Then transgene expression was induced by adding 1 µg/ml doxycycline for additional 24 hrs before performing the experiment.

5. Single molecule Q-dot labeling of surface TrkA in SK-N-BE cells

Forty-eight hours after the transduction, SK-N-BE cells were starved at 37°C for 2 hrs. Then, surface receptors were labelled with Qdot as described in (Amodeo et al., 2020a). Briefly, cells were first biotinylated with 0.5% BSA, 1 µM Sfp synthase, 10 mM $MgCl_2$ and 2 µM of coA-biotin in starvation medium, for 30 minutes at 37 °C. After two washes in PBS, cells were incubated for 2 min at room temperature (RT) with 2 nM Qdot® 655 streptavidin conjugate (Invitrogen) in borate buffer pH 8.3, 0.5% BSA and 215 mM sucrose. Cells were washed with PBS and left in medium. Finally, cells were stimulated with 125 ng/mL NGF or maintained in starvation medium. The addition of NGF was

performed directly on the dish at the microscope, and cells were imaged for a maximum of 15 min upon ligand addition.

6. Total internal reflection fluorescence (TIRF) imaging

Cells prepared as described above were imaged at 37 °C, 5% CO_2 with a Leica DM6000 microscope equipped with a TIRF-AM module, incubator chamber, electron multiplying charge-coupled-device (CCD) camera (ImagEM C9100-13, Hamamatsu), and 100× oil immersion objective (NA 1.47). For live cell imaging, time series were acquired on a ROI with constant size of 32.7 × 34.5 μm within the basal membrane of each cell. Qdot655 was imaged using the 488 nm laser line, FF01-655/15 Semrock emission filter and a penetration depth of 110 nm. The integration time per frame, corresponding to the lag time between two consecutive frames, was set at 21 ms and typical time series lasted 3000 frames. The analysis of TrkA membrane dynamics was performed as reported in (Marchetti et al., 2013). We also quantified the density of spots corresponding to labelled membrane receptors by manually counting them and dividing this number for the basal cell membrane area.

7. Single molecule internalization assay

The single molecule internalization assay of TrkA-WT versus TrkA-R649W mutant transduced in SHSY5Y cells was performed as previously described (Amodeo et al, 2020a). Briefly, cells seeded in glass-bottom WillCo dishes were starved for 2 hours, receptors labelled with Qdot and transferred at the TIRF microscope. The position of 4-5 fields displaying labelled cells was saved at the automatized stage. Then 125 ng/ml NGF was added to the medium and the cells of the selected fields were followed in a time course of eight points (0, 5, 10, 15, 30, 40, 50 and 60 min). For each cell and time point, we quantified the number of receptor spots per area. For comparing the internalization time-course of different cells, we normalized the spot density of each cell to its value at time 0. Cells with similar expression level were chosen, excluding those with a number of moving receptors below.

8. Cell surface labeling of TrkA by Qdots and immunofluorescence in DRG neurons

Forty-eight hours after the transduction, DRG neurons were starved at 37°C for 1 hour, then the membrane pool of receptors was biotinylated in two steps: i) 30 minutes at 37°C with 10 μM Coenzyme A-biotin, 10 mM $MgCl_2$ and 2 μM Sfp synthase resuspended in cell medium and ii) 60 minutes at 4°C with the same mix. Cells were washed two times with Hanks' Balanced Salt solution (HBSS, Sigma Aldrich-55021C) and incubated with 125-ng/ml NGF at 37°C for 60 minutes or were maintained in basal medium condition. After three washes with HBSS, cells were labelled at 4° for 15 minutes with 10 nM of streptavidin-Qdot (Qdot® 655 streptavidin conjugate; Invitrogen) in borate buffer at pH 8.3, 0.5% BSA and 215 mM sucrose. Cells were washed five times with HBSS and then fixed at RT for 15 minutes in PBS with 2% paraformaldehyde (PFA) and 5% of sucrose. After four washes with HBSS, neurons were permeabilized at RT for 5 minutes with a solution of PBS supplemented with 2.5% BSA and 0.1% Triton-X100. Neurons were blocked at RT for 1 hour with a solution of 5% BSA in PBS and incubated at RT for 2 hours with anti-TrkA (Millipore, 06-574, dilution 10 μg/ml) in PBS and 2.5% BSA. After three washes with PBS, cells were incubated at RT for 1 hour with anti-rabbit Alexa 488 antibody (Thermo Fisher, dilution 1:100). The coverslips were finally mounted using Fluoroshield mounting medium (Sigma-Aldrich). Qdot655 was imaged using the 488 nm laser line, FF01-655/15 Semrock emission filter and a penetration depth of 110 nm while Alexa-488 using the 488 nm laser line with a 482-510 excitation filter and a 525/20 Leica emission filter.

For the analysis of the TrkA membrane versus total pool, ImageJ software was used. In detail, the fluorescence value of the background was subtracted in both Qdot (surface TrkA) and Alexa647 (total TrkA) channels. Then, a mask was drawn around the cell and the fluorescence intensity of Qdot655 ($I_{Qdot655}$) and Alexa647 ($I_{Alexa647}$) was measured. The fraction of surface S6-TrkA was derived by calculating the $I_{Qdot655}$ / $I_{Alexa647}$ ratio, assuming a constant contribution of endogenous wt TrkA to the $I_{Alexa647}$ value.

9. Ethics statement on mouse experiments

All experimental procedures were performed in accordance with the Ministry of Health guidelines (Legislative Decree n°26/2014) and European Union 128 (Directive n°2010/63/UE) laws on animal research. The experimental protocol 45/2019-PR was previously approved by the Ministry of Health. The experiments were carried out in accordance with the ARRIVE guidelines (Animal Research: Reporting in Vivo Experiments) and the principles of the Basel Declaration, including the "3R" concept. Efforts were made to reduce the number and discomfort of animals throughout the study.

10. Generation of knock-in human TkrA$^{R649W/m}$ mice

To generate the human TrkA$^{R649W/m}$ mouse line, the starting point was the targeting vector AMB1-Tg-pA, containing the coding sequence of the human NTRK1 (TRKA) and from the corresponding humanized wild-type TrkA knock-in mouse line (AMB1-TrkA/170608), both of which were kindly provided by Glenmark Pharmaceuticals. This humanized TrkA^{WT} knock-in mouse line is based on the in-frame replacement of the exon 1 coding sequence, as well as part of intron 1, of the murine Ntrk1 gene by the complete coding sequence of the human ortholog *NTRK1* gene.

The mouse *Nrk1* gene is located on chromosome 3 and extends over 16.9 kb containing 17 exons separated by 16 introns, ATG translation initiation codon located in the exon 1 and the STOP codon located in exon 17, 5'-UTR and 3'-UTR are located at 20bp and 171bp respectively.

We performed a site-specific mutagenesis PCR to introduce the HSAN IV R649W mutation in the targeting vector AMB1-Tg-pA containing the coding sequence of the human NTRK1. The mutated AflII-FseI segment from the AMB-Tg-pA vector replaced the AflII-FseI DNA segment of the vector AMB1-HR containing the *TrkA* human cDNA, long and short homology regions and the positive selection neomycin gene flanked by *Lox*P sites. Both plasmids AMB1-Tg-pA and AMB1-HR were supplied by Glenmark Pharmaceuticals.

The final targeting vector AMB1-HR carrying the R649W mutation in the humanized TrkA, was linearized prior to electroporation, then transfected into R1p.15 cells (background SV129) and positive clones were selected using neomycin resistance. Then, positive clones were injected into blastocysts from C57BL/6 mice and chimeric animals were crossed with "Cre-deleter" mice expressing the Cre recombinase under the cytomegalovirus (CMV) promoter (Jackson Laboratories), to remove the neomycin selection cassette.

11. Southern Blot analysis and genotyping

Genomic DNA was extracted by means of phenol:chloroform:isoamyl alcohol from cell clones electroporated with $TrkA^{R649W}$ targeting vector. DNAs were first incubated with StuI (for 5' screening), then positive clones were confirmed by AflII digestion (for 3' screening). Digestions were run on a 0.8% agarose gel O/N at 50 V. After a mild depurination and denaturation, gels were blotted on nitrocellulose, and filters incubated with 5' or 3' probes. The internal 5' probe was located within the 5' homology sequence of TrkA targeting vector and detected an 11.3 kb band in the wild type allele and a 6.4 kb band in recombinant alleles. The external 3' probe was located downstream the 3' homology sequence of the TrkA targeting vector, and labeled a 6.4 kb band in the wild type allele and a 10.9 kb band in recombinant alleles.

Mice were genotyped by PCR. The following PCR primers were used:

fw_human: 5'-CTTGCTTGGCACTGTCCTCTCATGC-3'

rev_human: 5'- TGCACAGCTAACCACTCCTCCATGG-3'

fw_mouse: 5'- TGAGTGTGTGTCGTTCGGG-3'

rev_mouse: 5'- ATGGGCTTAGGAACTTGGGC-3'

Band sizes are: wild-type 343 bp, mutant 442 bp **(Figure 3B).**

12. Behavioral analyses

All behavioral experiments were performed on $TrkA^{R649W/m,}$, $TrkA^{h/m}$, $NGF^{R100W/m}$ and $NGF^{h/m}$ mice at two months of age. Both male and female mice were included. All animals were kept under a 12 h / 12 h light/dark cycle, with food and water *ad libitum*. The experimenter was always blind to the genotype of the mice.

1. Cold sensitivity test

Mice were habituated for 30 minutes on an elevated platform with mesh flooring. Acetone (50 µl; Sigma-Aldrich) was applied onto the plantar surface of the hindpaw using a Hamilton syringe and the responses were scored as a 4-point score: 0 = no response; 1 = paw withdrawal; 2 = repeated flicking of paw; 3 = licking of the paw. The acetone application was repeated six times by alternating the paw with an interval of 5 minutes. The frequency of response (expressed as a percentage) and the mean of the type of response (score response) were evaluated.[vii]

2. Hot plate test

Heat nociception was tested by hot plate test. After a habituation period (30 minutes), mice were placed on a surface heated at 48 °C and the response latency, expressed as flicking or licking of the hindpaw, was noted. In order to avoid injury to the mice, a cut-off of 20 seconds was fixed.

3. Capsaicin injection test

To test the response to a chemical noxious stimulus, capsaicin (catalog #141000, Abcam) was injected into the hindpaw of mice. Capsaicin was dissolved in dimethylsulfoxide (DMSO) and then diluted in saline to obtain the final concentration equal to 9 µg/µl. A total amount of 10 µl was injected in the ventral surface of the hindpaw using a Hamilton syringe. After the injection, mice were observed for 15 minutes and the time spent in licking the injected paw was measured. Control mice were injected with 10 µl of 0.1% DMSO in saline.

4. Tape response assay

Mice were acclimated in a plastic cage for 30 minutes. Then, a 3-cm piece of adhesive tape was placed gently on the mouse's back. Mice were observed for 5 minutes and the total number of bouts in response to the tape was recorded. Biting or scratching or "wet dog shake" motion was scored as a response (Ranade et al., 2014).

5. Mechanical sensitivity

Mechanical sensitivity was measured using the Von Frey Dynamic Plantar Aesthesiometer (Ugo Basile, Italy), which generates a mechanical force linearly increasing with time. The cutoff force was set at 20 grams. After habituation (30 minutes) on an elevated platform with mesh flooring, the plantar surface of the mice hindpaw was stimulated by a single non-flexible filament and the force intensity was scored.

6. Spontaneous alternation Y-Maze test

A PVC maze consisting of three identical arms (40 × 13 × 10 cm) that converged at an equal angle was employed. Each mouse was placed in the center of the maze and allowed to explore freely the arms during an 8-min session and recorded by video (Noldus Ethovision XT). An entry was scored when the mouse was at least halfway through an arm of the maze. An alternation was scored when all the three arms were entered in consecutive events. The percentage of spontaneous alternations (% SAP) was calculated according to the following formula: % SAP = number of alternations / total entries × 100.

7. Elevated plus maze

The elevated plus maze consisted of two closed arms and two open arms (each 30 × 5 cm) extending from a central platform at 90°. Mice were placed on the central platform and allowed to freely explore the maze. The times spent in the open arms, closed arms and center were measured.

8. Novel Object Recognition Test

The test was performed in 3 days. On day 1, mice were allowed to explore the empty arena (60 × 60 × 30 cm) for 10 minutes (habituation phase). On day 2, mice were exposed to two identical objects for 10 minutes to evaluate the total exploration time. On day 3, mice were exposed to a familiar object (namely A) and a new novel object (namely B) for 7 minutes (memory phase). The time spent exploring each object was recorded every day. We calculated a preference index by dividing the amount of time spent exploring the novel object by the total time spent exploring both objects. Mettere come è stato calcolato il preference index.

9. Three-chamber social approach test

Social approach behavior in mice was tested in the three-chamber apparatus as in Chadman et al., 2008, with minor modifications. First, the test mouse was habituated to the apparatus for 10-min in

the center chamber, and then for an additional 10-min with access to all three chambers. Then, the first stranger mouse (namely S1) and a novel object were placed into the two wire cups located in the opposite chambers of the apparatus (sociability phase). The test mouse was free to explore all three chambers for 10 minutes. The location of the novel object and stranger mouse was alternated across subjects. The time spent with the stranger mouse or the novel object was recorded. After the sociability phase, to evaluate the preference for social novelty, the test mouse was exposed for 10-min to a second novel stranger mouse (namely S2) which was placed into the wire cup where the object was previously located.

13. Sweat assay

A slightly modified standard method of sweat assay was performed (Tafari et al., 1997). Mice were anesthetized (Zolpidem/xylazine 80-10 mg/kg) and pilocarpine (catalog #P6503, Sigma) was injected subcutaneously into the plantar surface of the foot (50µg in 5µL 0.9% saline). The injected paw was painted with 3.5% iodine (catalog #207772, Sigma-Aldrich) in ethanol, followed by coating with 10% starch solution in costar oil (both from Sigma-Aldrich). Sweating induced by pilocarpine was revealed by black spot formations on the plantar surface of the paw. The number of dark spots was measured 2, 5 and 10 minutes later (Liu et al., 2017).

14. Immunohistochemistry

$TrkA^{h/m}$ and $TrkA^{R649W/m}$ mice were transcardially perfused with 4% PFA in PBS (pH 7.4%) and brains were dissected and post-fixed in the same solution, then cryoprotected in 30% sucrose in PBS for 72 hrs. Forty-five µm-thick coronal sections were obtained using a freezing sliding microtome (Leica) and were collected in PBS and then immediately processed for immunohistochemistry. In detail, brain sections were washed 3 times in TBS with 0.3% Triton X-100, then treated with 3.5% H_20_2 in TBS to inactivate endogenous peroxidases. Brain sections were treated with 10% FBS-0.3% Triton X-100 in TBS blocking solution for 30 minutes at RT. Then, the primary antibody (goat anti-

ChAT 1:500 #AB144P, Millipore) was diluted in the same blocking solution and incubated 4°C O/N. The following day, after washing in TBS, the biotinylated anti-goat secondary antibody (Vector Laboratories) was diluted in 10% FBS in TBS for 3 hrs at RT. Sections were incubated in Vectastain ABC HRP Kit (Vector Laboratories) in PBS for 1 h and subsequently incubated in 3,3'-diaminobenzidine HCl (DAB, Sigma-Aldrich) and the enzyme Glucose Oxidase Type VII (Sigma-Aldrich) in TBS solution. The reaction was stopped within 10 minutes. Lastly, stained sections were mounted on glass slides, coverslipped using DPX Medium and acquired with a Nikon Eclipse E600 optical microscope. ImageJ program was used to measure the density of immunoreactive cells.

15. Skin and DRG Immunofluorescence

DRG and skin (hairy and glabrous) were dissected and fixed with 4% PFA for 2h or O/N 4°C, respectively. Tissues were cryoprotected in 30% sucrose in PBS O/N at 4°C, then embedded in OCT medium (Leica). Both DRG and skin were sectioned at a thickness of 40 µm using a cryostat (Leica). Sections were then treated with cold 50% ethanol for 30 minutes, followed by 2% BSA-0.3% Triton-X in PBS blocking solution for 1h at RT, finally incubated with primary antibodies in blocking solution O/N at 4°C. Secondary antibodies were diluted in blocking solution and incubated for 2 hrs at RT. Sections were mounted using DAPI-Fluoroshield™ Medium (Sigma). All images were acquired at 40× with a Leica SP2 confocal microscope and analyzed with ImageJ-Fiji (NIH, MD, 312 USA).

The following primary antibodies were used: 1:300 mouse anti-TRPV1 (Neuromics), 1:50 mouse anti-TrkA (MNAC13, 5,4µg/µl) (Cattaneo et al., 1999), 1:200 mouse anti-CGRP (Immunostar), 1:100 isolectin GS-B4-biotin conjugate (Invitrogen), 1:300 guinea pig anti-parvalbumin (Synaptic System), 1:200 rabbit anti-tyrosine hydroxylase (Millipore), 1:500 mouse anti-NF200 (Sigma), 1:200 rabbit anti-PGP 9.5 (Dako-Agilent). All Alexa-conjugated secondary antibodies were used at 1:1000 concentration.

4. Results

1. Choice of HSAN IV mutation

HSAN IV is a rare autosomal recessive disorder linked to mutations in *NTRK1*. Genetic analyses of HSAN IV patients have identified more than 100 mutations in *NTRK1* (HGMD 2021.4). Mutations are distributed all along the protein sequence of TrkA, from the extracellular to the kinase domain, but most of them are missense and non-sense mutations in the cytoplasmic domain of the receptor. Missense mutations affecting the kinase domain disturb the ligand-induced kinase activity of TrkA. The functional analysis of a group of missense mutations in tyrosine kinase domains (TKD), including Arg649Trp (R649W), shows a diminished kinase activity and reduced autophosphorylation after NGF stimulation in transfected cells (Mardy et al., 1999; Miranda et al., 2002, and this manuscript). The importance of the Arg in 649 positions (R649-) has also been observed by Altassan and colleagues (Altassan et al., 2017), investigating HSAN IV patient carrying the TrkA R649Q mutation. The Arg649- substitution seems to alter the residue charge affecting the stability of TrkA receptor during NGF-binding and leading to altered phosphorylation. The neighbor mutations L694P and G571R cause a similar R649W phenotype leading to pain and temperature insensitivity, anhidrosis and speech delay in patients pointing out the robust link between TKD TrkAmutations and HSAN IV disease (Altassan et al., 2017; Greco et al., 2000; Indo et al., 1996). Indeed, structural mapping of HSAN IV TrkA variants has recently indicated that mutations located in TKD can affect the TrkA interaction with substrates, such as PLCγ, and these damages in TrkA-PLCγ interactions may have an analgesic effect on pain states in mice (Moraes et al., 2022), given that the recruitment of PLCγ to TrkA is essential for NGF-mediated sensitization (Chuang et al., 2001). In addition to mutations in the TKD mutations, other HSAN IV TrkA mutations are located in the extracellular domain, such as L213P. This class of mutations causes TrkA retention in the ER, impairing the export of TrkA to the membrane and its trafficking (Mardy et al. 2001; Miranda et al. 2002). There are, as well, nucleotide deletions that cause a frameshift introducing a premature stop codon, as with the Gly181fsX58

mutation located in the extracellular domain that results in a truncated TrkA impairing the NGF-TrkA binding (Verpoorten et al., 2006). Finally, other mutations such as the supposed HSAN IV-linked C752S mutation do not affect the TrkA autophosphorylation and trafficking, nor neurite outgrowth in cell assays (Shaikh et al., 2016). Since the diversity of these HSAN IV mutations determines variable degrees of clinical phenotype and intellectual disabilities in affected individuals, we have decided to investigate in depth the missense Arg649Trp (R649W) mutation, located in TrkA TKD (**Fig. 1A**), first identified in a Spanish family (Mardy et al., 1999), with the ultimate goal to shed light on the contribution of TrkA TKD mutations to HSAN IV disease.

After a biochemical and biophysical characterization of the mutant TrkA receptor, we generated the HSAN IV $TrkA^{R649W/m}$ knock-in mouse line and, as the control group, we used the heterozygous (AMB1-TrkA/170608) $TrkA^{h/m}$ mice, which represent the most proper control as they underwent the same knock-in strategy as $TrkA^{R649W/m}$ mice, to achieve the expression of the wild type human NTRK1 in the corresponding mouse locus.

2. The TrkA^{R649W} mutant receptor shows reduced NGF-induced phosphorylation

We first aimed to analyze whether the selected R649W mutation may affect the response of TrkA to the neurotrophin NGF. In line with previous findings (Mardy et al., 1999, 2001; Miranda et al., 2002), we found that NGF binding to human TrkA^{R649W} mutant receptors, expressed by transfection in HEK293 cells, resulted in a significantly reduced phosphorylation in comparison to TrkA^{WT}, whereas, the total amount of protein was not altered (**Fig. 1B**).

In addition, we tested TrkA ubiquitination (Arevalo et al., 2006; Sánchez-Sánchez & Arévalo, 2017), a physiologically important mechanism regulating TrkA function *in vivo*, also in nociceptive neurons (Yu et al., 2014; Kiris et al., 2014). We found that the R649W mutation resulted in a pronounced reduction of constitutive ubiquitination in HEK293 cells, compared to control TrkA^{WT} (**Fig. 1C**). We also observed that the TrkA immature form (approx. 110 kDa and represented by the lower band) appears less intense than the TrkA mature form (approx. 140 kDa and indicated as the upper band), suggesting possible damages in N-linked glycosylation events. However, further studies are needed to clarify whether and how the R649W mutation affects glycosylation events on the receptor.

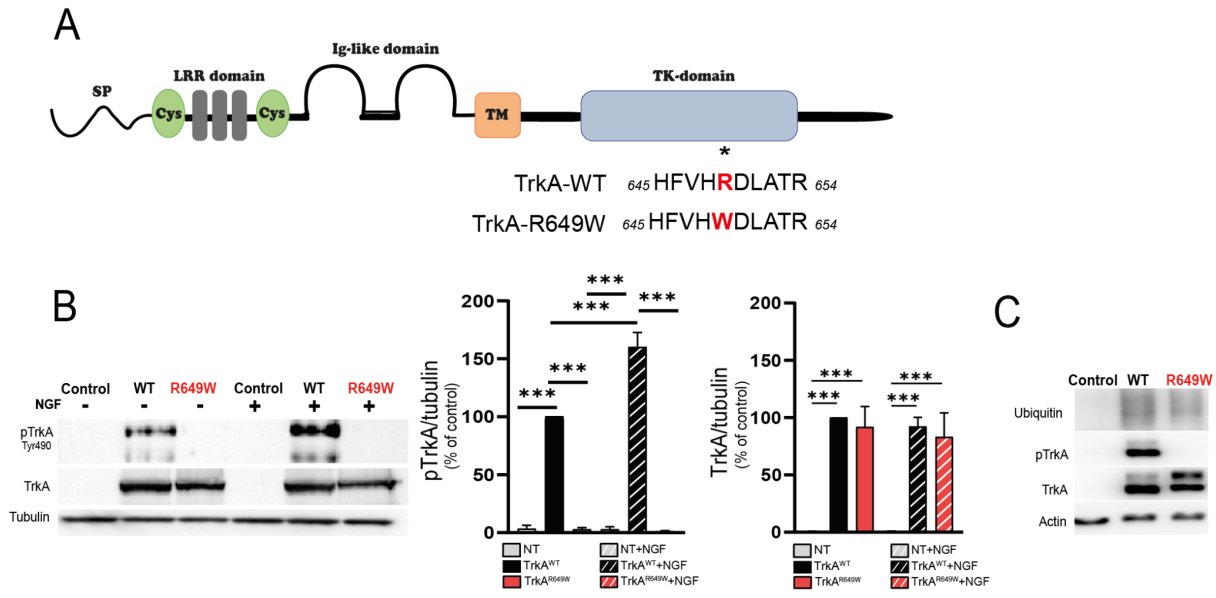


Figure 1: The R649W mutation affects TrkA phosphorylation and ubiquitination. (A) Schematic cartoon of TrkA domains. The asterisk shows the position of the R649W mutation. Bottom: Amino acid sequence alignment of the tyrosine kinase domain of human TrkA. Mutated residue is indicated in red. (SP: signal peptide. Cys: cysteine-rich domains. LRR: Leucine-rich region.

Ig: immunoglobulin-like domain. TM: transmembrane domain. TK: tyrosine kinase domain). **(B)** Representative western blot (WB) and quantification showing TrkA phosphorylation and total TrkA levels in Hek293 cells transfected with human $TrkA^{WT}$ and human $TrkA^{R649W}$, in the presence (+) or absence (-) of stimulation with 100ng/ml of NGF for 30 minutes. Tubulin was used as a loading control in WB. The mutation severely affects the response of mutant TrkA to NGF stimulation. Two-way ANOVA $F_{(2, 12)} = 22{,}264$, p=<0,001 followed by Bonferroni post-hoc test (*** p= <0,001); TrkA: Two-way ANOVA $F_{(2, 9)} = 0{,}226$, p=<0,001 followed by Bonferroni post-hoc test (*** p= <0,001). (**C**) Representative WB with anti-ubiquitin antibodies showing that constitutive ubiquitination of $TrkA^{R649W}$ is significantly reduced with respect to $TrkA^{WT}$.

3. R649W mutation alters TrkA membrane dynamics in transfected cells

To elucidate the biophysical properties of the TrkA mutant receptor, $TrkA^{R649W}$, we have taken advantage of the expertise of Laura Marchetti and Rosy Amodeo, researchers at the National Enterprise for nanoScience and nanoTechnology Center (NEST) of Scuola Normale Superiore.

Indeed, given that NGF-induced membrane mobility and tyrosine kinase activity of TrkA are correlated (Amodeo et al., 2020a), the mobility of the $TrkA^{R649W}$ receptor on the membrane of living cells was investigated by single-particle tracking and TIRF microscopy (Marchetti et al., 2013). This method relies on the site-directed stoichiometric labeling of the TrkA with brilliant organic dyes or quantum dots via the S6 chemical tag inserted at its N-terminus (Amodeo et al., 2020b). The S6-tagged TrkA receptor remains fully functional (Marchetti et al., 2013; Amodeo et al., 2020a). The mutant $TrkA^{R649W}$ receptor was therefore tagged with the S6 tag and labeled, in order to visualize and record its activity. To do so, we performed single-particle tracking (SPT) measurements of $TrkA^{WT}$ and $TrkA^{R649W}$ membrane diffusion in living human neuroblastoma SK-N-BE cells, in order to investigate if and how the R649W mutation influences the receptor dynamics. Quantitative analysis of the diffusion coefficient (D) confirmed the typical bimodal distribution of D values of $TrkA^{WT}$ trajectories, and the inversion of the relative population of the fast and slow D peaks induced by NGF stimulation (Marchetti et al., 2013; Amodeo et al., 2020a). Conversely, $TrkA^{R649W}$ trajectories appeared to diffuse almost 4 times more slowly than $TrkA^{WT}$ ones, both in the absence and in the

presence of NGF **(Fig. 2A).** This result was further supported by the analysis of the 2D distribution of D and γ coefficients, calculated using the MSS-TAD algorithm (Callegari et al., 2012; Marchetti et al., 2013). This analysis showed that the trajectories of single $TrkA^{R649W}$ receptors were not modulated by NGF stimulation, as opposed to the strong modulation of single-particle membrane dynamics induced by NGF in wild-type TrkA receptors **(Fig. 2B).** This shows that the main determinant of the membrane dynamics of TrkA receptors is not the NGF binding event, but its NGF-induced phosphorylation.

We then analysed by TIRF microscopy the membrane pools of $TrkA^{WT}$ and $TrkA^{R649W}$ receptors, labeled with Qdots. Quantification of the density of labeled receptors per cell area (n. spots/μm^2) highlighted an increased membrane pool in cells expressing $TrkA^{R649W}$ compared to wild-type human TrkA **(Fig. 2C),** despite the total amount of TrkA protein being the same, as shown in **figure 1B**.

The kinetics of NGF-induced internalization of $TrkA^{WT}$ and $TrkA^{R649W}$ receptors, was investigated in SK-N-BE cells by quantifying the density of Qdot-labeled receptors exposed on the cell membrane at different times after NGF addition (**Fig. 2D**). The normalized densities of Qdot-labeled spots were almost superimposable for $TrkA^{WT}$ and $TrkA^{R649W}$ receptors, at all time points analysed, showing that the kinetics of NGF-induced internalization of $TrkA^{WT}$ and $TrkA^{R649W}$ receptors are the same (**Fig. 2D**). In order to have a more comprehensive view of the membrane trafficking of the two TrkA receptors, these were transfected in primary Dorsal Root Ganglia (DRG) neurons. S6-tagged $TrkA^{WT}$ and $TrkA^{R649W}$ were labelled with Qdots, while immunofluorescence using a-TrkA antibody was used to mark the whole receptor pool. Importantly, S6 labelling was performed either in resting conditions or after a 1-hour incubation of the neurons with 125 ng/ml NGF. This time window allows us to appreciate both ligand-induced TrkA internalization and recycling back to the membrane (Chen et al., 2005). Quantification of the Qdot/TrkA ratio in DRG neurons showed that the $TrkA^{R649W}$ membrane pool, normalized to the total amount of receptor, is increased with respect to that of $TrkA^{WT}$ **(Fig. 2E)** also in DRG neurons. However, upon NGF stimulation for one hour, the increased membrane pool of $TrkA^{R649W}$ was drastically reduced, with respect to the membrane pool of $TrkA^{WT}$,

which remained constant **(Fig. 2E)**. This effect was even stronger when the same quantification was selectively performed at the level of the growth cones, accounting for a ~72% average reduction of $TrkA^{R649W}$ membrane pool, while no effect is observed in cells expressing $TrkA^{WT}$ **(Fig. 2F)**. Taken together, the data obtained in SK-N-BE and DRG cells show that the mutant $TrkA^{R649W}$ displays a higher membrane abundance than $TrkA^{WT}$, which can be a consequence of alterations in recycling due to the reduced basal ubiquitination. Altogether, these profound alterations deserved a detailed investigation of the *in vivo* consequences of $TrkA^{R649W}$ mutation, prompting us to develop a genetic mouse model of the HSAN IV disease.

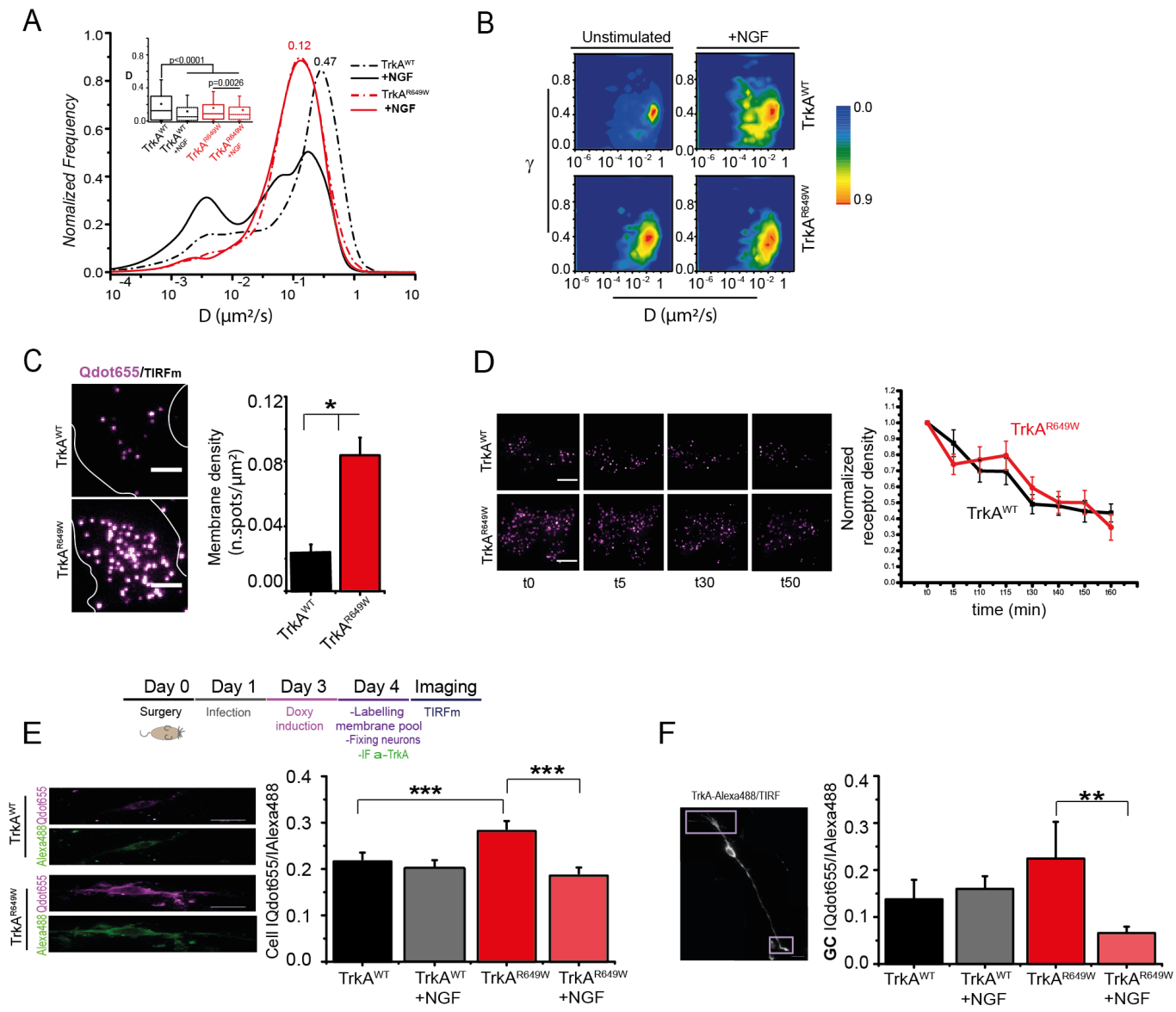


Figure 2: Effect of R649W mutation on TrkA membrane dynamics: biophysical studies. (A) Single Particle Tracking of $TrkA^{WT}$ and $TrkA^{R649W}$ receptors on the membrane of living human neuroblastoma SK-N-BE cells, viewed by TIRF microscopy. Distribution of diffusion coefficient (D) obtained from $TrkA^{WT}$ trajectories before (black solid curve, n=3369 trajectories from 25 cells) and after NGF administration (125 ng/mL for 15 minutes) (black dotted curve, n= 3283 trajectories from 28 cells) and for $TrkA^{R649W}$ trajectories before (red solid curve, n= 3638 trajectories from 31 cells) and after NGF administration (red dotted curve, n= 3375 trajectories from 27 cells). On the left: box-plot for D values retrieved from the same trajectories (at least 6 frames long) of $TrkA^{WT}$ before (black solid) and after NGF administration (black dotted) and for $TrkA^{R649W}$ before (red solid) and after NGF administration (red dotted). Trajectories are pooled from two independent measures. Boxes: 25^{th}-75^{th} percentiles; whiskers: 10^{th}-90^{th} percentile; line: median; square: mean. $P<0.0001$ and $p=0.0026$ according to Kruskal-Wallis test, with Dunn's means comparison. (**B)** Total D-γ distributions according to MSS-TAD analysis of the same trajectories reported in panel A, for $TrkA^{WT}$ and $TrkA^{R649W}$ before and after NGF administration. On the right, logarithmic-scale color code for the frequency of the total D-γ distributions, normalized to 1 at the peak. (**C)** TIRF images of Qdot-labelled single receptor molecules of $TrkA^{WT}$ (upper panel) and $TrkA^{R649W}$ (lower panel); scale bar=10 μm. On the right: quantification of density of labeled receptors per cell area (n.spots/μm^2) in SHSY5Y cells, obtained from three experimental replicates (n=22 cells for $TrkA^{WT}$ n=12 cells for $TrkA^{R649W}$). *$p<0.05$ according to two-tailed Mann-Whitney test. **(D) Left**: Representative TIRF images of single receptor spots of $TrkA^{WT}$ (top) and $TrkA^{R649W}$ (bottom) during a time-course after NGF stimulation. Every image corresponds to a time point of the same cell: t0 (time of NGF administration), t5, t30, t50 minutes. Scale bar=10 μm. **Right**: Normalized membrane density for $TrkA^{WT}$ (black) and $rTrkA^{R649W}$ (red) is reported as mean±sem from cells acquired at each time point normalized for the respective density at time 0. $p_{construct}>0.05$, $p_{time}<0.001$, and $p_{constructxtime}>0.05$ according to a two-way ANOVA test. All data are pools from 36 ($TrkA^{WT}$) and 59 ($TrkA^{R649W}$) cells, collected in two independent replicas. **(E)** Schematic timeline of the experimental procedure for the detection of the TrkA membrane pool in DRG neurons. **Left**: TIRF images of Qdot-labelled membrane $TrkA^{WT}$ and $TrkA^{R649W}$ receptors (magenta) and corresponding immunostained DRG neurons (green) infected with lentiviral particles bearing S6-tagged $TrkA^{WT}$ and $TrkA^{R649W}$ transgenes. Scale bar: 20 m. **Right**: Quantification of the membrane pool fraction on the total pool of $TrkA^{WT}$ and $TrkA^{R649W}$ in DRG neurons before and after 1h of NGF stimulation (125ng/ml). Quantification is obtained from the ratio between the intensity of Qdot signal (membrane receptors) against the intensity of Alexa488 (TrkA immunostaining) measured in the whole neuron ($TrkA^{WT}$ n= 102 neurons; $TrkA^{WT}$+NGF n=21 neurons; $TrkA^{R649W}$ n=87 neurons, $TrkA^{R649W}$+NGF n=43 neurons). *** $p<0,001$ according to Kruskal-Wallis Test. **(F) Left**: representative TIRF image of DRG neuron immunostained against TrkA with secondary antibody conjugated with Alexa488; growth cones are within the light purple boxes; scale bar: 20nm. **Right**: quantification of TrkA membrane pool at growth cones before ($TrkA^{WT}$ n=17 neurons and $TrkA^{R649W}$ m=11 neurons) and after 1h of NGF stimulation (125 ng/ml) ($TrkA^{WT}$ n=10 neurons and $TrkA^{R649W}$ n=10 neurons); **$p<0,01$ according to Kruskal-Wallis test.

4. Generation of TrkA^{R649W} knock-in mice: early postnatal lethality of homozygous mice

In order to generate a knock-in mouse line for HSAN IV carrying the R649W mutation in the human *NTRK1* (*TRKA*) gene, we adopted a targeted gene approach based on the in-frame replacement of the exon 1 coding sequence, as well as of part of intron 1, of the murine NTRK1 gene with the complete coding sequence of the human ortholog *NTRK1* gene, yielding the humanized wild-type TrkA knock-in mouse line (AMB1-TrkA/170608). To generate the human TrkA^{R649W} knock-in mouse line, the starting point was the targeting vector AMB1-Tg-pA, containing the coding sequence of the human NTRK1 (TRKA), that was used to generate the humanized TrkA knock-in mouse line (AMB1-TrkA/170608, or TrkA$^{h/m}$ mice). We used the targeting vector AMB1-Tg-pA, containing the human *NTRK1* cDNA coding sequence, to introduce the HSAN IV R649W mutation in human *NTRK1* cDNA by site-specific mutagenesis PCR. As shown in Figure **3A**, the *NTRK1* cDNA cassette, followed at the 3' end by an exogenous hGH polyA cassette and a loxP-neomycin-loxP cassette, replaced the exon 1 as well as part of intron 1 of the murine Ntrk1 gene. The resulting mouse line was mated with "Cre-deleter" mice to remove the neomycin selection cassette (**Fig. 3A**), thus generating heterozygous mice carrying the human mutant TrkA R649W allele (TrkA$^{R649W/m}$ mice). Homozygous mice (TrkA$^{R649W/R649W}$) were obtained by cross-breeding heterozygous mice (TrkA$^{R649W/m}$ mice) carrying one human TrkA^{R649W} allele and one murine allele. While heterozygous mice thrive to adulthood, TrkA$^{R649W/R649W}$ mice die within the first week of life. Representative pictures of pups at P0 show that at birth TrkA$^{R649W/R649W}$ pups were normal and the body size was comparable to that of heterozygous littermates, whereas at P4 the body size of TrkA$^{R649W/R649W}$ mice was lower than controls (**Fig. 3C)**.

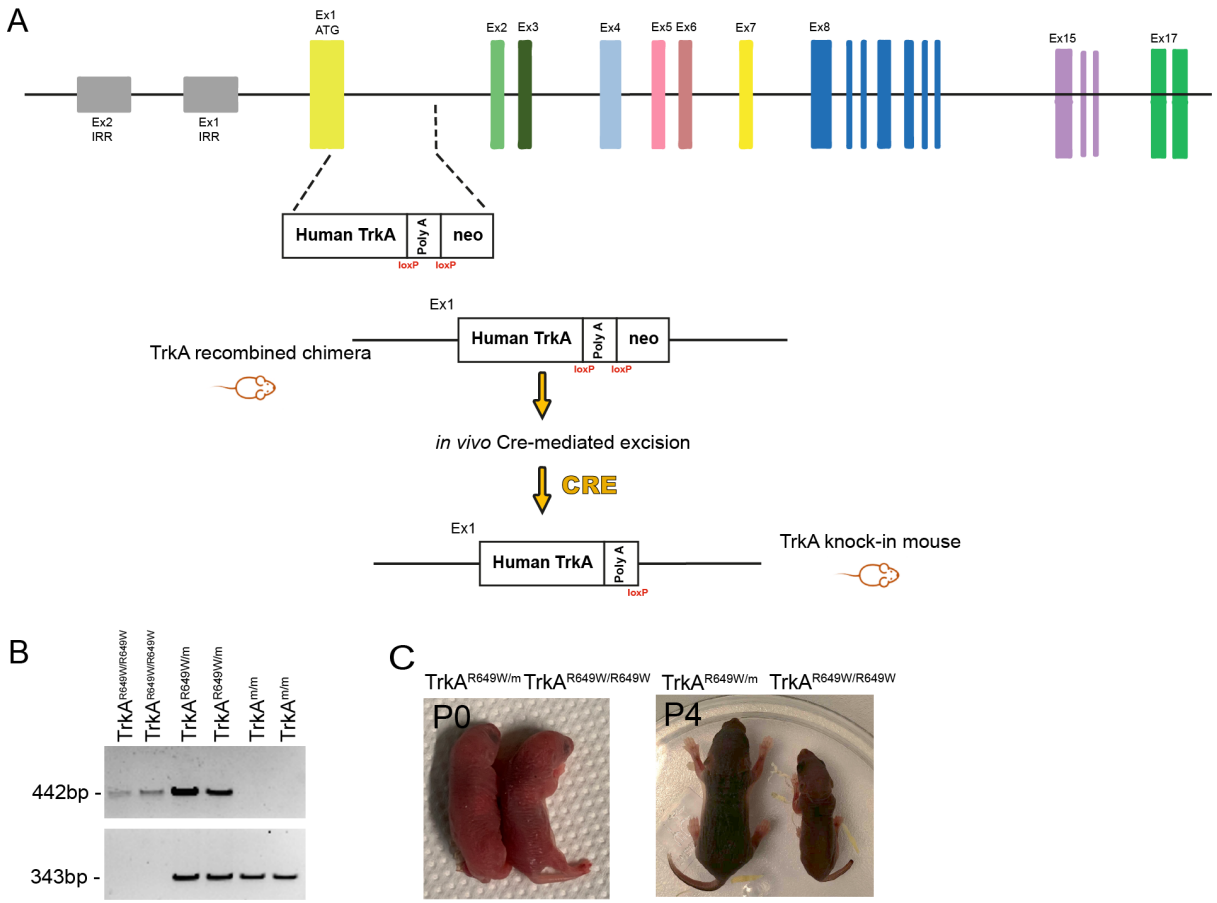
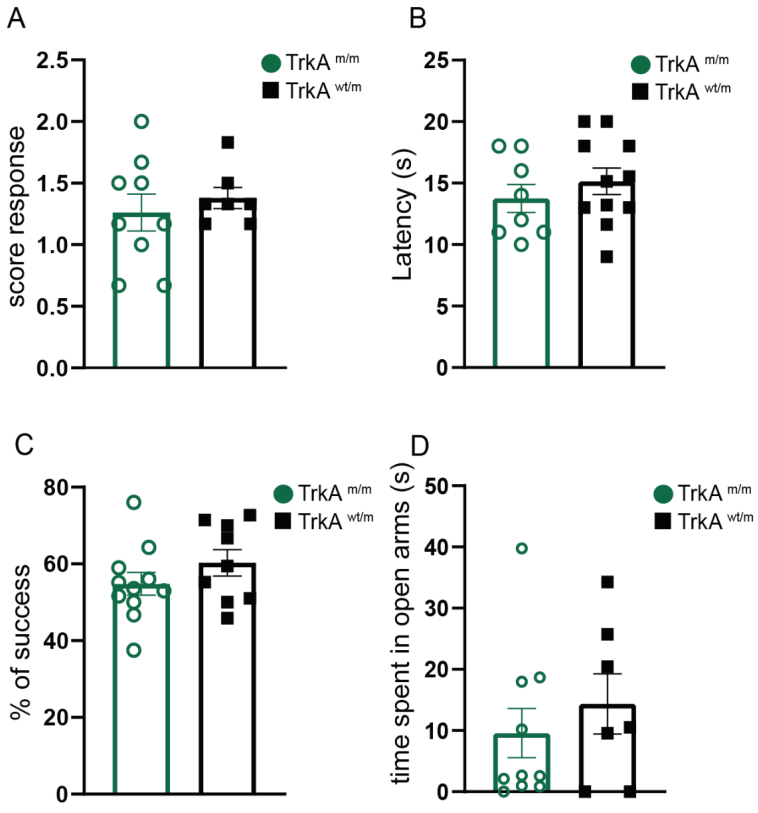


Figure 3: Generation of human TrkA^{R649W} knock-in mice. (A) Molecular strategy to generate the human TrkA^{R649W} knock-in mouse**.** Diagram illustrating the gene-targeting strategy to produce the TrkA^{R649W} knock-in mice. Human TrkA cDNA cassette harbouring the missense C-to-T mutation at the 1945 position replaced the murine TrkA exon 1 locus (corresponding to the R649W amino acid substitution). **(B)** PCR genotyping of homozygous (TrkA$^{R649W/R649W}$), heterozygous (TrkA$^{R649W/m}$) and wild-type (TrkA$^{m/m}$) mice; wild-type band: 343bp, mutant band: 442bp. **(C)** Representative pictures of TrkA^{R649W} knock-in mice. Homozygous mice appeared normal at birth (P0), but failed to grow during early postnatal life (e.g., at P4) compared to TrkA$^{R649W/m}$.

The homozygous condition of TrkA$^{R649W/R649W}$ mice matches the lethality observed in fully TrkA-deficient (TrkA$^{-/-}$) mice (Smeyne et al., 1994). This suggests that the loss of productive NGF-TrkA signaling in TrkA-expressing cells is a main consequence of the R649W mutation, in line with the experiments in cultured cells (Fig. 1).

5. Comparison between knock-in TrkA$^{h/m}$ and wild-type mice TrkA$^{m/m}$

Since homozygous TrkA$^{R649W/R649W}$ mice do not thrive to adulthood, I analyzed the phenotypic consequences of the R649W mutation in heterozygous TrkA$^{R649W/m}$ mice, in comparison to the control line TrkA$^{h/m}$ mice. First of all, I observed the general health state of both heterozygous TrkA$^{R649W/m}$ mice and their control monitoring specific phenotypic features such as gait, hind-limb clasping, tremor, irregular breathing and poor general condition and scoring them as absent, present, or severe (scores of 0, 1, and 2, respectively) (Guy et al., 2007). TrkA$^{R649W/m}$ mice always scored zero (data not shown) indicating that the TrkA^{R649W} does not affect the overall health condition of animals. Since these mice have never been phenotypically characterized, before comparing them to TrkA$^{R649W/m}$ I verified whether they behave similarly to wild-type mice (TrkA$^{m/m}$). As shown in panels A and B of figure 4, the response to noxious thermal stimuli (Acetone drope score response and the latency in the hot plate test), as well as and cognitive abilities measured in the Y-Maze and Elevated Plus Maze, in panels C and D, of TrkA$^{h/m}$ mice and wild-type mice (TrkA$^{m/m}$) were similar.



I also evaluated the expression of the most representative DRG nociceptive markers founding that the percentage of TrkA, TRPV1, CGRP and IB4 do not change across both groups (**Fig 4E**).

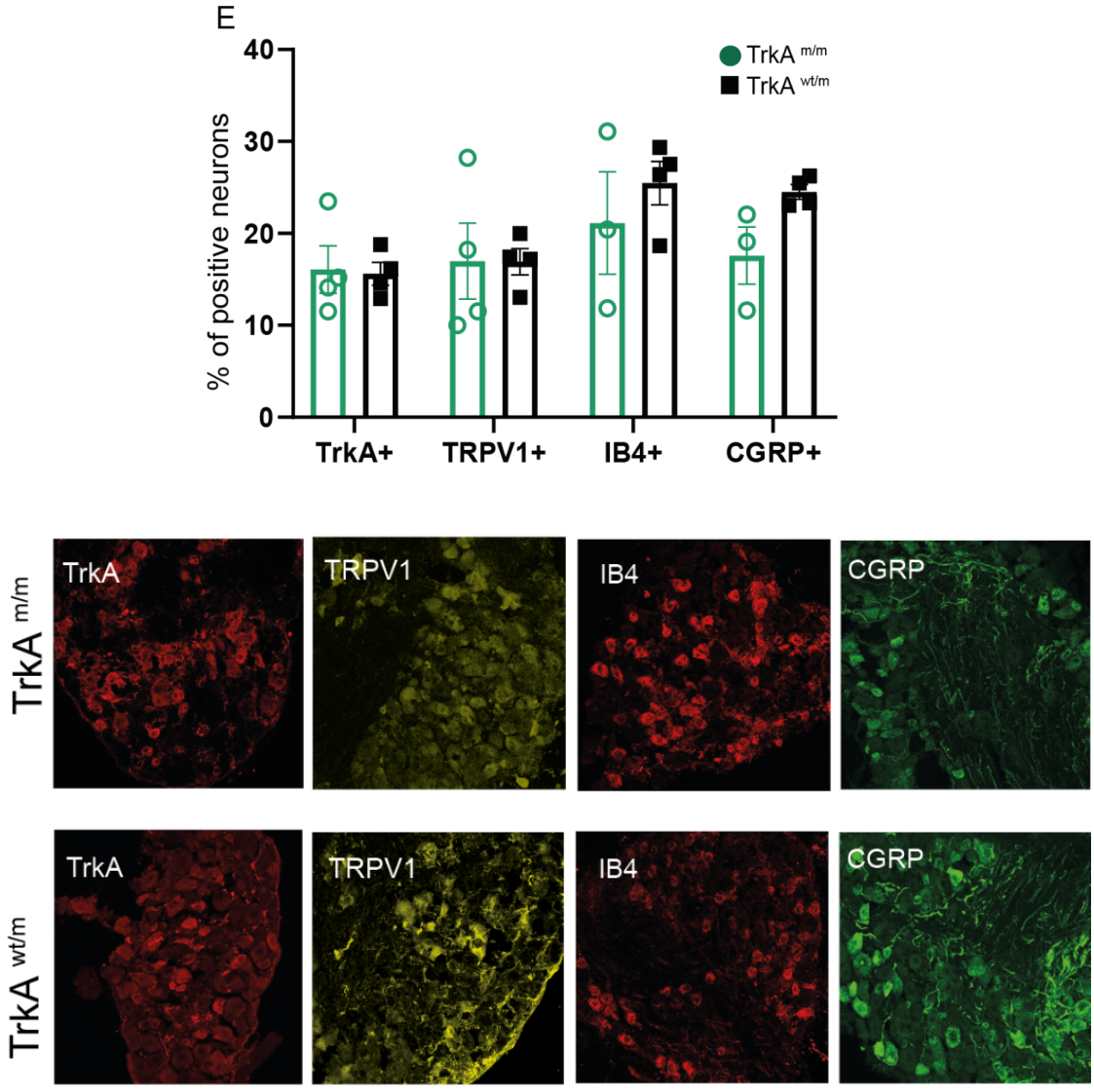
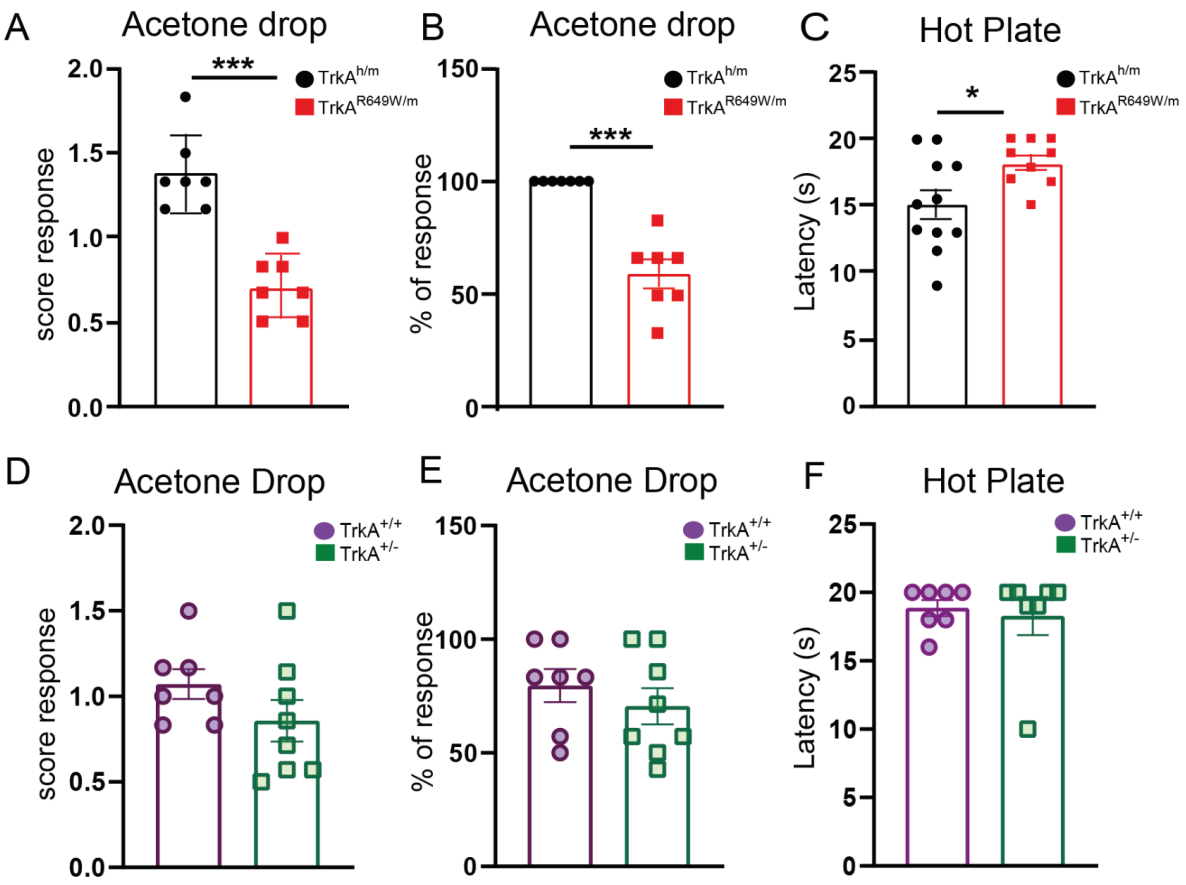


Figure 4. Comparison between wild-type TrkA$^{m/m}$ and humanized TrkA$^{h/m}$ mice. (A-B) No differences in the response to noxious cold and heat stimuli, as shown in acetone drop and hot plate. Student's t two-tailed test: Acetone Drop t=-0,636, p=0,535 wild-type TrkA$^{m/m}$ n=9, TrkA$^{h/m}$ n=7; Hot-Plate t=-0,870 , p=0,396 wild-type TrkA$^{m/m}$ n=8, TrkA$^{h/m}$ n=11. (C-D) Comparable responses of wild-type TrkAm/m and TrkAh/m mice during cognitive behavioral tests. Student's t two-tailed test: Y-Maze t=-1,218, p=0,239; EPM t= -0,756, p=0,462; wild-type TrkA$^{m/m}$ n=11, TrkA$^{h/m}$ n=10. (E)

Left. Quantification of the percentage of the most relevant nociceptive subpopulations of sensory neurons (TrkA+, TRPV1+, IB4+ and CGRP+) showed no significant differences between groups. Right. Representative images of DRG sections of wild-type $TrkA^{m/m}$ and $TrkA^{h/m}$ mice. Student's t two-tailed test: TrkA t =0,161 p= 0,877; TRPV1 t =0,0149, p=0,989; IB4 t = -0,804 , p=0,458; Mann-Whitney Rank Sum Test CGRP p=0,057; wild-type $TrkA^{m/m}$ n=3, $TrkA^{h/m}$ n=4.

6. Defective responses to pain and mechanical stimuli in $TrkA^{R649W/m}$ mice

One of the most characteristic and salient manifestations of HSAN IV is a generalized insensitivity to pain and thermal stimuli (Swanson, 1963; Haga et., 2015). To elucidate the consequences of the R649W mutation in peripheral somatosensation, I analysed the phenotype of heterozygous $TrkA^{R649W/m}$ at 2 months of age. I performed an array of behavioral tests to evaluate the sensory responses to thermal, chemical and mechanical stimuli. I first tested the response to cold noxious stimuli induced by acetone application on the hindpaw. A significant decrease was observed both in the score and in the percentage of responses **(Fig. 5A-B).** The response threshold to a noxious high-temperature stimulus, was also significantly reduced in $TrkA^{R649W/m}$ compared to the control group **(Fig. 5C)**. To test if the altered thermal sensation was specifically due to the R649W mutation or to functional haploinsufficiency, I compared $TrkA^{R649W/m}$ mice with heterozygous knock-out mice lacking one TrkA allele ($TrkA^{+/-}$). Strikingly, $TrkA^{+/-}$ mice showed comparable behavioral responses to that of wild-type $TrkA^{+/+}$ mice **(Fig. 5D-F)**.



Moreover, intraepidermal injection of capsaicin, which evokes a prolonged pain sensation in TrkA$^{h/m}$ mice, via activation of TRPV1 receptors (Caterina et al. 1997), failed to do so in TrkA$^{R649W/m}$ mice **(Fig. 5G)**. To test the response to innocuous mechanical stimuli, I used the tape response assay (Ranade et al., 2014) and the von Frey test. I found a decrease in the number of attempts to remove the adhesive tape attached to the back of TrkA$^{R649W/m}$ mice, compared to TrkA$^{h/m}$ **(Fig. 5H).** On the other hand, no differences in the response to mechanical stimuli applied on the plantar surface of the hindpaw were observed between TrkA$^{R649W/m}$ and TrkA$^{h/m}$ mice **(Fig. 5I)**. The different effect of the R649W mutation on mechanosensation is possibly due to distinct functional and anatomical properties of hairy and glabrous skin (L. Li et al., 2011) and to deficits in the growth of hair follicles (Peters et al., 2006)**.** These behavioral data indicate that TrkA$^{R649W/m}$ mice, unlike heterozygous TrkA$^{+/-}$ mice, show a significant inability to sense thermal and chemical noxious stimuli and to

respond to innocuous touch. This suggests a mutation-specific effect, distinct from a simple effect of wild-type TrkA haploinsufficiency.

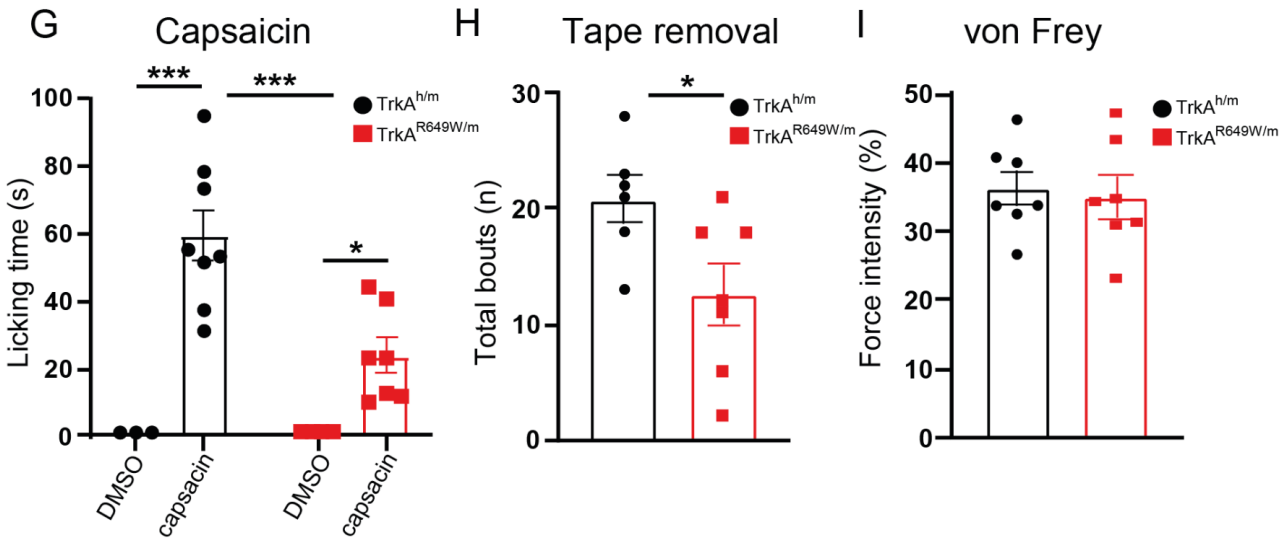


Figure 5: The R649W mutation specifically affects the response to noxious-thermal stimuli and the response to mechanical innocuous stimulus. Comparable response to noxious thermal stimuli in TrkA$^{+/-}$ mice. (A-B) Decreased cold sensitivity of TrkA$^{R649W/m}$ mice, analyzed as score and percentage (%) of responses on six trials A: Student's t two-tailed test (t = 5,970, p = <0,001); B: Mann-Whitney Rank Sum Test (p = <0,001) n=7 per group **(C)** Increased latency in TrkA$^{R649W/m}$ mice to respond to thermal stimulus of 48°C. Student's t two-tailed test (t = -1,281, p =0,025); TrkA$^{h/m}$ n=11; TrkA$^{R649W/m}$ n=9. **(D-E)** Comparable response to cold sensitivity between TrkA$^{+/+}$ and TrkA$^{+/-}$, both in the score and percentage (%) of responses D: Student's t two-tailed test (t = 1,391, p = 0,188); E: Student's t two-tailed test (t = 0,831, p = 0,421); TrkA$^{+/+}$ n=7 and TrkA$^{+/-}$ n=8. **(F)** No differences between TrkA$^{+/+}$ and TrkA$^{+/-}$ in the response to thermal stimulus of 48°C Mann-Whitney Rank Sum Test (p = 0,902) n=7 per group. **(G)** Decreased nociceptive behaviour in TrkA$^{R649W/m}$ mice after intraplantar injection of capsaicin (9µg/µl) compared to the control group. Two-way ANOVA ($F_{(1, 18)}$ = 6,190, p=0,023 followed by Holm-Sidak test (* p= 0,032; *** p= <0,001); TrkA$^{h/m}$ n=8; TrkA$^{R649W/m}$ n=7. **(H)** Reduced number of bouts in response to a piece of adhesive tape applied to the back neck of HSAN IV mice, compared to controls. Student's t two-tailed test (t = 2,419, p =0,034); TrkA$^{h/m}$ n=6; TrkA$^{R649W/m}$ n=7. **(I)** No differences between wild type and TrkA$^{R649W/m}$ mice in the response to mechanical stimulation measured by von Frey test.Student's t two-tailed test (t = -0,274, p =0,789); TrkA$^{h/m}$ n=7; TrkA$^{R649W/m}$ n=7). Data are presented as mean ± SEM.

7. Alteration of neuronal subpopulations in Dorsal Root Ganglia from TrkA$^{R649W/m}$ mice

In order to obtain a phenotypic portrait of DRGs in the TrkA$^{R649W/m}$ HSAN IV transgenic model, we analyzed the expression of protein markers characterizing the main sensory neuron subtypes.

First of all, no differences were found in the total DRG cell number between TrkA$^{R649W/m}$ and control mice (TrkA$^{h/m}$ 1182,53 cells/mm^2, TrkA$^{R649W/m}$ 1057,33 cells/mm^2; Student's t two-tailed test t=1,396 p= 0,212; n= 4 for both groups)**.** I then investigated by immunofluorescence the number of neurons expressing the TrkA receptor and the transient receptor channel V1 (TRPV1) **(Fig. 6A-B)**. Quantification revealed a loss of TRPV1 expression in primary sensory neurons, whereas no differences were observed in the expression of TrkA. Moreover, the number of DRG neurons co-expressing TRPV1 and TrkA in TrkA$^{R649W/m}$ mice was reduced **(Fig. 6E)**. Then, I examined the numbers of small-diameter neurons, divided into two main groups: the peptidergic Calcitonin Gene-Related Peptide (CGRP)-positive and non-peptidergic isolectin-B4 (IB4)-positive populations respectively **(Fig. 6C-D)**. The number of neurons expressing CGRP was normal in TrkA$^{R649W/m}$ mice, whereas the numbers of IB4-positive CGRP/IB4 co-expressing neurons were strongly affected by R649W mutation **(Fig. 6F)**.

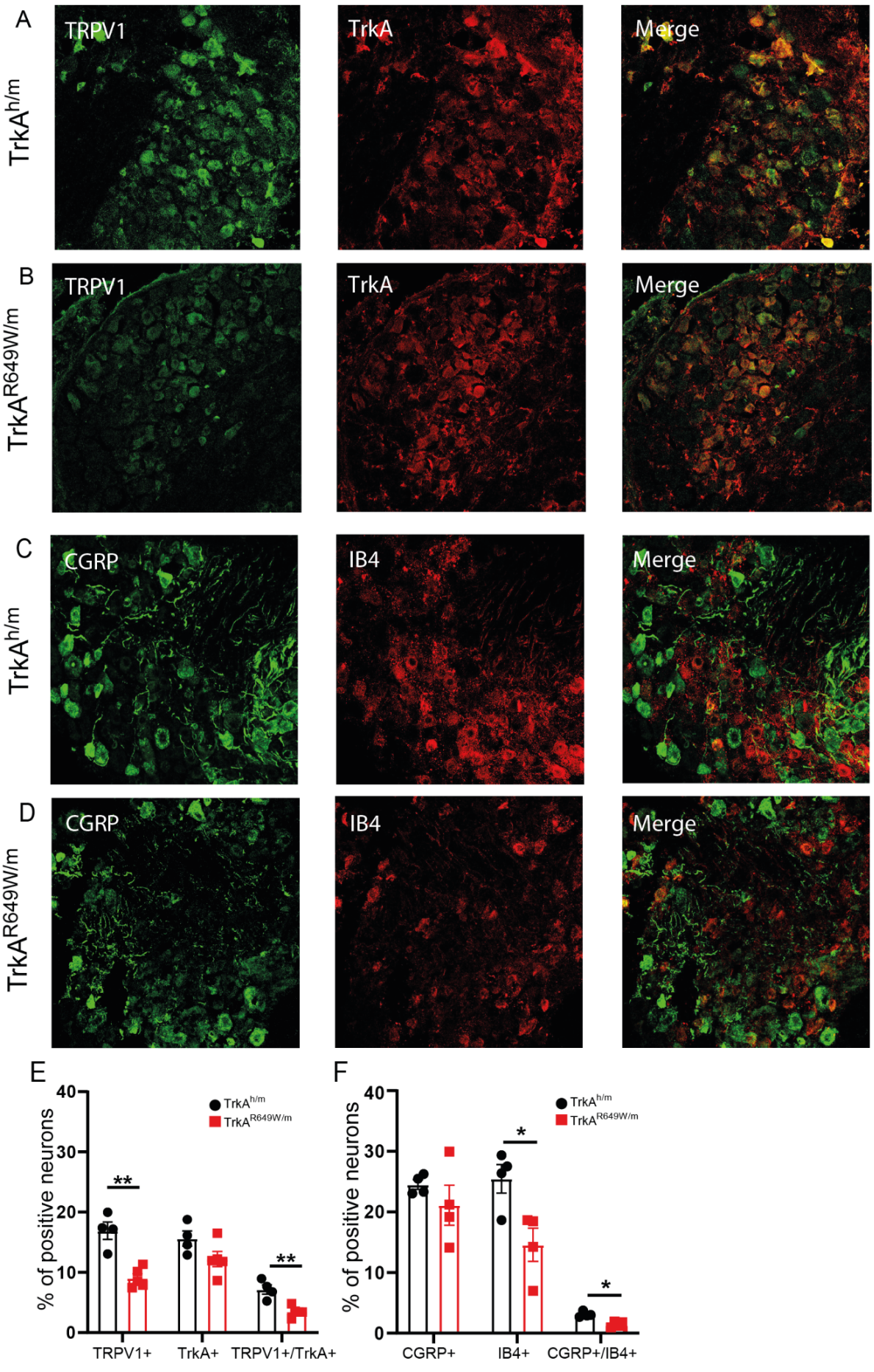

A
TrkA h/m
TRPV1
TrkA
Merge
B
TrkA R649W/m
TRPV1
TrkA
Merge
C
TrkA h/m
CGRP
IB4
Merge
D
TrkA R649W/m
CGRP
IB4
Merge
E
% of positive neurons
TrkA h/m
TrkA R649W/m
40
30
20
10
0
**
**
TRPV1+
TrkA+
TRPV1+/TrkA+
F
% of positive neurons
TrkA h/m
TrkA R649W/m
40
30
20
10
0
*
*
CGRP+
IB4+
CGRP+/IB4+

Figure 6: Analysis of nociceptive markers in DRG: reduced expression of TRPV1 and IB4 expression. (A-D) Double immunofluorescence of DRG cryosections: for (A-B) TRPV1 and TrkA, (C-D) CGRP and IB4. **(E**) Significant decrease of TRPV1+ /TrkA+ sensory neurons in $TrkA^{R649W/m}$ mice. Student's t two-tailed test: TRPV1 t = 5,207 Pp= 0,001; TrkA t = 1,881, p= 0,102; TRPV1/TrkA t = 4,456, p=0,003; $TrkA^{h/m}$ n=4, $TrkA^{R649W/m}$ n=5. (**F**) Reduced number of IB4-positive neurons in $TrkA^{R649W/m}$ mice. Student's t two-tailed test: CGRP t= 1,089, p= 0,318; Mann-Whitney Rank Sum Test: IB4 p = 0,03; Student's t two-tailed test CGRP/IB4 t=4,933, p=0,003; $TrkA^{h/m}$ n=4, $TrkA^{R649W/m}$ n=4.

8. R649W mutation does not affect proprioceptors and C-LTMRs

I further investigated the neuronal subpopulations in DRGs from $TrkA^{R649W/m}$ mice, using known markers of proprioceptive sensory neurons and of non-nociceptive C-low-threshold mechanoreceptors (C-LTMRs). In addition to Neurofilament 200 (NF200), a marker of myelinated sensory neurons (Usoskin et al., 2015), I also used Parvalbumin (PV) as a marker of proprioceptors (Usoskin et al., 2015) **(Fig. 7A-B)**, while unmyelinated c-LTMRs were identified using Tyrosine Hydroxylase (TH) (L. Li et al., 2011) **(Fig. 7C-D)**. I found no changes in the percentage of sensory neurons expressing PV-NF200 and TH-NF200 **(Fig. 7E-F)**.

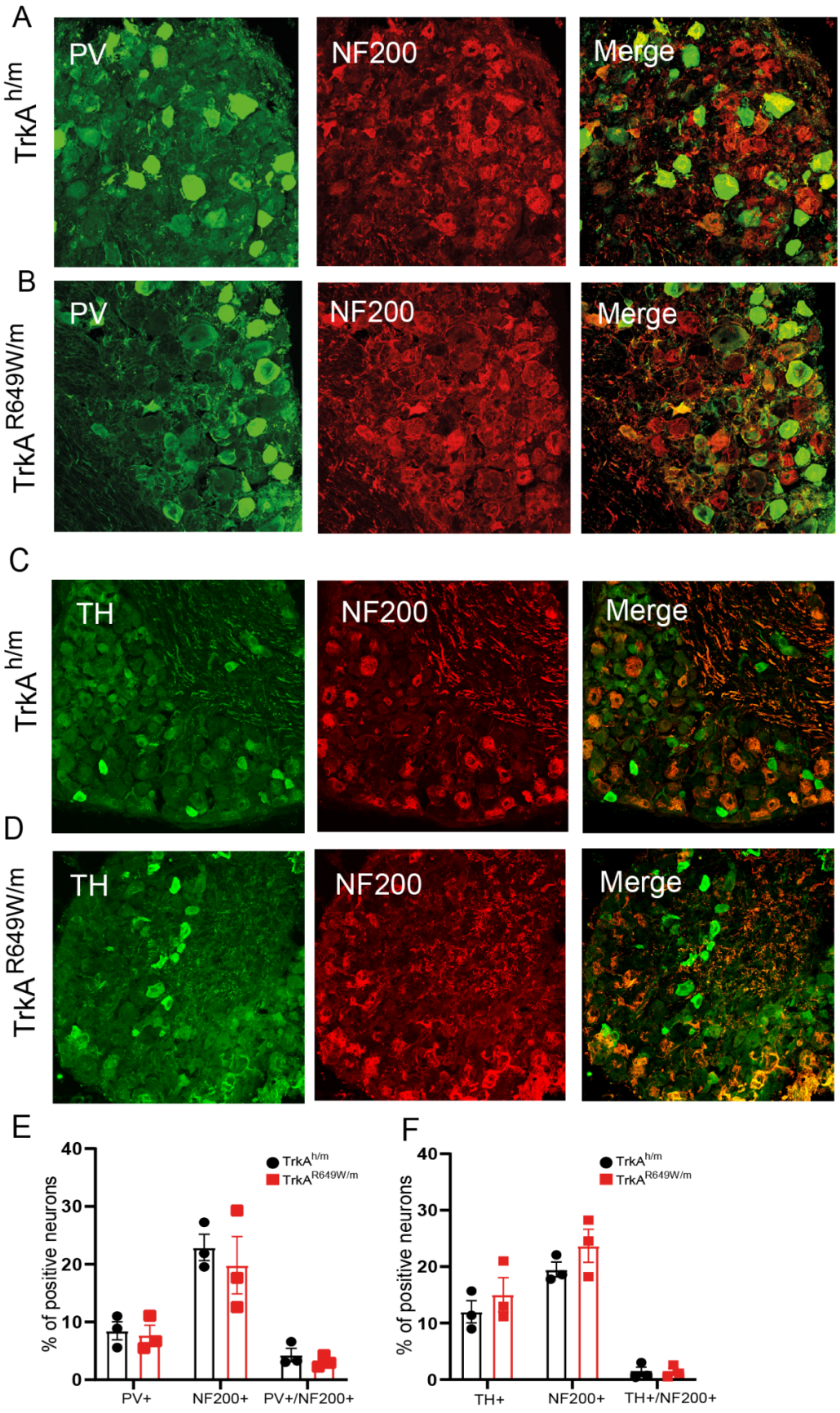


Figure 7. The R649W mutation does not alter proprioceptors and mechanoreceptors. (A-D) Representative images of DRG cryosections stained for (A-B) PV and NF200, (C-D) TH and NF200

antibodies. **(E-F)** Normal expression of PV, TH and NF200 in DRG neurons in TrkA$^{R649W/m}$ mice compared to TrkA$^{h/m}$ controls. Student's t two-tailed test: PV t = 0,318 p = 0,767; NF200 t = 0,562, p= 0,604; PV/NF200PV-NF200 t = 0,892, p=0,423; TH t = -0,838, Pp= 0,449; NF200 t = -1,313, p= 0,259; TH-NF200 t = -0,00210, P=0,998; TrkA$^{h/m}$ n=3, TrkA$^{R649W/m}$ n=3.

Histological analysis of DRG sensory neurons suggest that the R649W mutation specifically impacts the TRPV1+ and IB4+ subsets of DRG neurons, known to be essential for pain sensation, without affecting other neurons involved in mechanical or proprioceptive functions.

9. Severe lack of PGP9.5-positive fibers in TrkA$^{R649W/m}$ mice

In HSAN IV patients, the lack of pain sensation is associated with the loss of sensory afferents in the skin (Indo, 2001). For this reason, I investigated skin sensory innervation in TrkA$^{R649W/m}$ and control mice (**Fig. 8A, C).** The area and number of PGP9.5-immunoreactive terminals were decreased in the glabrous skin sections of TrkA$^{R649W/m}$ mice compared to control mice **(Fig. 8B).** In addition, I observed a diminished innervation in the hairy skin of TrkA$^{R649W/m}$ mice **(Fig. 8D)**. In agreement with the clinical features of HSAN IV disease, TrkA$^{R649W/m}$ mice show a severe lack of innervation in both glabrous and hairy skin.

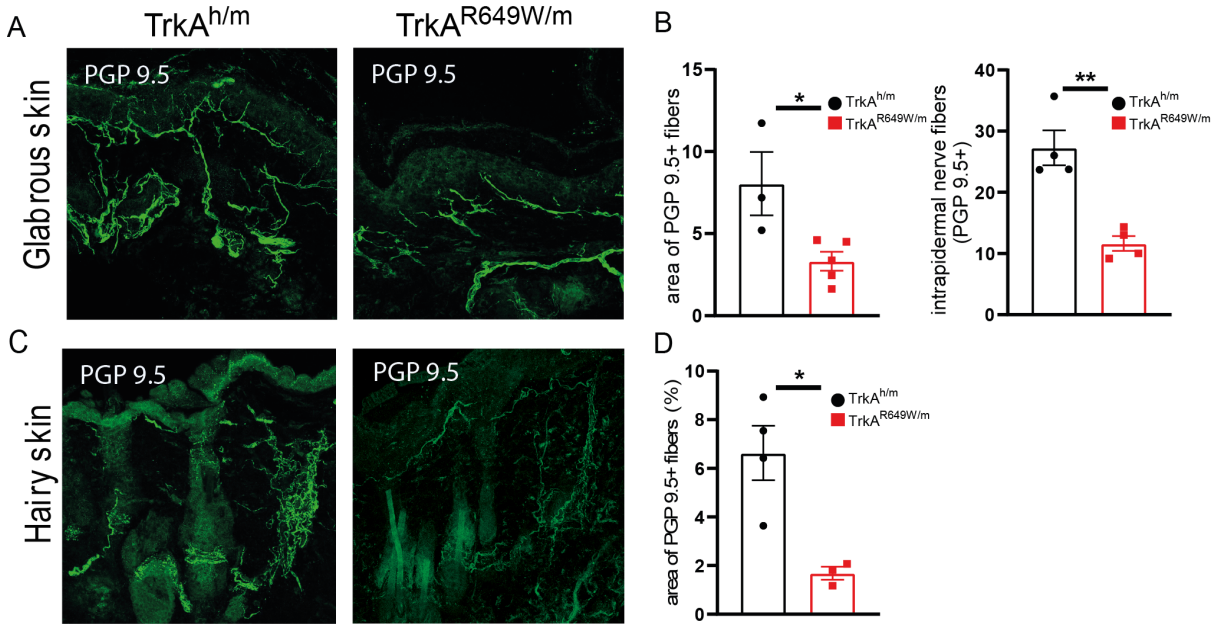


Figure 8: Loss of innervation in hairless and hairy skin in TrkA$^{R649W/m}$ mice. (A, C) Representative images and **(B, D)** quantification of PGP9.5 expression in glabrous and hairy skin sections. (**B**) **Left:** significant reduction of hairless skin innervation measured as area occupied by PGP9.5-positive fibers. Student's t two-tailed test: t =2,947 p= 0,026 TrkA$^{h/m}$ n=3, TrkA$^{R649W/m}$ n=5. **Right**: reduction of PGP9.5-postive intraepidermal fibers in TrkA$^{R649W/m}$ compared to TrkA$^{h/m}$ mice. Student's t two-tailed test: t = 5,045 p= 0,002 TrkA$^{h/m}$ n=4, TrkA$^{R649W/m}$ n=4. **(D)** TrkA$^{R649W/m}$ mice exhibit a diminished hairy skin innervation measured as area occupied by PGP9.5-positive fibers. Student's t two-tailed test: t = 3,670 p= 0,014 TrkA$^{h/m}$ n=4, TrkA$^{R649W/m}$ n=3.

10. Characterization of somatosensation of 1-year-old TrkA$^{R649W/m}$ mice

To obtain a more detailed and deep analysis of the sensory phenotype of HSAN IV TrkA$^{R649W/m}$ mice, I performed a behavioral and histological characterization of 1-year-old mice. As expected, the inability to respond to noxious stimuli observed at two months of age was present also in old TrkA$^{R649W/m}$ mice compared to their control **(Fig. 9A-B)** as well as the reduced response to non-noxious mechanical stimulation applied on the back skin **(Fig. 9C).**

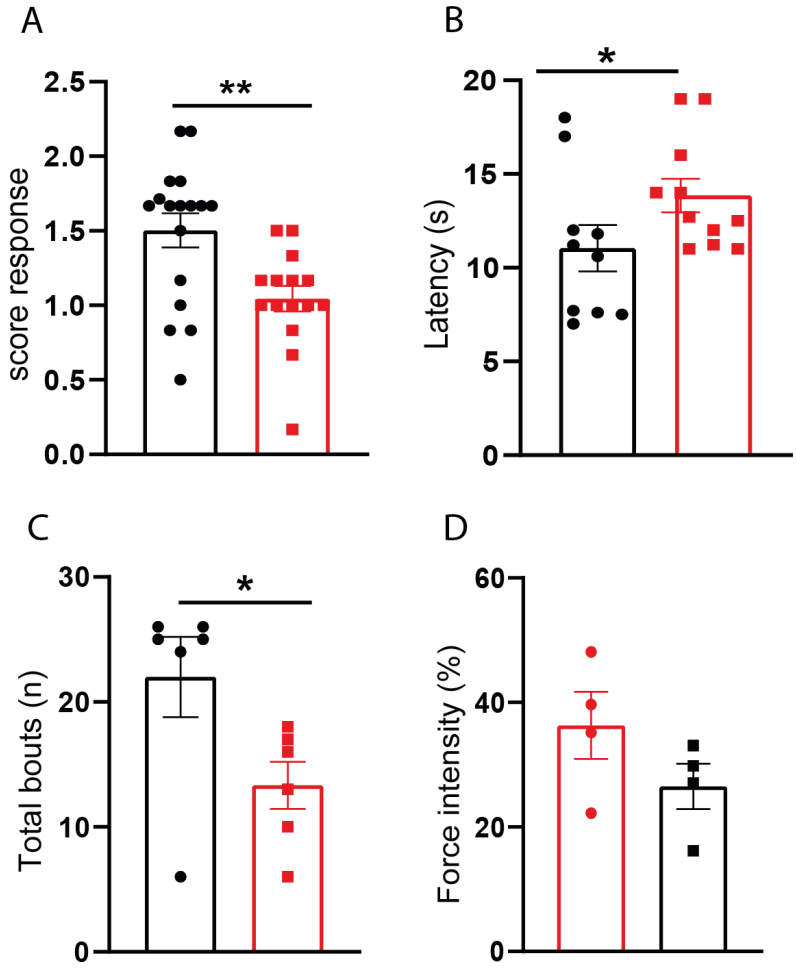


Figure 9: Reduced response to noxious and non-noxious stimuli in HSAN IV TrkA$^{R649W/m}$ mice. **(A-B)** TrkA$^{R649W/m}$ mice show a reduced score response in the Acetone drop test and increased latency in the hot plate test. Mann-Whitney Rank Sum Test: Acetone drop p=0.006 TrkA$^{h/m}$ n=17, TrkA$^{R649W/m}$ n=15; Hot plate 48°C p=0,048 TrkA$^{h/m}$ n=10, TrkA$^{R649W/m}$ n=11. (**C**) Reduced number of attempts of TrkA$^{R649W/m}$ mice in the tape test removal test. Mann-Whitney Rank Sum Test p=0,041 n=6 per group. **(D)** No difference in the non-noxious mechanical response to von Frey stimulation. Student's t two-tailed test t = 0,185, p=0,0925 n=4 per group.

As expected, changes in the expression of DRG molecular markers were found in 1-year-old TrkA$^{R649W/m}$ mice. The percentage of TRPV1+ and the non-peptidergic IB4+ neurons was reduced in TrkA$^{R649W/m}$ mice compared to old control mice, whereas the expression of TrkA and the peptidergic DRG marker, CGRP, did not change **(Fig. 10A-B)**. In addition, the analysis of the sensory fiber target tissues, glabrous and hairy skins, was done using the neuronal marker PGP9.5. A significant reduction was observed at 1 year both in the glabrous and hairy skin of HSAN IV **(Fig. 10C-E)**. Despite a

possible loss of sensory innervation age-related, these findings suggest that the TrkA^{R649W} mutation robustly affects the somatosensation causing the loss of pain sensation in HSAN IV mice.

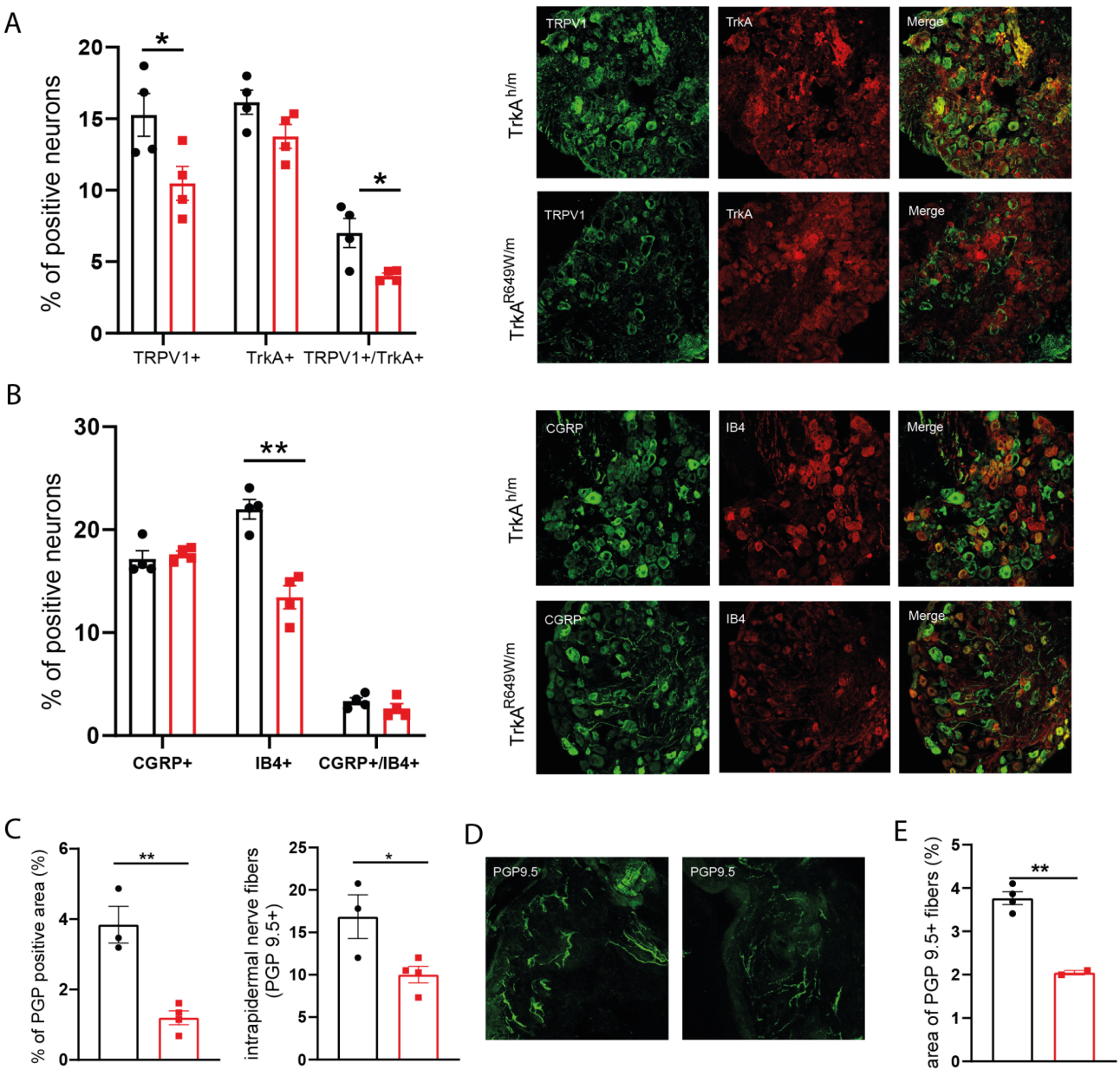
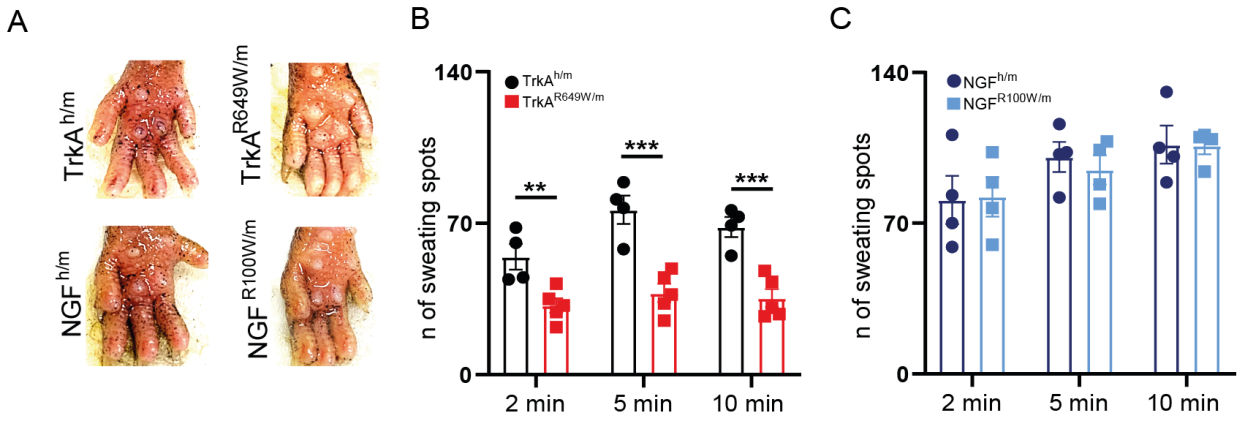


Figure 10: Histological analysis of DRG sensory neurons and skin innervation in 1-year-old TrkA$^{R649W/m}$ mice. (A-B) Quantification and representative images of nociceptive markers TRPV1, TrkA, CGRP and IB4. Student's t two-tailed test: TRPV1 t=0,0456 p=0,0461; TrkA t=0,0893, p= 0,089; TRPV1/TrkA t = 0,0274, p=0,027 n=4 per group. Student's t two-tailed test: CGRP t=0,730, p=0,730; IB4 t=0,00115, p=0,001; CGRP/IB4 t=0,266, p=0,266 n=4 per group. (**C**) Quantification of sensory fibers PGP9.5+ in glabrous skin. **Left:** significant reduction of hairless skin innervation measured as area occupied by PGP9.5-positive fibers. Student's t two-tailed test t =0,00302 p= 0,026 TrkA$^{h/m}$ n=3, TrkA$^{R649W/m}$ n=4. **Right**: reduction of PGP9.5-postive intraepidermal fibers in TrkA$^{R649W/m}$ compared to TrkA$^{h/m}$ mice. Student's t two-tailed test: t =0,0381 p= 0,038 TrkA$^{h/m}$ n=3, TrkA$^{R649W/m}$ n=4. (**D**) Representative images of glabrous skin sections of 1-year-old TrkA$^{h/m}$ and

TrkA$^{R649W/m}$ mice. (**E**) Quantification of the hairy skin area occupied by PGP9.5+ fibers. Student's t two-tailed test: t =0,00149 p= 0,001 TrkA$^{h/m}$ n=4, TrkA$^{R649W/m}$ n=2.

11. Anhidrosis in TrkA^{R649W} but not in HSAN V NGF$^{R100W/m}$ mice: a distinctive hallmark of HSAN IV disease

The absence of sweating is a distinctive clinical trait of HSAN IV patients (Indo, 2001), unlike HSAN V patients, which harbor mutations in the NGF1 gene (Axelsson et al., 2009; Einarsdottir et al., 2004). For this reason, by using a pilocarpine-induced sweat assay (Liu et al., 2017), I examined if TrkA$^{R649W/m}$ mice were affected by abnormalities in sweat production. Having recently developed an HSAN V mouse model (NGF$^{R100W/m}$) (Testa et al., 2019a; Testa et al., 2019b), I had the opportunity to compare the sweating phenotype in heterozygous HSAN IV TrkA$^{R649W/m}$ and HSAN V NGF$^{R100W/m}$ mice (NGF$^{R100W/m}$), in order to evaluate the specific response of an HSAN IV mouse model in this specific assay. In the pilocarpine-induced assay, sweating appears as dark precipitates on iodine and starch-coated footpads (**Fig. 11A**). After pilocarpine injection, the formation of dark spots in footpads was monitored and recorded by a digital camera at 2, 5 and 10 minutes. The iodine-starch sweat test revealed striking anhidrosis in TrkA$^{R649W/m}$ mice as also shown in representative pictures (**Fig. 11B**). On the other hand, the sweat assay performed in NGF$^{R100W/m}$ and their corresponding control mice revealed no differences in the number of black spots, confirming normal sweating in the HSAN V model and further validating the specificity of our HSANIV model **(Fig. 11C)**. This is fully consistent with human studies reporting sweating alterations in HSAN IV but not in HSAN V patients.



Since sweat glands are innervated with adrenergic and cholinergic terminals, I investigated if reduced sweating was associated with an altered sympathetic innervation of these populations. The area occupied by TH-immunoreactive positive fibers appeared normal in both TrkA$^{R649W/m}$ and controls, as well as in HSAN V sweat glands **(Fig. 11D-E).** No differences were also found in the mean immunofluorescence signal intensity of the cholinergic fibers labeled with the vesicular acetylcholine transporter (VAChT) between TrkA$^{R649W/m}$ and controls, as well as in HSAN V mice **(Fig. 11F-G)**. I can conclude that the differential anhidrosis sweating phenotype, observed in HSAN IV versus HSN V mice is not due to a differential innervation of the adrenergic and cholinergic innervation of the sweat glands in the two mouse lines.

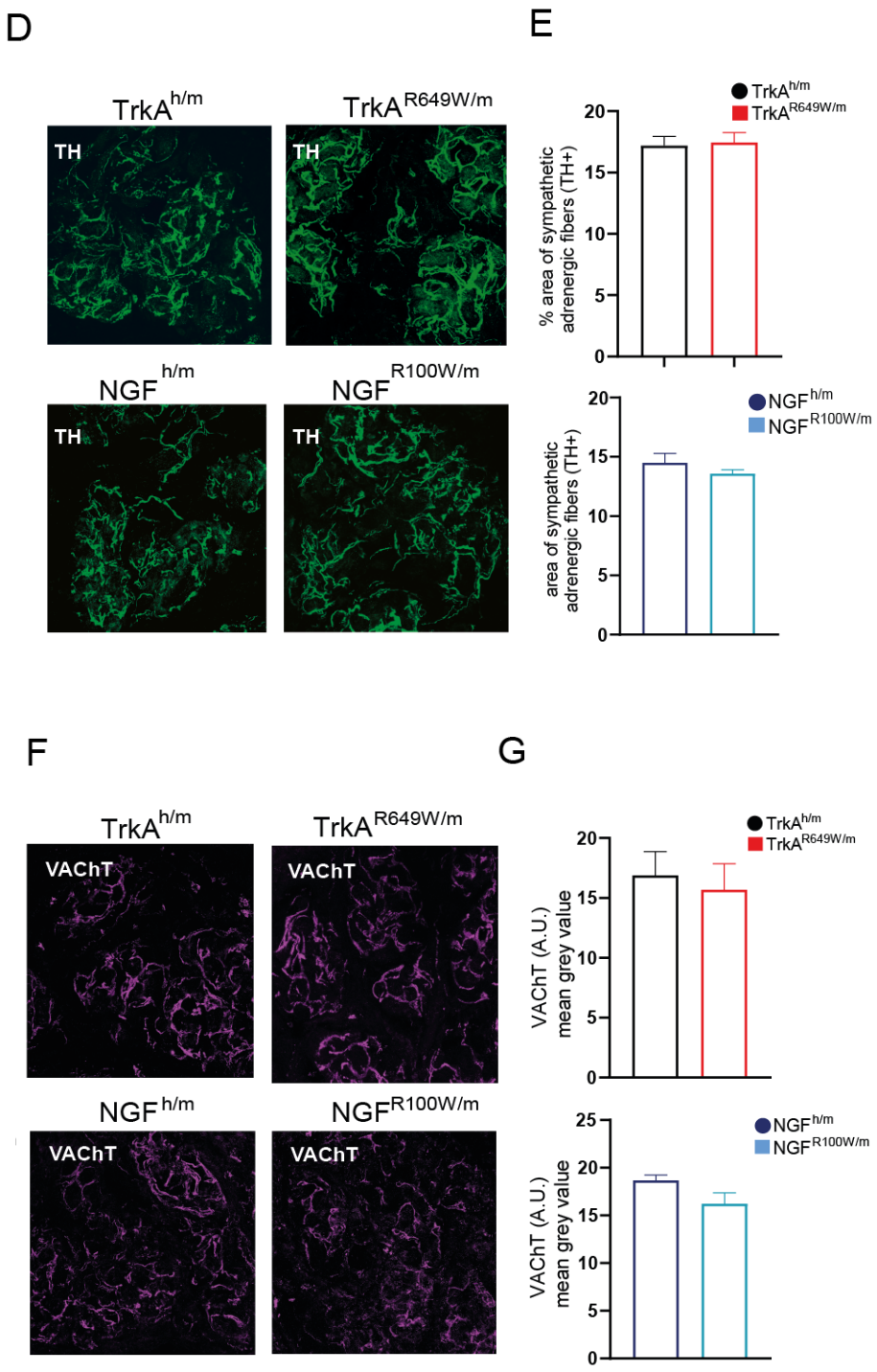


Figure 11: Impaired sweating in HSAN IV TrkA$^{R649W/m}$ but not in HSAN V NGF$^{R100W/m}$ mice. **(A)** Representative images of sweat droplets (dark precipitates from iodine/starch assay) on footpads at 5 minutes and quantification of sweat droplets at 2, 5 and 10 minutes. **(B)** TrkA$^{R649W/m}$ mice show a significant reduction of the number of sweat droplets compared to control mice. Two-way RM ANOVA ($F_{(2,14)}$=2,61 p=0,109), followed by Holm-Sidak test: 2min p=0,005; 5min p= <0,001; 10min p= *<0,001;* TrkA$^{h/m}$ n=4, TrkA$^{R649W/m}$ n=5. (**C**) Normal sweating in NGF$^{h/m}$ and NGF$^{R100W/m}$ Two-way RM ANOVA ($F_{(2,12)}$=0,084 p=0,920), followed by Holm-Sidak test: 2min *p*=0,940; 5min p=0,652; 10min p=0,821; NGF$^{h/m}$ n=4, NGF$^{R100W/m}$ n=4). **(D)** Representative images showing the innervation of sweat glands in the footpad, revealed by TH immunofluorescence. **(E)** Unaffected

sympathetic innervation of sweat glands in $TrkA^{h/m}$ and $TrkA^{R649W/m}$ mice and in $NGF^{h/m}$ and $NGF^{R100W/m}$. $TrkA^{h/m}$ and $TrkA^{R649W/m}$ Student's t two-tailed test t =-0,228, p= 0,826; $TrkA^{h/m}$ n=5, $TrkA^{R649W/m}$ n=4. While, $NGF^{h/m}$ and $NGF^{R100W/m}$ Student's t two-tailed test t =1,116, p= 0,297 n=5 per group. **(F)** Representative images showing the sympathetic innervation of sweat glands, revealed by VAChT immunofluorescence. **(G)** VAChT (A.U) mean grey value revealed no differences of sympathetic innervation in $TrkA^{h/m}$ and $TrkA^{R649W/m}$ mice and in $NGF^{h/m}$ and $NGF^{R100W/m}$. Histograms summarize the mean immunofluorescence signal intensity measured as the subtraction of the mean gray values and the background. $TrkA^{h/m}$ and $TrkA^{R649W/m}$ Student's t two-tailed test t = 0,411, p= 0,702; $TrkA^{h/m}$ n=3, $TrkA^{R649W/m}$ n=3. While, $NGF^{h/m}$ and $NGF^{R100W/m}$ Student's t two-tailed test t = 1,924, p= 0,127 n=3 per group.

12. $TrkA^{R649W}$ specifically impairs the working-spatial memory and the anxiety in $TrkA^{R649W/m}$ mice.

Another distinctive feature of the HSAN IV patients is the presence of mental retardation with variable severity (Indo et al., 1996), which include aspects that have been classified as attention deficit hyperactivity disorder (ADHD) (Levy-Erez et al., 2010). Thus, to evaluate cognitive abilities in $TrkA^{R649W/m}$ mice, I performed a battery of tests that have been often used in mouse models of ADHD to evaluate working spatial memory and inattention, anxiety and sociability (Kawade et al., 2020; Jeon et al., 2021). First, I tested the tendency of rodents to alternately explore new environments, reproduced by the arms of the Y-maze. The spontaneous alternations evaluated in Y-maze apparatus were reduced in $TrkA^{R649W/m}$ mice compared to the control group **(Fig. 12A**). In addition, when tested in an elevated plus maze, $TrkA^{R649W/m}$ mice exhibit less anxious behavior than wild-type mice **(Fig. 12B)**. Importantly, these alterations in both Y-Maze and Elevated Plus Maze test were not detected in $TrkA^{+/-}$ mice (**Fig. 12C-D**), suggesting that these behavioral impairments are strictly related to the R649W mutation. In the novel object recognition test, in which a specific form of learning and memory abilities is assessed, no differences were found between $TrkA^{R649W/m}$ mice and their controls (**Fig. 12E**).

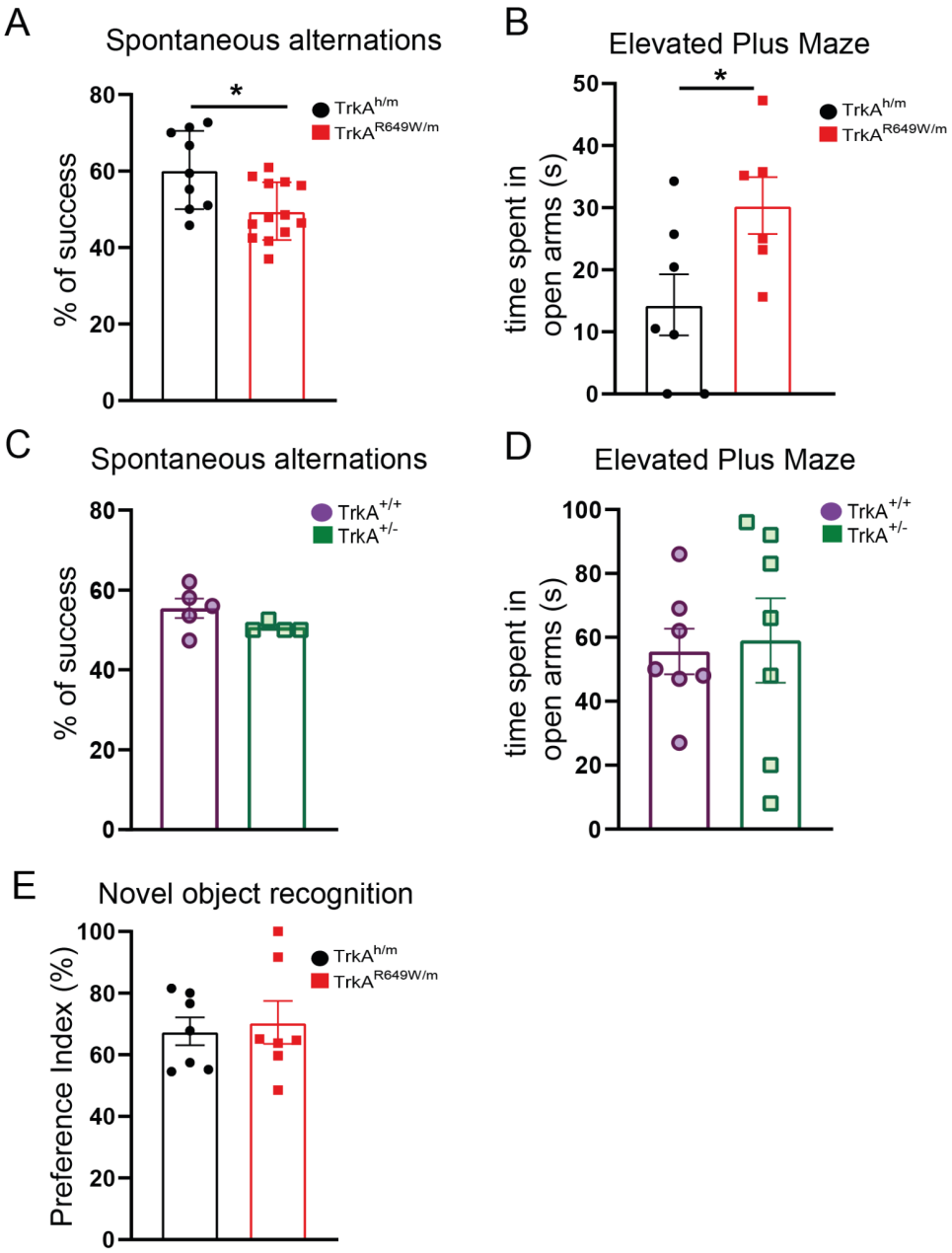


Figure 12: Impaired cognitive abilities in TrkA$^{R649W/m}$ mice and not in TrkA$^{+/-}$ mice. (A) Decreased percentage (%) of success in Y-maze test in TrkA$^{R649W/m}$ mice. Student's t two-tailed test, (t = 2,519, p= 0,026), TrkA$^{h/m}$ n=8, TrkA$^{R649W/m}$ n=7. **(B)** Decrease of anxiety-related behaviour in HSAN IV mice, evaluated in the elevated plus maze. Student's t two-tailed test, (t = -2,349, p= 0,039); TrkA$^{h/m}$ n=7, TrkA$^{R649W/m}$ n=6. **(C-D)** Percentage (%) of success in Y-maze test (C) and anxiety-related behavior (D) are not affected in both TrkA$^{+/+}$ and TrkA$^{+/-}$ mice. C: Student's t two-tailed test (t = -1,683, p = 0,136); TrkA$^{+/+}$ n=5 and TrkA$^{+/-}$ n=4; D: Student's t two-tailed test (t = -0,229, p = 0,823) n=7 per group. **(E)** No differences in novel object recognition test (Student's t two-tailed test, t = 0,351, p = 0.732; n=7 per group).

13. The sociability is negatively influenced only by TrkA^{R649W} mutation.

Another hallmark of HSAN IV patients is an altered social interaction and the propensity to avoid eye contact (Axelrod & Gold-Von Simson, 2007). To investigate the effect of R649W mutation on social behavior, I tested the performance of HSAN IV mice in the three-chamber sociability test (Chadman et al., 2008; Yang et al., 2011). During the socialization phase, TrkA$^{R649W/m}$ mice displayed a comparable exploration time between the unfamiliar mouse cage (namely stranger 1 – S1) and the inanimate object cage, indicating altered sociability compared to controls (**Fig. 13A left**) while, as expected, HSAN V NGF$^{R100W/m}$ mice and wild type mice, showed a significant preference for the mouse cage rather than the object (**Fig. 13A right**). When tested in the social novelty preference test, in which a new unfamiliar mouse (namely Stranger 2 – S2) replaced the object into the wire cup, TrkA$^{R649W/m}$ mice, compared to TrkA$^{h/m}$ mice, do not show a significant preference for the unfamiliar S2 mouse (**Fig. 13B left**). Interestingly, I found that sociability was unaltered in NGF$^{R100W/m}$ mice (**Fig. 13B right**), suggesting that this characteristic behavior is strictly related to the HSAN IV clinical phenotype, as reported in human patients.

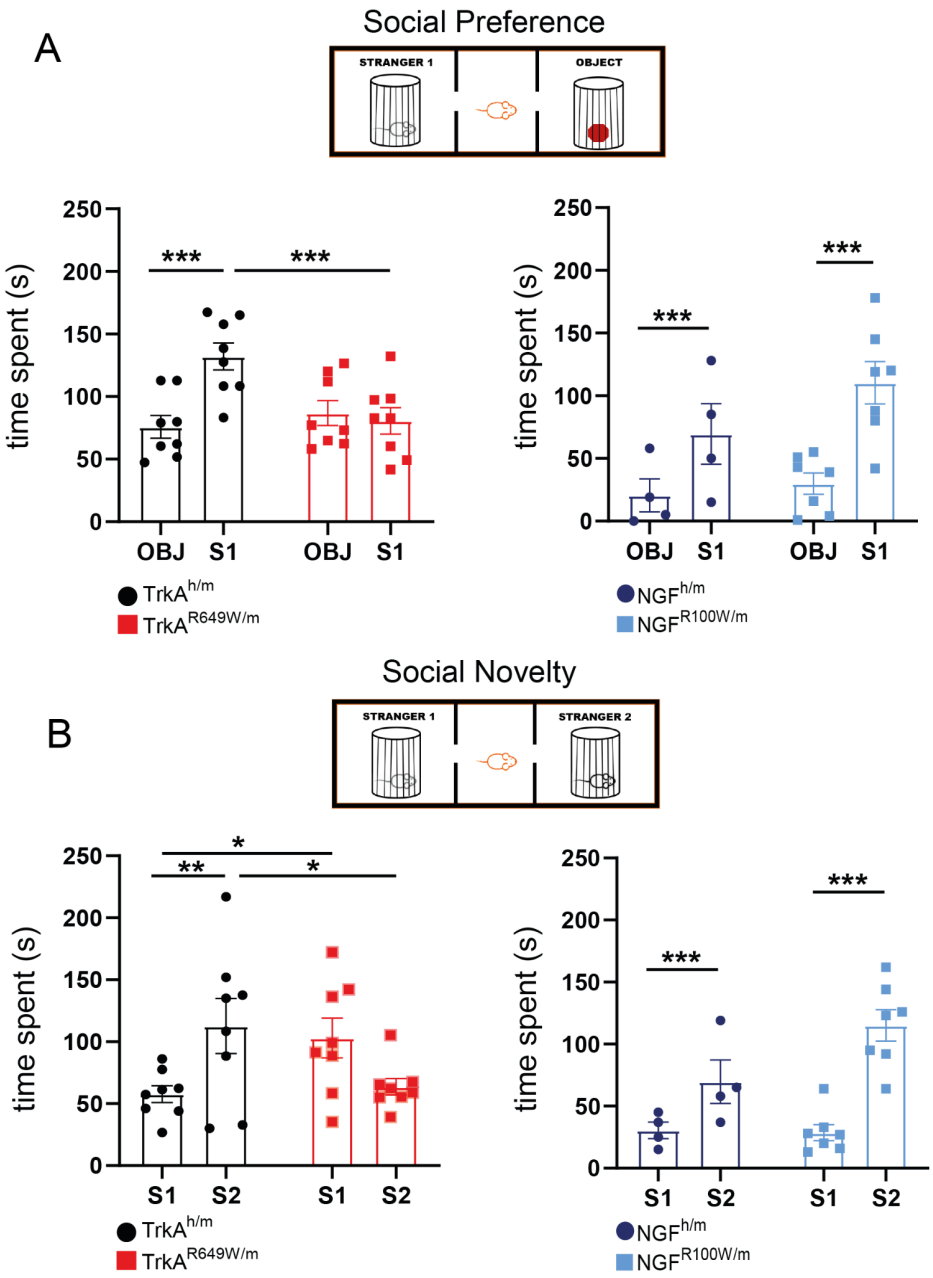


Figure 13: Reduced sociability in TrkA$^{R649W/m}$ and not in NGF$^{R100W/m}$ mice. (A) Left: TrkA$^{R649W/m}$ mice display reduced social preference in three-chamber test. Two-way ANOVA ($F_{(1,28)}$=9,56 p=0,004), followed by Holm−Sidak method *** p= ≤0,001 TrkA$^{h/m}$ n=8, TrkA$^{R649W/m}$ n=8. **Right:** no differences in social preference in HSAN V mouse model. Two-way ANOVA ($F_{(1,18)}$=9,37 p=0,346), ***p=<0,001 NGF$^{h/m}$ n=4, NGF$^{R100W/m}$ n=7. **(B) Left:** Social novelty behavior is impaired in TrkA$^{R649W/m}$ mice Two-way ANOVA, ($F_{(1,28)}$=10,63 p=0,003), followed by Holm-Sidak method ** p=0,012; * p=0,035; * p=0,023 TrkA$^{h/m}$ n=8, TrkA$^{R649W/m}$ n=8. **Right:** normal social novelty exploration in NGF$^{h/m}$ and NGF$^{R100W/m}$ Two-way ANOVA, ($F_{(1,18)}$=4,01 p=0,060) *** p= <0,001 NGF$^{h/m}$ n=4, NGF$^{R100W/m}$ n=7.

This comprehensive array of behavioral tests shows that TrkA$^{R649W/m}$ mice are characterized by deficits in working memory, reduced anxiety and decreased social interactions.

Overall, I report that the TrkA$^{R649W/m}$ mice recapitulate key phenotypes associated with HSANIV disease and thus they could represent an important model to study pain insensitivity.

5. Discussion

1. TrkA as an in vivo pain modulator

The importance of pain has been highlighted in all animals as a universal warning system to prevent and avoid injuries. The nociceptive system, highly conserved in animal species, requires specialized primary sensory neurons that are responsive to a wide variety of noxious stimuli (Sherrington, 1906). The ability to detect noxious stimuli is due to different types of membrane receptors and, among these, a key role is played by TrkA (Basbaum et al., 2009; Woolf & Ma, 2007). Indeed, a subpopulation of sensory neurons called peptidergic-nociceptors are NGF-dependent and express TrkA receptors (Snider & Mcmahon, 1998). The role of the NGF-TrkA system has been abundantly explored in the survival and maintenance of neurons both in the development and in adulthood (Crowley et al., 1994), and for its crucial contribution to NGF-induced pain sensitization and hyperalgesia (Lewin et al., 1993). Consistently, mice deprived of the NGF-TrkA signaling during embryonic life fail to develop sensory neurons properly, thus losing their response to noxious stimuli and dying within the first month, hampering behavioral and physiological studies (Crowley et al., 1994; Smeyne et al., 1994). The necessity of a functioning NGF-TrkA system strikingly emerges also from the existence of two rare genetic conditions of pain insensitivity called HSAN IV and V that affect TrkA and NGF, respectively (Indo, 2018; Rosemberg et al., 1994). In particular, HSAN IV is due to mutations in the *TRKA* gene (*NTRK1*) and it is specifically characterized by the loss of pain sensation, anhidrosis and variable degree of mental retardation (Indo et al., 1996). Genetic analyses of HSAN IV patients have identified more than 100 mutations in *TRKA* (HGMD 2021.4), suggesting that mutations in TrkA functional domains, such as Tyrosine Kinase Domain (TKD), can correlate with more severe clinical manifestations (Indo, 2001; Z. Liu et al., 2018). No suitable animal model for HSAN IV has been developed so far, making the search for disease-relevant biomarkers, the

investigation of the underlying mechanisms and the development of therapeutic strategies, particularly challenging.

2. R649W mutation influences TrkA biochemical and biophysical functions

Missense and nonsense mutations located in TrkA functional domains, such as the tyrosine kinase domain (TKD), are thought to contribute to greater clinical manifestations in HSAN IV patients (Indo, 2001; Z. Liu et al., 2018). In this regard, we selected the missense mutation Arg649Trp (R649W) located in Exon 15 of *TRKA,* firstly identified in a Spanish HSAN IV patient (Mardy et al., 1999). Since it has been previously shown that the substitution of the Arginine-649 residue causes a strong reduction in the receptor phosphorylation in response to the binding of NGF (Mardy et al., 1999; Miranda et al., 2002), we have replicated and extended biochemical experiments confirming that the R649W mutation prevents the receptor auto-phosphorylation resulting in the TrkA inactivation, without affecting the total amount of protein. Interestingly, we also found that the $TrkA^{R649W}$-mutant receptor shows a lower level of ubiquitination compared to $TrkA^{WT}$. All Receptor Tyrosine Kinases (RTKs) exhibit a similar organization of intracellular cytoplasmic domain that conformationally changes after the ligand engagement, resulting in the activation of the receptor, in the downstream signal transduction and, lastly, in the degradation based on a ligand-dependent ubiquitination mechanism (Hicke & Dunn, 2003). In addition to degradation, the ubiquitination machinery also modulates protein transport and acts as a sorting signal to direct RTKs to different cellular compartments (Hicke & Dunn, 2003). For instance, the Epidermal Growth Factor Receptor (EGFR), upon ligand activation, is rapidly directed to lysosomes and degraded by the ubiquitination machinery (Thien & Langdon, 2001; Huang et al., 2006) in a threshold-controlled way that allows cells to convert different stimuli into threshold-dependent biological responses (Sigismund et al., 2013).

Regarding TrkA, it has been observed that Nedd4-2, a member of the Nedd4 E3 HECT Ub-ligases family, specifically ubiquitinates TrkA promoting the neuronal apoptosis NGF-dependent in DRGs overexpressing Nedd4-2 (Arevalo et al., 2006). The highly conserved Nedd4-2 binding domain of

TrkA is involved in the *in vivo* response to noxious stimuli. Indeed, the $TrkA^{P782S}$ mutation in the TrkA binding motif for Nedd4-2 leads to a decreased ubiquitination and degradation, resulting in an increased number of peptidergic nociceptors CGRP+ and SP+ and a consequent higher sensitivity to thermal and inflammatory pain in animals (Yu et al., 2014). A similar behavioral phenotype has been observed also in animals in which the TrkA 3 aa (KFG) domain has been deleted, resulting in a reduced TrkA ubiquitination that leads to an increase in TrkA protein levels and activity, without affecting DRGs density (Kiris et al., 2014).

Interestingly, our data show that the R649W mutation in the TKD is important for the constitutive ubiquitination of TrkA, suggesting a potential relevance of this residue in the trafficking of TrkA receptors (Yu et al., 2011). However, as shown in the results, this does not lead to enhanced sensitivity to thermal and inflammatory pain in knock-in $TrkA^{R649W}$ mice, as instead occurred with other TrkA mutant proteins (Yu et al., 2014; Kiris et al., 2014). In the future, it will be of interest to clarify how the TrkA mutations modulating constitutive or NGF-induced ubiquitination differentially affect pain sensitivity in patients with pathological pain conditions.

Furthermore, using TIRF microscopy combined with Single Particle Tracking (SPT), we observed a reduced membrane mobility of $TrkA^{R649W}$, possibly dependent on the kinase inactivity of TrkA and, surprisingly, a significant increase in the membrane density of $TrkA^{R649W}$. SPT had previously revealed that the mobility of the TrkA receptor is highly NGF-dependent (Marchetti et al., 2013). The SPT performed in this paper shows that mutations in TKD, such as R649W, could be sufficient to modify the receptor-membrane dynamics. Moreover, since post-translational modifications are critical for function and localisation of membrane proteins, such as TrkA, it is of great interest to study whether the mutation could impair the traffic of the mutant $TrkA^{R649W}$ to the cell surface by interfering with the N-linked glycosylation events. Glycosylation starts in the Endoplasmic Reticulum (ER) and culminates in the Golgi before the mature glycosylated protein transits to the plasma membrane. In several transmembrane proteins and ion channels, the fully glycosylated protein, usually indicated by the upper band in western blot, is an indicator of correct membrane trafficking,

whereas the lower band primarily represents the immature protein mainly located in the ER and Golgi (Ohtsubo & Marth, 2006). As shown in the Fig. 1C, the TrkA immature form (approx. 110 kDa and represented by the lower band) appears less intense than the TrkA mature form (approx. 140 kDa and indicated as the upper band), suggesting possible damage in N-linked glycosylation events. By treating HEK TrkA^{R649W}-expressing cells and controls with enzymatic methods for removing N-linked glycans, such as PNGase F or tunicamycin, I could be able to determine if the two bands in Fig. 1C resulted from different levels of glycosylation. Notably, N-linked glycosylation on TrkA sites is reported to prevent the NGF-independent activation that otherwise leads to a mislocalized intracellular expression of the receptor (Watson et al., 1998). Further experiments aimed to clarify possible changes in the glycosylation state of the mutant TrkA^{R649W} receptor may be a predictor of efficient membrane trafficking and functionality.

The *in vitro* data, collected both in a neuroblastoma cell line and in DRG primary cultures, reinforce the conclusion that the R649W mutation substantially alters both the kinase activity and the subcellular trafficking of TrkA receptors. Importantly, these alterations are maintained when TrkA^{R649W} is expressed in DRG neurons from mice expressing endogenously wild-type TrkA, a model which better recapitulates what occurs in TrkA^{R649W} heterozygous mice. However, whether and how the R649W mutation influences the formation of homo- and/ or hetero-dimers and corresponding signal transduction pathways, remains to be seen. Several lines of evidence indicate that functional interactions between TrkA and p75^{NTR} may play a critical role in NGF-triggered signaling, especially by promoting the formation or stabilization of TrkA active homodimers (Franco et al., 2021) and by reducing the TrkA ubiquitination and internalization boosting prolonged signaling (Makkerh et al., 2005). It is thus tempting to hypothesize that the presence of R649W mutation might limit p75^{NTR}-TrkA interactions in NGF-triggered signal transduction. This will need to be investigated with a dedicated study that is beyond the scope of this manuscript.

3. TrkA^{R649W} mutant mice as HSAN IV mouse model

Despite efforts to understand congenital insensitivity pain disorders, only a few transgenic mouse models of pain insensitivity have been reported that fully reproduce human conditions (Monteiro et al., 2019). The early lethality observed in homozygous TrkA-deficient (TrkA$^{-/-}$) mice (Smeyne et al., 1994) has hampered the use of this animal model to study HSAN IV disease. Similarly to TrkA$^{-/-}$ mice, also NGF$^{-/-}$ mice (Crowley et al., 1994) show a postnatal lethality, suggesting a critical role of the NGF-TrkA signaling pathway in the development and maintenance of sensory neurons. Generating a transgenic mouse line harboring the human 661C>T mutation in the human NGF gene allowed to clarify the involvement of the neurotrophin in HSAN V disease and to contribute in developing of a useful model to study painless syndromes (Testa et al., 2019). As for HSAN V, mechanistic investigations and treatment of HSAN IV is nowadays still challenging. Thus, we have generated and characterized a novel knock-in mouse line carrying the mutation R649W in the human *TRKA* that displays the key features of the disease, also including the distinctive aspects with respect to the related HSAN V disease. In homozygosity, the R649W mutation causes a postnatal lethal phenotype within the first week of life, similarly to the R100W mutation in the NGF gene (Testa et al., 2019). The lethality observed for homozygous TrkA$^{R649W/R649W}$ mice is only seemingly in contrast with the homozygosity of the R649W mutation in HSAN IV patients (Mardy et al. 1999). In fact, individuals affected by HSAN IV often survive into adulthood also thanks to careful medical treatments (Indo, 2018). Moreover, this was rather expected due to the observed lethality also in TrkA$^{-/-}$ mice (Smeyne et al., 1994) and might be possibly explained by i) redundant mechanisms that have developed during evolutionary processes for the NGF-TrkA pathway in humans or by ii) the lower affinity of endogenous mouse NGF for the human TrkA receptors (Paoletti et al., 2015) and hence also for the homozygous TrkA$^{R649W/R649W}$ mice. In this respect, by crossing homozygous wild-type human NGF mice (NGF$^{wt/wt}$) (Testa et al., 2019) with homozygous TrkA$^{R649W/R649W}$ mice will allow testing the latter explanation, and thus if increasing the affinity of the ligand for the receptor might overtake the lethality observed in homozygous TrkA$^{R649W/R649W}$ mice.

4. Effect of TrkA^{R649W} mutation on sensation

In line with HSAN IV human symptoms, TrkA$^{R649W/m}$ mice failed to react to thermal and chemical noxious stimuli. I found a diminished licking behavior after capsaicin injection and, interestingly, I found a decrease in the number of TRPV1+ neurons that are involved in the mediation of noxious stimuli (Caterina et al., 1997). The loss of TRPV1+ neurons, crucial in avoiding tissue damage and in the development of NGF-induced heat hyperalgesia (Chuang et al., 2001; Omerbašić et al., 2016), and the behavioral correlate of pain insensitivity in HSAN IV TrkA$^{R649W/m}$ mice, may represent a feasible explanation for the inability of HSAN IV patients to avoid injuries caused by noxious-thermal stimuli. This hypothesis could also explain the lower responsiveness of TrkA$^{R649W/m}$ mice in the hot plate test. TrkA$^{R649W/m}$ mice also show a decreased reaction to cold, which might suggest an abnormal regulation of the cold-activated channels TRPA1, expressed in subsets of heat-sensitive, TRPV1-positive and NGF-dependent neurons (Story et al., 2003; Babes et al., 2004; Obata et al., 2005; Diogenes et al., 2007). The neuronal reduction observed in non-peptidergic nociceptors IB4+ may be due to developmental alterations. Indeed, during embryonic stages and in the early postnatal period, about 50% of developing nociceptors down-regulate TrkA and begin to express the receptor for glial cell-derived growth factor (GDNF), Ret. This neuronal population is destined to become non-peptidergic nociceptors that bind isolectin B4 (IB4+), while the remaining nociceptors expressing TrkA but not Ret (TrkA^{+}/Ret^{-}) become the peptidergic subtype CGRP^{+} (Luo et al., 2007; Woolf & Ma, 2007). The quantification of these two major classes of nociceptors, peptidergic (CGRP^{+}) and non-peptidergic (IB4^{+}) nociceptors (Snider & Mcmahon, 1998), showed a significant decrease in IB4^{+} neurons in TrkA$^{R649W/m}$ mice, both in young and adult mice. Defects in the development of nociceptors, similarly to mutations in the epigenetic regulator PRDM12 causing developmental changes in nociceptors (Chen et al., 2015), can explain several congenital insensitivity to pain disorders (Drissi et al., 2020), as also mutations in $Na_v1.9$ linked to a sustained depolarization of nociceptors (Leipold et al., 2013), and inactivating mutations in Nav1.7 altering the transmission of tissue-damage signal (Minett et al., 2014). Conversely, the R100W mutation in NGF in heterozygous

$NGF^{R100W/m}$ mice does not affect the development of nociceptors during the early postnatal stage , since NGF^{R100W} maintains its neurotrophic activity unaltered (Testa et al., 2019). In view of these considerations, I speculate that R649W mutation could alter the developmental transition of non-peptidergic. DRG neurons from $TrkA^{+}$ to Ret^{+} in $TrkA^{R649W/m}$ mice, resulting in a strong reduction of IB4 expression in the $TrkA^{R649W/m}$ DRG. The inability of $TrkA^{R649W/m}$ mice to perceive thermal and chemical noxious stimuli was accompanied by a strong reduction of cutaneous nerves in both hairless and hairy skin. R649W mutation seems to be sufficient to affect skin innervation through a possible change in the early stages of sensory neuron development (Davies et al., 1987). Interestingly, I found that $TrkA^{R649W}$ reduces the response to non-noxious mechanical stimulation in the hairy skin. This result could suggest that $TrkA^{R649W}$ impairs the growth of hair follicles in the hairy skin by damaging the NGF-TrkA signaling (Peters et al., 2006). A functional TrkA trafficking and signaling during the development are both necessary for the innervation of NGF-target organs/tissues and crucial for the axon growth of sensory neurons (Ascano et al., 2009). Indeed, a continuous supply of TrkA at axon tips is fundamental during axon elongation of sensory neurons (Vaegter et al., 2011) and dysregulation of TrkA axonal trafficking has been causally linked to peripheral neuropathies (Zhang et al., 2013).

As already mentioned, though the pain-related behavioral phenotype of $TrkA^{R649W/m}$ mice is quite similar to that of HSAN V $NGF^{R100W/m}$ mice (Testa et al., 2019b), only HSAN IV $TrkA^{R649W/m}$ mice show a specific lack of the IB4+ DRG subpopulation, suggesting that the developmental consequences of the HSAN IV $TrkA^{R649W}$ mutation on sensory neurons appear to be more severe than those observed in HSAN V mice (Testa et al., 2019b).

Interestingly, a similar HSAN IV-painless phenotype is also found in patients affected by Familial dysautonomia (FD) or HSAN III/Riley-Day syndrome, a debilitating disorder caused by mutations in ELP1/*IKBKAP* gene that results in the skipping of exon 20 in the mRNA of patients with FD. Although the loss of ELP1 appears not to affect the specification and/or migration of neural crest cells, it is linked to the accumulation of DNA damages in differentiated TrkA+ neurons during the

development, leading to sensory dysfunction such as pain and temperature insensitivity (George et al., 2013; Goffena et al., 2018). Interestingly, the lack of Elp1 in Elp1-cKO mice seems to be caused by impaired retrograde transport of NGF in sensory neurons. Elp1 seems to facilitate the protein-protein interaction required for the axonal retrograde transport of the NGF-TrkA complex (Tourtellotte, 2016; Jackson et al., 2014). In this respect, mutations in ELP1 and TrkA genes might have a (partial) common consequence in a defect in sensory neurons. As well as FD, impairment in NGF-retrograde transport and signaling may contribute to explaining the HSAN IV pathological phenotype. Indeed, our results suggest deficits in the NGF-TrkA trafficking in sensory neurons in HSAN IV TrkA^{R649W} mice. Thus, potential involvement of ELP1, possibly via a disrupted direct or indirect interaction with the mutant TrkA, might be postulated. However, whether and how Elp1and TrkA overlap in a functional manner requires further investigation that may help to highlight similarities and differences between these two congenital conditions.

5. TrkA^{R649W} mutation induces defects in thermoregulation

By using a murine-adapted version of Minor's starch iodine test, usually performed in humans to assess the severity of sweat-production, I determined in our transgenic model the presence of anhidrosis, a distinctive trait of HSAN IV disease (Minor V, 1928). Quantification of "dark spots" strongly remarked that the sweating in TrkA$^{R649W/m}$ mice requires functional NGF-TrkA signaling and that the TrkA^{R649W}-inactive receptor can lead to anhidrosis. These results were supported by comparing TrkA$^{R649W/m}$ mice to HSAN V NGF$^{R100W/m}$ mice that showed normal sweating. In line with HSAN V heterozygous patients (Axelsson et al., 2009), I highlighted that HSAN V NGF$^{R100W/m}$ mice, although impaired temperature perception (Testa et al., 2019), showed normal sweating and unaffected sweat glands innervation. Regarding HSAN IV, sweat glands innervation appears to be variable among patients. Indeed, skin biopsies in some patients revealed normal sweat glands innervation (Itoh et al., 1986; Pinsky & DiGeorge, 1966; Swanson, 1963), while skin samples of other patients reported non-innervated sweat glands (Nolano et al., 2000; Langer et al., 1981). The analysis of noradrenergic and cholinergic innervation of TrkA$^{R649W/m}$ sweat glands revealed no differences compared to control. These data may suggest that a possible cause of anhidrosis in HSAN IV could be found in brain regions, such as the preoptic hypothalamic area, that control thermoregulation (Morrison, 2016). However, I can not completely exclude the peripheral contribution of nerve fibers to anhidrosis, and further experiments aimed to elucidate the electrophysiological function of these fibers is necessary. An inactive-TrkA, such as TrkA^{R649W}, might lead to changes in the regulation of the body temperature in HSAN IV patients (Indo, 2018; Loewenthal et al., 2005). Indeed, cold and warmth, detected in the skin by primary sensory nerve endings, many of which express TrkA, are transmitted to the hypothalamic areas that represent the main integration site for thermoregulation, including sweating (Nakamura & Morrison, 2008).

Interestingly, one of the most important players of thermosensation, TRPV1, has been found also outside of sensory nerves, such as in skeletal muscle, heart and adipose tissues contributing to blood

flow control (Phan et al., 2020). Researchers found that as easily observed in humans after consumption of chili peppers, the capsaicin injections activate several thermoregulatory processes in rodents, including vasodilation and sweating in order to promote a decrease in the body temperature (Phan et al., 2020). The loss of TRPV1, indeed, leads to defective control of the temperature homeostasis in rodents contributing also to an altered itch and pain sensation (Mishra et al., 2010). Although the still debated effect of TRPV1 on body temperature limits the therapeutic use of TRPV1-antagonists for the treatment of pain conditions, recent findings have suggested that the neuronal TRPV1 is necessary for body temperature modulation (Yue et a., 2022).

Altogether these observations allow us to speculate that the $TrkA^{R649W}$ mutation may affect the thermoregulation acting on both the peripheral and central nervous system. However, how the R649W mutation may cause thermoregulatory perturbations in HSAN IV patients and in an animal model leading to sweating deficiency, needs to be clarified in future work.

6. TrkA contributes to cognitive abilities

Among several symptoms, HSAN IV patients are affected by variable degrees of mental retardation (Rosemberg et al., 1994), usually associated with ADHD (Levy-Erez et al., 2010). Contrary to TrkA$^{-/-}$ mice, cognitive performances of TrkA$^{R649W/m}$ mice were strongly influenced by the R649W mutation. Specifically, the exploratory behavior observed in working-spatial memory-dependent tests was found altered in TrkA$^{R649W/m}$ mice. In addition, the much higher time spent in open arms was predictive of a reduced anxiety-like phenotype of TrkA$^{R649W/m}$ mice. Importantly, the social memory, evaluated in the Three-Chamber test that assesses cognition as general sociability and interest in social novelty (Chadman et al., 2008), was strongly reduced, contrary to HSAN V mice. Indeed, difficulty establishing interpersonal relationships is considered an exclusive trait of children with HSAN IV (Indo, 2018).

Our data suggest that an inactive receptor, as TrkA^{R649W}, might have a stronger influence on cognitive performance than the total loss of the protein, as observed in TrkA ko mice (Muller et al., 2012; Sanchez-Ortiz et al., 2012). Moreover, these data might indicate that cognitive abilities are more influenced by TrkA than NGF. Indeed, NGF^{R100W} mutation associated with HSAN V disease causes a milder pathological phenotype with a loss of pain and temperature sensation but with unaffected sweating and cognitive abilities (Einarsdottir et al., 2004; Testa et al., 2019).

As discussed in a previous chapter, pain is nowadays not simply considered a consequence of intensive stimulation (nociception), but it is also determined by psychological factors such as the emotional, motivational and social components (IASP, 1978). Several theories have suggested that social bondings, such as couple relationships, may have an important role in the pain perception in chronic pain conditions, meaning that partner(s) may positively contribute to pain severity (Leonard et al., 2006). On the other hand, pain itself has a robust influence on social behavior in laboratory animals, leading to approach conspecifics in pain (Smith et al., 2021; Ferretti et al., 2017). Similarly to human “empathy” that is the ability of others to perceive such social cues, this mouse behavior is

referred to as “social transfer of pain” (Smith et al., 2021). Interestingly, the observation of pain in one mouse is demonstrated to affect the responses of its conspecifics, the observer, to painful stimuli, resulting in a pain contagion (Langford et al. 2006). These observations highlight the importance of pain perception not only in avoiding damage but also in establishing interpersonal relationships. The insensitivity to painful stimuli can thus affect the sociability and interest in the social novelty of $TrkA^{R649W}$ HSAN IV mice, contributing to their reduced cognitive performances. However, to understand how TrkA and its mutations may compromise the social aspects will need further experiments. Interestingly, the NGF-target brain regions expressing TrkA, such as the basal forebrain, are linked to cortical and subcortical areas involved in empathetic responses to pain in humans (Lamm et al., 2011) and in rodents (Smith et al., 2021). TrkA and its related mutation may also influence cognitive performances, mostly related to sociability, acting on non-neuronal cells. Indeed, microglia is an NGF-target cell type in the brain (Rizzi et al., 2018) possibly contributing to its scavenger function involved in neuropathic pain (Batti et al., 2016). Anterior cingulate cortex (ACC) microglia is reported to participate in the maintenance of chronic conditions, especially by influencing affective-motivational components (Miyamoto et al., 2017). Further analysis of microglia in emotional-related brain areas, such as ACC and Nucleus Accumbens may clarify the role of $TrkA^{R649W}$ mutation in HSAN IV sociability defects.

6. Conclusion and future perspectives

The experimental approach and the results described in this thesis emphasize the necessity of a transgenic animal model that can strongly help to broaden the knowledge about the causes, mechanisms, onset and progression of rare genetic pain-related diseases, such as HSAN IV.

The lack of TrkA, as shown in TrkA fully knock-out mice, causes a more severe phenotype than that observed in HSAN IV patients even though humans probably have identical mutations to the knock-out mice and should thus have similar effects (Monteiro et al., 2019; Smeyne et al., 1994). This could be possibly due to the lack of compensatory mechanisms that mitigate the severity of the human mutation. The development of $TrkA^{R649W}$ HSAN IV mice can thus contribute to gain knowledge about pain transmission, possibly identifying different contributions of the receptor TrkA and the ligand NGF to the physiology of sensory neurons.

Since $TrkA^{R649W}$ HSAN IV mice represent a very useful and promising tool to dissect the multifaceted roles of NGF-TrkA in the nervous system, I can suggest three main aspects to investigate:

1. One of the most intriguing results concerns the absence of sweating that might suggest damage in thermoregulation in $TrkA^{R649W}$ HSAN IV mice. In spite of the impossibility of excluding the TrkA peripheral contribution to anhidrosis (as discussed above), it is reasonable to suggest performing a broader analysis of the central role of TrkA in thermoregulation. Indeed, a possible cause of anhidrosis in HSAN IV patients may be related to a failure in the central mechanism(s) controlling body temperature homeostasis and thus involving TrkA effect on warm-sensitive neurons in POA. Since the sweating defects and possible thermoregulatory consequences might affect the body temperature, I could record the external $TrkA^{R649W}$ mice's body temperature using a thermal camera. Then, using a model of fever induced by lipopolysaccharide (LPS), I could provide insights into the mechanisms leading to recurrent

and, at times, lethal episodes of fever caused by anhidrosis in HSAN IV patients. Additionally, to dissect the peripheral impact of $TrkA^{R649W}$ mutation on central thermoregulation, I could perform a thermal challenge to test if the influence of the altered response to environmental temperature changes or chemical agents (capsaicin), already observed in $TrkA^{R649W}$ HSAN IV mice, on POA neurons.

2. Exploring the contribution of the $TrkA^{R649W}$ receptor in neuropathic pain conditions in order to give an answer to an interesting question. This follow-up project aims to clarify if the absence of a functional NGF-TrkA system is required for the onset and maintenance of neuropathic pain conditions, and if it is thus possible to experience neuropathic pain in the absence of acute pain, as in HSAN IV patients. Nassar and colleagues have demonstrated that mice lacking both $Na_v1.7$ and $Na_v1.8$, two voltage gated sodium channels highly expressed in nociceptors and involved in inflammatory pain, develop normal levels of neuropathic pain (Nassar et al., 2005). Indeed, even though double knock-outs of both $Na_v1.7$ and $Na_v1.8$ showed reduced response to noxious stimuli, after peripheral nerve injury, mice developed robust mechanical allodynia (Nassar et al., 2005). Interestingly, a diagnosed HSAN II patient, carrying the painless null mutations in *SCN9A* that encodes for $Na_v1.7$, has developed neuropathic pain (Wheeler et al., 2014), suggesting that neuropathic pain can be initiated and maintained in the absence of Nav1.7 channel both in humans and in rodents (Nassar et al., 2005; Wheeler et al., 2014). As profoundly discussed in this thesis, the double relevance of the NGF-TrkA signaling on both painful state retention and therapeutic target, makes the $TrkA^{R649W}$ mutation a key candidate to study of neuropathic pain conditions. Among several types of peripheral neuropathies, painful diabetic neuropathy (PDN) has been reported to have a higher incidence affecting 25% of diabetic patients (Abbott et al., 2011). An obvious question thus comes to mind, may $TrkA^{R649W}$ HSAN IV mice develop PDN, or does the

TrkA^{R649W} mutation exert a protective effect on animals? The nociceptors hyperexcitability PDN-induced is linked to high intracellular calcium and it is found to affect mitochondrial morphology leading to mechanical allodynia and sensory nerve degeneration (George et al., 2022). Interestingly, TrkA^{R649W} mutation could reduce the hyperexcitability PDN-induced by driving mitochondrial calcium flux changes in sensory neurons and thus blocking the genesis of chronic state. TrkA, indeed, has been reported to be localized in the mitochondrial compartment of neuronal and non-neuronal cells contributing to the modulation of intracellular calcium (Carito et al., 2012). Moreover, the NGF-TrkA signaling seems to influence the electrophysiological properties of some Aβ- rapidly adapting fibers involved in the response to mechanical stimuli (Fang et al., 2005) by regulating the expression of Nav1.8 in nociceptors (Dib-Hajj et al., 1998). Based on these observations, trying to understand if the TrkA^{R649W} mutation may confer any resistance to the development of neuropathic pain in HSAN IV animal model, and possibly in patients, can result in a captivating question.

3. As discussed in the previous chapter, pain perception is critical in establishing interpersonal relationships and thus, the pain insensitivity of HSAN IV patients may compromise social abilities contributing to reduced cognitive performance. It is known that psychological factors, such as fear and anxiety, are linked to pain, and several animal studies suggest that responses to pain stimuli are strongly reduced by fear (Gross & Canteras, 2012). On the other hand, pain itself influences social behavior in animals, including humans (Smith et al., 2021; Ferretti et al., 2017). However, although I found deficits in spatial-working and social memory in TrkA^{R649W} HSAN IV mice, it is not yet known whether the reduction of pain perception may also affect central emotional and motivational responses to stimuli.

   First of all, I want to evaluate the fear system in our painless animal model. Does exposure to pain, which was never experienced before, influence learning of the fear response of

TrkA^{R649W} HSAN IV mice? Can the inability to feel pain impair the capacity to avoid an adverse stimulus, such as a foot shock? In order to give an answer to these questions, I could perform a fear-conditioning protocol on TrkA^{R649W} HSAN IV mice and explore brain regions involved in the "pain-fear circuit", especially the amygdala and PAG. Additionally, I could investigate the role of microglia non-neuronal cells in establishing and maintaining the pain-fear circuit in the painless animal model.

Afterward, I want to understand if TrkA^{R649W} HSAN IV mice' sensory impairment compromises the ability to feel the pain in "others". As reported by Langford, pain sensation in rodents, as well as in humans, can be modulated solely by exposure to other conspecifics in pain (Langford et al., 2006). In order to observe the "pain contagion" in TrkA^{R649W} HSAN IV mice, I want to perform a slightly modified version of the "social transfer of pain" protocol (Smith et al., 2021). The test consists of two main sessions, "social-interaction" and "pain-transfer" parts. The first session is based on the interaction of "bystanders" (TrkA^{R649W} HSAN IV mice) and "demonstrators" (wild-type cagemate mice). Tested TrkA^{R649W} HSAN IV mouse, the bystander, will be free to interact with one demonstrator "in pain" and one "neutral". The "in-pain demonstrator" will be previously injected with capsaicin, contrary to the "neutral demonstrator". The interaction time between tested bystander TrkA^{R649W} HSAN IV mouse and demonstrators will be measured. At the end of this session, TrkA^{R649W} HSAN IV bystander mouse will be tested for a mechanical response. Indeed, the observation of pain in a conspecific can sensitize the observer, leading to an increased response to non-noxious stimuli. In order to obtain a better understanding of this behavior, I will perform a histological analysis of brain areas involved in "empathetic" responses, such as ACC and Nucleus Accumbens. Interestingly, I aim to characterize the microglia profile that is involved in the maintenance of pain state, especially by influencing affective-motivational components (Miyamoto et al., 2017).

7. References

Abbott, C. A., Malik, R. A., Van Ross, E. R. E., Kulkarni, J., & Boulton, A. J. M. (2011). Prevalence and characteristics of painful diabetic neuropathy in a large community-based diabetic population in the U.K. *Diabetes Care*, *34*(10), 2220–2224.

Abdiche, Y. N., Malashock, D. S., & Pons, J. (2008). Probing the binding mechanism and affinity of tanezumab, a recombinant humanized anti-NGF monoclonal antibody, using a repertoire of biosensors. *Protein Science*, *17*(8), 1326–1335.

Abrahamsen, B., Zhao, J., Asante, C. O., Cendan, C. M., Marsh, S., Martinez-Barbera, J. P., Nassar, M. A., Dickenson, A. H., & Wood, J. N. (2008). References and Notes. *SCIENCE*, *321*, 702–705.

Abraira, V. E., & Ginty, D. D. (2013). The sensory neurons of touch. *Neuron* 79, 618–639.

Alleva, E., Petruzzi, S., Cirulli, F., & Aloe, L. (1996). NGF regulatory role in stress and coping of rodents and humans. *Pharmacology Biochemistry and Behavior*, *54*(1), 65–72.

Altassan, R., Saud, H. Al, Masoodi, T. A., Dosssari, H. Al, Khalifa, O., Al-Zaidan, H., Sakati, N., Rhabeeni, Z., Al-Hassnan, Z., Binamer, Y., Alhashemi, N., Wade, W., Al-Zayed, Z., Al-Sayed, M., Al-Muhaizea, M. A., Meyer, B., Al-Owain, M., & Wakil, S. M. (2017). Exome sequencing identifies novel NTRK1 mutations in patients with HSAN-IV phenotype. *American Journal of Medical Genetics, Part A*, *173*(4), 1009–1016.

Apkarian, A. V., & Baliki, M. N. (2015). Nociception, pain, negative moods and behavior selection. *Neuron*, *83*(5), 474–491.

Apkarian, A. V., Hashmi, J. A., & Baliki, M. N. (2011). Pain and the brain: Specificity and plasticity of the brain in clinical chronic pain. *NIH Public Access*, *152*(1), 1–35.

Arévalo, J. C., Waite, J., Rajagopal, R., Beyna, M., Chen, Z. Y., Lee, F. S., & Chao, M. V. (2006). Cell Survival through Trk Neurotrophin Receptors Is Differentially Regulated by Ubiquitination. *Neuron*, *50*(4), 549–559.

Arnett, M. G., Ryals, J. M., & Wright, D. E. (2007). pro-NGF, sortilin, and p75NTR: Potential mediators of injury-induced apoptosis in the mouse dorsal root ganglion. *Brain Research*, *1183*(1), 32–42.

Ascano, M., Richmond, A., Borden, P., & Kuruvilla, R. (2009). Axonal targeting of Trk receptors via transcytosis regulates sensitivity to neurotrophin responses. *Journal of Neuroscience*, *29*(37), 11674–11685.

Axelrod, F. B., & Gold-Von Simson, G. (2007). Hereditary sensory and autonomic neuropathies: Types II, III, and IV. *Orphanet Journal of Rare Diseases*, *2*(1).

Bai, L., Lehnert, B. P., Liu, J., Neubarth, N. L., Dickendesher, T. L., Nwe, P. H., Cassidy, C., Woodbury, C. J., & Ginty, D. D. (2015). Genetic Identification of an Expansive Mechanoreceptor Sensitive to Skin Stroking. *Cell*, *163*(7), 1783–1795.

Bandell, M., Story, G. M., Hwang, S. W., Viswanath, V., Eid, S. R., Petrus, M. J., Earley, T. J., & Patapoutian, A. (2004). Three other TRPV ion channels with distinct thresholds (mTRPA1) show a sharp increase in intracellular calcium. *Neuron*, *41*, 849–857.

Barde, Y. A., Edgar, D., & Thoenen, H. (1982). Purification of a new neurotrophic factor from mammalian brain. *The EMBO Journal*, *1*(5), 549–553.

Bardoni, R., Tawfik, V. L., Wang, D., François, A., Solorzano, C., Shuster, S. A., Choudhury, P., Betelli, C., Cassidy, C., Smith, K., deNooij, J. C., Mennicken, F., O'Donnell, D., Kieffer, B. L., Woodbury, C. J.,

Basbaum, A. I., MacDermott, A. B., & Scherrer, G. (2014). Delta opioid receptors presynaptically regulate cutaneous mechanosensory neuron input to the spinal cord dorsal horn. *Neuron*, *81*(6), 1312–1327.

Barker, P. A., Mantyh, P., Arendt-Nielsen, L., Viktrup, L., & Tive, L. (2020). Nerve growth factor signaling and its contribution to pain. *Journal of Pain Research* 13, 1223–1241.

Basbaum, A. I., Bautista, D. M., Scherrer, G., & Julius, D. (2009). Cellular and Molecular Mechanisms of Pain. *Cell* 139, 267–284

Bastuji, H., Frot, M., Perchet, C., Magnin, M., & Garcia-Larrea, L. (2016). Pain networks from the inside: Spatiotemporal analysis of brain responses leading from nociception to conscious perception. *Human Brain Mapping*, *37*(12), 4301–4315.

Batti, L., Sundukova, M., Murana, E., Pimpinella, S., De Castro Reis, F., Pagani, F., Wang, H., Pellegrino, E., Perlas, E., Di Angelantonio, S., Ragozzino, D., & Heppenstall, P. A. (2016). TMEM16F Regulates Spinal Microglial Function in Neuropathic Pain States. *Cell Reports*, *15*(12), 2608–2615.

Bautista, D. M., Jordt, S. E., Nikai, T., Tsuruda, P. R., Read, A. J., Poblete, J., Yamoah, E. N., Basbaum, A. I., & Julius, D. (2006). TRPA1 Mediates the Inflammatory Actions of Environmental Irritants and Proalgesic Agents. *Cell*, *124*(6), 1269–1282.

Bautista, D. M., Movahed, P., Hinman, A., Axelsson, H. E., Sterner, O., Högestätt, E. D., Julius, D., Jordt, S. E., & Zygmunt, P. M. (2005). Pungent products from garlic activate the sensory ion channel TRPA1. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(34), 12248–12252.

Bautista, D. M., Siemens, J., Glazer, J. M., Tsuruda, P. R., Basbaum, A. I., Stucky, C. L., Jordt, S. E., & Julius, D. (2007). The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature*, *448*(7150), 204–208.

Bautista, D. M., Wilson, S. R., & Hoon, M. A. (2014). Why we scratch an itch: the molecules, cells and circuits of itch. *Nat Neuroscience*, *17*(2), 175–182.

Beggs, S., & Salter, M. W. (2016). SnapShot: Microglia in Disease. *Cell*, *165*(5), 1294-1294.e1.

Bennett, D. L., Clark, X. A. J., Huang, J., Waxman, S. G., & Dib-Hajj, S. D. (2019). The role of voltage-gated sodium channels in pain signaling. *Physiological Reviews*, *99*(2), 1079–1151.

Bennett, D. L. H., Averill, S., Clary, D. O., Priestley, J. V., & McMahon, S. B. (1996). Postnatal changes in the expression of the trkA high-affinity NGF receptor in primary sensory neurons. *European Journal of Neuroscience*, *8*(10), 2204–2208.

Berardi, N., Domenici, L., Parisi, V., Pizzorusso, T., Cellerino, A., & Maffei, L. (1993). Monocular deprivation effects in the rat visual cortex and lateral geniculate nucleus are prevented by nerve growth factor (NGF): I - Visual cortex. *Proceedings of the Royal Society B: Biological Sciences*, *251*(1330), 17–23.

Bhatnagar, S., & Dallman, M. F. (1999). The paraventricular nucleus of the thalamus alters rhythms in core temperature and energy balance in a state-dependent manner. *Brain Research*, *851*(1–2), 66–75.

Bonkowsky, J. L., Johnson, J., Carey, J. C., Smith, A. G., & Swoboda, K. J. (2003). An infant with primary tooth loss and palmar hyperkeratosis: a novel mutation in the NTRK1 gene causing congenital insensitivity to pain with anhidrosis. *Pediatrics*, *112*(3 Pt 1).

Brown, B. Y. A. G., & Iggo, A. (1967). *From the Department of Veterinary Physiology , University of Edinburgh*. 707–733.

Burgess, P. R., Petit, D., & Warren, R. M. (1968). Receptor types in cat hairy skin supplied by myelinated fibers. *Journal of Neurophysiology*, *31*(6), 833–848.

Caleo, M., Lodovichi, C., & Maffei, L. (1999). Effects of nerve growth factor on visual cortical plasticity require afferent electrical activity. *European Journal of Neuroscience*, *11*(8), 2979–2984.

Camino, D. Del, Murphy, S., Heiry, M., Barrett, L. B., Earley, T. J., Cook, C. A., Petrus, M. J., Zhao, M., D'Amours, M., Deering, N., Brenner, G. J., Costigan, M., Hayward, N. J., Chong, J. A., Fanger, C. M., Woolf, C. J., Patapoutian, A., & Moran, M. M. (2010). TRPA1 contributes to cold hypersensitivity. *Journal of Neuroscience*, *30*(45), 15165–15174.

Campbell, J. N., Raja, S. N., Meyer, R. A., & Mackinnon, S. E. (1988). Myelinated afferents signal the hyperalgesia associated with nerve injury. *Pain*, *32*(1), 89–94.

Capsoni, S. (2014). From genes to pain: Nerve growth factor and hereditary sensory and autonomic neuropathy type V. *European Journal of Neuroscience*, *39*(3), 392–400.

Capsoni, S., Giannotta, S., & Cattaneo, A. (2002). β-amyloid plaques in a model for sporadic Alzheimer's disease based on transgenic anti-nerve growth factor antibodies. *Molecular and Cellular Neuroscience*, *21*(1), 15–28.

Capsoni, S., Malerba, F., Carucci, N. M., Rizzi, C., Criscuolo, C., Origlia, N., Calvello, M., Viegi, A., Meli, G., & Cattaneo, A. (2017). The chemokine CXCL12 mediates the antiamyloidogenic action of painless human nerve growth factor. *Brain*, *140*(1), 201–217.

Carito, V., Pingitore, A., Cione, E., Perrotta, I., Mancuso, D., Russo, A., Genchi, G., & Caroleo, M. C. (2012). Localization of nerve growth factor (NGF) receptors in the mitochondrial compartment: Characterization and putative role. *Biochimica et Biophysica Acta - General Subjects*, *1820*(2), 96–

Carniglia, L., Ramírez, D., Durand, D., Saba, J., Turati, J., Caruso, C., Scimonelli, T. N., & Lasaga, M. (2017). Neuropeptides and Microglial Activation in Inflammation, Pain and Neurodegenerative Diseases. *Mediators of Inflammation*, *2017*.

Caterina, M. J., Leffler, A., Malmberg, A. B., Martin, W. J., Trafton, J., Petersen-Zeitz, K. R., Koltzenburg, M., Basbaum, A. I., & Julius, D. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science*, *288*(5464), 306–313.

Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., & Julius, D. (1997). *The capsaicin receptor: a heat-activated ion channel in the pain pathway*.

Cattaneo, A., & Calissano, P. (2012). Nerve growth factor and Alzheimer's disease: New facts for an old hypothesis. *Molecular Neurobiology*, *46*(3), 588–604.

Cattaneo, A., Capsoni, S., Margotti, E., Righi, M., Kontsekova, E., Pavlik, P., Filipcik, P., & Novak, M. (1999). *Functional Blockade of Tyrosine Kinase A in the Rat Basal Forebrain by a Novel Antagonistic Anti-Receptor Monoclonal Antibody*.

Cellerino, A., Pinzón-Duarte, G., Carroll, P., & Kohler, K. (1998). Brain-derived neurotrophic factor modulates the development of the dopaminergic network in the rodent retina. *Journal of Neuroscience*, *18*(9), 3351–3362.

Centeno, M. V., Procissi, D., Contini, M., Baria, A. T., Martina, M., Apkarian, V., & Apkarian1. (2014). Role of Nucleus Accumbens in Neuropathic Pain: Linked Multi_Scale Evidence in the Rat Transitioning to

Neuropathic PainRole of Nucleus Accumbens in Neuropathic Pain: Linked Multi_Scale Evidence in the Rat Transitioning to Neuropathic Pain. *Pain*, *155*(1), 1128–1139.

Ceredig, R. A., Pierre, F., Doridot, S., Alduntzin, U., Hener, P., Salvat, E., Yalcin, I., Gaveriaux-Ruff, C., Barrot, M., & Massotte, D. (2020). Peripheral Delta Opioid Receptors Mediate Formoterol Anti-allodynic Effect in a Mouse Model of Neuropathic Pain. *Frontiers in Molecular Neuroscience*, *12*(2), 1–11.

Chao, M. V. (2003). Neurotrophins and their receptors: A convergence point for many signalling pathways. *Nature Reviews Neuroscience*, *4*(4), 299–309.

Chen, A. I., De Nooij, J. C., & Jessell, T. M. (2006). Graded activity of transcription factor Runx3 specifies the laminar termination pattern of sensory axons in the developing spinal cord. *Neuron*, *49*(3), 395–408.

Chen, C. L., Broom, D. C., Liu, Y., De Nooij, J. C., Li, Z., Cen, C., Samad, O. A., Jessell, T. M., Woolf, C. J., & Ma, Q. (2006). Runx1 determines nociceptive sensory neuron phenotype and is required for thermal and neuropathic pain. *Neuron*, *49*(3), 365–377.

Chen, G., Zhang, Y. Q., Qadri, Y. J., Serhan, C. N., & Ji, R. R. (2018). Microglia in Pain: Detrimental and Protective Roles in Pathogenesis and Resolution of Pain. *Neuron* 100, 1292–1311.

Chen, K. S., Nishimura, M. C., Armanini, M. P., Crowley, C., Spencer, S. D., & Phillips, H. S. (1997). Disruption of a single allele of the nerve growth factor gene results in atrophy of basal forebrain cholinergic neurons and memory deficits. *Journal of Neuroscience*, *17*(19), 7288–7296.

Chen Y.C, Auer-Grumbach M., Matsukawa S., Zitzelsberger M., Themistocleous A.C, Strom TM, Samara C, Moore AW, Cho LT, Young GT, Weiss C, Schabhüttl M, Stucka R, Schmid AB, Parman Y, Graul-Neumann L, Heinritz W, Passarge E, Watson RM, Hertz JM, Moog U, Baumgartner M, Valente EM, Pereira D, Restrepo CM, Katona I, Dusl M, Stendel C, Wieland T, Stafford F, Reimann F, von Au K, Finke C, Willems PJ, Nahorski MS, Shaikh SS, Carvalho OP, Nicholas AK, Karbani G, McAleer MA, Cilio MR, McHugh JC, Murphy SM, Irvine AD, Jensen UB, Windhager R, Weis J, Bergmann C, Rautenstrauss B, Baets J, De Jonghe P, Reilly MM, Kropatsch R, Kurth I, Chrast R, Michiue T, Bennett DL, Woods CG, Senderek J. Transcriptional regulator PRDM12 is essential for human pain perception. *Nat Genet.* 2015 Jul;47(7):803-8.

Chen, X. J., & Sun, Y. G. (2020). Central circuit mechanisms of itch. *Nature Communications*, *11*(1), 1–10.

Chen, Z., Donnelly, C. R., Dominguez, B., Harada, Y., Lin, W., Halim, A. S., Bengoechea, T. G., Pierchala, B. A., & Lee, K. F. (2017). p75 Is Required for the Establishment of Postnatal Sensory Neuron Diversity by Potentiating Ret Signaling. *Cell Reports*, *21*(3), 707–720.

Cheng, L., Duan, B., Huang, T., Zhang, Y., Chen, Y., Britz, O., Garcia-Campmany, L., Ren, X., Vong, L., Lowell, B. B., Goulding, M., Wang, Y., & Ma, Q. (2017). Identification of spinal circuits involved in touch-evoked dynamic mechanical pain. *Nature Neuroscience*, *20*(6), 804–814.

Chuang, H.-H., Prescott, E. D., Kong[3], H., Shields, S., Jordt, S.-E., Basbaum, A. I., Chao, M. V, & Julius, D. (2001). Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P 2-mediated inhibition. *Nature*, 411.

Clark, A. K., & Malcangio, M. (2014). Fractalkine/CX3CR1 signaling during neuropathic pain. *Frontiers in Cellular Neuroscience*, *8(5)*, 1–7.

Cohen, S., & Levi-Montalcini, R. (1954). a Nerve Growth-Stimulating Factor Isolated From Snake Venom. *Proceedings of the National Academy of Sciences*, *42*(9), 571–574.

Cosens, D. J., & Manning, A. (1969). *Abnormal electroretinogram from drosophila*. *224*, 3–5.

Coste, B., Mathur, J., Schmidt, M., Earley, T. J., Ranade, S., Petrus, M. J., Dubin, A. E., & Patapoutian, A. (2010). Activated Cation Channels. *Science, 330*(10), 7–12.

Cottingham, John et al. (1985). *Descartes, René, Philosophical Writings*.

Covaceuszach, S., Marinelli, S., Krastanova, I., Ugolini, G., Pavone, F., Lamba, D., & Cattaneo, A. (2012). Single cycle structure-based humanization of an anti-nerve growth factor therapeutic antibody. *PLoS ONE*, *7*(3).

Cox, J. J., Reimann, F., Nicholas, A. K., Thornton, G., Roberts, E., Springell, K., Karbani, G., Jafri, H., Mannan, J., Raashid, Y., Al-Gazali, L., Hamamy, H., Valente, E. M., Gorman, S., Williams, R., McHale, D. P., Wood, J. N., Gribble, F. M., & Woods, C. G. (2006). An SCN9A channelopathy causes congenital inability to experience pain. *Nature*, *444*(7121), 894–898.

Crowley, C., Spencer, S. D., Nishlmum, M. C., Chen, K. S., Pitts-meek, S., Armanlni, M. P., Llng, L. H., Mcmahon, S. B., Shelton, D. L., Levinson, A. D., & Phillips, H. S. (1994). *Mice Lacking Nerve Growth Factor Display Perinatal Loss of Sensory and Sympathetic Neurons yet Develop Basal Forebrain Cholinergic Neurons*. *76*, 1001–1011.

De Carlos, F., Cobo, J., Germanà, G., Silos-Santiago, I., Laurà, R., Haro, J. J., Fariñas, I., & Vega, J. A. (2006). Abnormal development of pacinian corpuscles in double trkB;trkC knockout mice. *Neuroscience Letters*, *410*(3), 157–161.

De Andrade, D. C., Baudic, S., Attal, N., Rodrigues, C. L., Caramelli, P., Lino, A. M. M., Marchiori, P. E., Okada, M., Scaff, M., Bouhassira, D., & Teixeira, M. J. (2008). Beyond neuropathy in hereditary sensory and autonomic neuropathy type V: Cognitive evaluation. *European Journal of Neurology*, *15*(7), 712–719.

De Nadai, T., Marchetti, L., Di Rienzo, C., Calvello, M., Signore, G., Di Matteo, P., Gobbo, F., Turturro, S., Meucci, S., Viegi, A., Beltram, F., Luin, S., & Cattaneo, A. (2016). Precursor and mature NGF live tracking: One versus many at a time in the axons. *Scientific Reports*, *6*(2), 1–11.

Dahlhamer, J., Lucas, J., Zelaya, C., Nahin, R., Mackey, S., DeBar, L., Kerns, R., Von Korff, M., Porter, L., & Helmick, C. (2018). Prevalence of Chronic Pain and High-Impact Chronic Pain Among Adults — United States, 2016. *MMWR. Morbidity and Mortality Weekly Report*, *67*(36), 1001–1006.

Dallenbach, K. M. (1939). *Pain : History and Present Status*. *52*(3), 331–347.

Daneshjou, K., Jafarieh, H., & Raaeskarami, S. R. (2012). Congenital insensitivity to pain and anhydrosis (CIPA) syndrome; A report of 4 cases. *Iranian Journal of Pediatrics*, *22*(3), 412–416.

Davis, J. B., Gray, J., Martin J. Gunthorpe, J. P. H., Davey, P. T., Overend, P., Harries, M. H., Latcham, J., Clapham, C., Atkinson, K., Hughes, S. A., Rance, K., Grau, E., Harper, A. J., Pugh, P. L., Rogers, D. C., Bingham, S., Randall, A., & Sheardown, S. A. (2000). Vanilloid receptor-1 is essential for infammatory thermal hyperalgesia. *Nature*, *405*, 183–187.

Davies, A. M. (1996). The neurotrophic hypothesis: Where does it stand? *Philosophical Transactions of the Royal Society B: Biological Sciences*, *351*(1338), 389–394.

Dearborn, "A case of congenital general pure analgesia," Journal of Nervous & Mental Disease, vol. 75, no. 6, pp. 612–615, 1932.

Denk, F., Bennett, D. L., & Mcmahon, S. B. (2017). *Nerve Growth Factor and Pain Mechanisms*.

Deppmann, C. D., Mihalas, S., Sharma, N., Lonze, B. E., Niebur, E., & Ginty, D. D. (2008). A model for neuronal competition during development. *Science*, *320*(5874), 369–373.

Dhandapani, R., Arokiaraj, C. M., Taberner, F. J., Pacifico, P., Raja, S., Nocchi, L., Portulano, C., Franciosa, F., Maffei, M., Hussain, A. F., De Castro Reis, F., Reymond, L., Perlas, E., Garcovich, S., Barth, S., Johnsson, K., Lechner, S. G., & Heppenstall, P. A. (2018). Control of mechanical pain hypersensitivity in mice through ligand-targeted photoablation of TrkB-positive sensory neurons. *Nature Communications*, *9*(1).

Dib-Hajj, S. D., Black, J. A., Cummins, T. R., Kenney, A. M., Kocsis, J. D., & Waxman, S. G. (1998). Rescue of α-SNS sodium channel expression in small dorsal root ganglion neurons after axotomy by nerve growth factor in vivo. *Journal of Neurophysiology*, *79*(5), 2668–2676.

Dib-Hajj, S. D., Rush, A. M., Cummins, T. R., Hisama, F. M., Novella, S., Tyrrell, L., Marshall, L., & Waxman, S. G. (2005). Gain-of-function mutation in Nav1.7 in familial erythromelalgia induces bursting of sensory neurons. *Brain*, *128*(8), 1847–1854.

Diogenes, A., Akopian, A. N., & Hargreaves, K. M. (2007). NGF Up-regulates TRPA1 : Implications for Orofacial Pain. *J Dent Res*, *86*(6), 550–555.

Domenici, L., Cellerino, A., & Maffei, L. (1993). Monocular deprivation effects in the rat visual cortex and lateral geniculate nucleus are prevented by nerve growth factor (NGF): II - Lateral geniculate nucleus. *Proceedings of the Royal Society B: Biological Sciences*, *251*(1330), 25–31.

Dong, X., & Dong, X. (2018). Peripheral and Central Mechanisms of Itch Xintong. *Neuron*, *98*(12), 482–494.

Dray, A., & Perkins, M. (1993). Bradykinin and inflammatory pain. *Agents and Actions*, *16*, 65–73.

Duan, B., Cheng, L., & Ma, Q. (2018). Spinal Circuits Transmitting Mechanical Pain and Itch. *Neuroscience Bulletin*, *34*(1), 186–193.

Duan, G., Han, C., Wang, Q., Guo, S., Zhang, Y., Ying, Y., Huang, P., Zhang, L., Macala, L., Shah, P., Zhang, M., Li, N., Dib-Hajj, S. D., Waxman, S. G., & Zhang, X. (2016). A SCN10A SNP biases human pain sensitivity. *Molecular Pain*, *12*, 1–16.

Dyck, P. J., Mellinger, J. F., Reagan, T. J., Horowitz, S. J., Mcdonald, J. W., Litchy, W. J., Daube, J. R., Fealey, R. D., Go, V. L., Kao, P. C., Brimijoin, W. S., & Lambert, E. H. (1983). Not "indifference to pain" but varieties of hereditary sensory and autonomic neuropathy. *Brain*, *106*(2), 373–390.

Dyck, P. J., Peroutka, S., Rask, C., Burton, E., Baker, M. K., Lehman, K. A., Gillen, D. A., Hokanson, J. L., & O'Brien, P. C. (1997). Intradermal recombinant human nerve growth factor induces pressure allodynia and lowered heat-pain threshold in humans. *Neurology*, *48*(2), 501–505.

Edvardson, S., Cinnamon, Y., Jalas, C., Shaag, A., Maayan, C., Axelrod, F. B., & Elpeleg, O. (2012). Hereditary sensory autonomic neuropathy caused by a mutation in dystonin. *Annals of Neurology*, *71*(4), 569–572.

Eijkelkamp, N., Linley, J. E., Torres, J. M., Bee, L., Dickenson, A. H., Gringhuis, M., Minett, M. S., Hong, G. S., Lee, E., Oh, U., Ishikawa, Y., Zwartkuis, F. J., Cox, J. J., & Wood, J. N. (2013). A role for Piezo2 in EPAC1-dependent mechanical allodynia. *Nature Communications*, *4*(4).

Eijkelkamp, Niels, Linley, J. E., Baker, M. D., Minett, M. S., Cregg, R., Werdehausen, R., Rugiero, F., & Wood, J. N. (2012). Neurological perspectives on voltage-gated sodium channels. *Brain*, *135*(9), 2585–2612.

Einarsdottir, E., Carlsson, A., Minde, J., Toolanen, G., Svensson, O., Solders, G., Holmgren, G., Holmberg, D., & Holmberg, M. (2004). A mutation in the nerve growth factor beta gene (NGFB) causes loss of pain perception. *Human Molecular Genetics*, *13*(8), 799–805.

Eippert, F., & Tracey, I. (2014). Pain and the PAG: Learning from painful mistakes. *Nature Neuroscience*, *17*(11), 1438–1439.

Ernfors, P., Lee, K., & Jaenisch, R. (1994). Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature*, *368*(3), 147–150.

Faber, C. G., Lauria, G., Merkies, I. S. J., Cheng, X., Han, C., Ahn, H. S., Persson, A. K., Hoeijmakers, J. G. J., Gerrits, M. M., Pierro, T., Lombardi, R., Kapetis, D., Dib-Hajj, S. D., & Waxman, S. G. (2012). Gain-of-function Nav1.8 mutations in painful neuropathy. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(47), 19444–19449.

Fagan, A. M., Garber, M., Barbacid, M., Silos-Santiago, I., & Holtzman, D. M. (1997). *A Role for TrkA during Maturation of Striatal and Basal Forebrain Cholinergic Neurons In Vivo*.

Fagan, A. M., Zhang, H., Landis, S., Smeyne, R. J., Silos-Santiago, I., & Barbacid, M. (1996). *TrkA, But Not TrkC, Receptors Are Essential for Survival of Sympathetic Neurons In Vivo*.

Fahnestock, M., Yu, G., & Coughlin, M. D. (2004). ProNGF: A neurotrophic or an apoptotic molecule? *Progress in Brain Research*, *146*, 101–110.

Fang, X., Djouhri, L., Black, J. A., Dib-Hajj, S. D., Waxman, S. G., & Lawson, S. N. (2002). The presence and role of the tetrodotoxin-resistant sodium channel Nav1.9 (NaN) in nociceptive primary afferent neurons. *Journal of Neuroscience*, *22*(17), 7425–7433.

Fang, X., Djouhri, L., McMullan, S., Berry, C., Okuse, K., Waxman, S. G., & Lawson, S. N. (2005). trkA is expressed in nociceptive neurons and influences electrophysiological properties via Nav1.8 expression in rapidly conducting nociceptors. *Journal of Neuroscience*, *25*(19), 4868–4878.

Fertleman, C. R., Baker, M. D., Parker, K. A., Moffatt, S., Elmslie, F. V., Abrahamsen, B., Ostman, J., Klugbauer, N., Wood, J. N., Gardiner, R. M., & Rees, M. (2006). SCN9A Mutations in Paroxysmal Extreme Pain Disorder: Allelic Variants Underlie Distinct Channel Defects and Phenotypes. *Neuron*, *52*(5), 767–774.

Ferretti, V., Maltese, F., Contarini, G., Nigro, M., Bonavia, A., Huang, H., Gigliucci, V., Morelli, G., Scheggia, D., Managò, F., Castellani, G., Lefevre, A., Cancedda, L., Chini, B., Grinevich, V., & Papaleo, F. (2019). Oxytocin Signaling in the Central Amygdala Modulates Emotion Discrimination in Mice. *Current Biology*, *29*(12), 1938-1953.

Finger, S., Hustwit, M. P., Meyerson, B., Elias, W. J., Goodrich, J. T., Adams, C. B. T., & Philippon, J. H. (2003). Five early accounts of phantom limb in context: Paré Descartes, Lemos, Bell, and Mitchell. In *Neurosurgery*, 52,3.

Finnerup, N. B., Haroutounianb, S., Kamermanc, P., Barond, R., Bennette, D. L. H., Bouhassiraf, D., Cruccuh, G., Freemani, R., Hanssonj, P., Nurmikkol, T., Rajam, S. N., Ricen, A. S. ., Serrap, J., Smithq, B. H., Treeder, R.-D., & Jensena, T. S. (2016). Neuropathic pain: an updated grading system for research and clinical practice. *Pain*, *157*(8), 30–37.

Fox, A., Wotherspoon, G., McNair, K., Hudson, L., Patel, S., Gentry, C., & Winter, J. (2003). Regulation and function of spinal and peripheral neuronal B1 bradykinin receptors in inflammatory mechanical hyperalgesia. *Pain*, *104*(3), 683–691.

Franco, M. L., Nadezhdin, K. D., Goncharuk, S. A., Mineev, K. S., Arseniev, A. S., & Vilar, M. (2020). Structural basis of the transmembrane domain dimerization and rotation in the activation mechanism of the TRKA receptor by nerve growth factor. *Journal of Biological Chemistry*, *295*(1), 275–286.

Franco, M. L., Nadezhdin, K. D., Light, T. P., Goncharuk, S. A., Soler-Lopez, A., Ahmed, F., Mineev, K. S., Hristova, K., Arseniev, A. S., & Vilar, M. (2021a). Interaction between the transmembrane domains of neurotrophin receptors p75 and TrkA mediates their reciprocal activation. *Journal of Biological Chemistry*, *297*(2), 100926.

Franco, M. L., Nadezhdin, K. D., Light, T. P., Goncharuk, S. A., Soler-Lopez, A., Ahmed, F., Mineev, K. S., Hristova, K., Arseniev, A. S., & Vilar, M. (2021b). Interaction between the transmembrane domains of neurotrophin receptors p75 and TrkA mediates their reciprocal activation. *Journal of Biological Chemistry*, *297*(2), 100926.

George, D. S., Hackelberg, S., Jayaraj, N. D., Ren, D., Edassery, S. L., & Rathwell, C. A. (2022). Mitochondrial calcium uniporter deletion prevents painful diabetic neuropathy by restoring mitochondrial morphology and dynamics. Pain, 163, 560–578.

Ginty, D. D., & Segal, R. A. (2002). Retrograde neurotrophin signaling: Trk-ing along the axon. *Current Opinion in Neurobiology*, *12*(3), 268–274.

Goodwin, G., & McMahon, S. B. (2021). The physiological function of different voltage-gated sodium channels in pain. *Nature Reviews Neuroscience*, *22*(5), 263–274.

Greco, A., Villa, R., Fusetti, L., Orlandi, R., & Pierotti, M. A. (2000). The Gly571arg mutation, associated with the autonomic and sensory disorder congenital insensitivity to pain with anhidrosis, causes the inactivation of the NTRK1/nerve growth factor receptor. *Journal of Cellular Physiology*, *182*(1), 127–133.

Grills, B. L., & Schuijers, J. A. (1998). *Grills and Schuijers, 1998*. *69*(4), 5–9.

Gross, C. T., & Canteras, N. S. (2012). The many paths to fear. *Nature Reviews Neuroscience*, *13*(9), 651–658

Guy, J., Gan, J., Selfridge, J., Cobb, S., Bird, A. (2007). Reversal of neurological defects in a mouse model of Rett syndrome. *Science*. 315(5815), 1143-7.

Handwerker, H. O. (2014). Chapter 1 - Itch Hypotheses. In *Itch Mechanisms and Treatment* (pp. 1–6).

Harding, E. K., Fung, S. W., & Bonin, R. P. (2020). Insights Into Spinal Dorsal Horn Circuit Function and Dysfunction Using Optical Approaches. *Frontiers in Neural Circuits*, *14*(June), 1–21.

Häring, M., Zeisel, A., Hochgerner, H., Rinwa, P., Jakobsson, J. E. T., Lönnerberg, P., La Manno, G., Sharma, N., Borgius, L., Kiehn, O., Lagerström, M. C., Linnarsson, S., & Ernfors, P. (2018). Neuronal atlas of the dorsal horn defines its architecture and links sensory input to transcriptional cell types. *Nature Neuroscience*, *21*(6), 869–880.

Harrington, A. W., & Ginty, D. D. (2013). Long-distance retrograde neurotrophic factor signalling in neurons. In *Nature Reviews Neuroscience* (Vol. 14, Issue 3, pp. 177–187).

Hefti, F. F., Rosenthal, A., Walicke, P. A., Wyatt, S., Vergara, G., Shelton, D. L., & Davies, A. M. (2006). *Novel class of pain drugs based on antagonism of NGF*. *27*(2).

Hefti, F., Knusel, B., & Lapchak, P. A. (1993). Protective effects of nerve growth factor and brain-derived neurotrophic factor on basal forebrain cholinergic neurons in adult rats with partial fimbrial transections. *Progress in Brain Research*, *98*(C), 257–263.

Hempstead, B. L. (2002). The many faces of p75NTR. *Current Opinion in Neurobiology*, *12*(3), 260–267.

Hempstead, B. L., Martin-Zanca, D., Kaplan, D. R., Parada, L. F., & Chao, M. V. (1991). High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. *Nature*, *350*(6320), 678–683.

Hendry, A. I., Stöckel, K., Paravicini, U., & Thoenen, H. (1974). Specificity of the retrograde axonal transport of nerve growth factor. *Brain Research*, *76*(3), 413–421.

Hicke L, Dunn R. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol*., 2003; 141-72.

Hillier, S., Immink, M., & Thewlis, D. (2015). Assessing Proprioception: A Systematic Review of Possibilities. *Neurorehabilitation and Neural Repair*, *29*(10), 933–949.

Himmel, N. J., & Cox, D. N. (2020). "Transient receptor potential channels: current perspectives on evolution. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1933), 1–9.

Hjerling-Leffler, J., AlQatari, M., Ernfors, P., & Koltzenburg, M. (2007). Emergence of functional sensory subtypes as defined by transient receptor potential channel expression. *Journal of Neuroscience*, *27*(10), 2435–2443.

Hodgkin, A. L., & Huxley, A. F. (1952). Propagation of electrical signals along giant nerve fibers. *Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character. Royal Society (Great Britain)*, *140*(899), 177–183.

Howe, C. L., & Mobley, W. C. (2004). Signaling Endosome Hypothesis: A Cellular Mechanism for Long Distance Communication. *Journal of Neurobiology*, *58*(2), 207–216.

Howe, C. L., Valletta, J. S., Rusnak, A. S., & Mobley, W. C. (2001). NGF signaling from clathrin-coated vesicles: Evidence that signaling endosomes serve as a platform for the Ras-MAPK pathway. *Neuron*, *32*(5), 801–814.

Huang, E. J., & Reichardt, L. F. (2001). Neurotrophins: Roles in neuronal development and function. *Annual Review of Neuroscience*, *24*, 677–736.

Iannetti, G. D., & Mouraux, A. (2010). From the neuromatrix to the pain matrix (and back). *Experimental Brain Research*, *205*(1), 1–12.

Iggo, A., & Andres, K. H. (1982). Morphology of cutaneous receptors. *Annual Review of Neuroscience*, *5*, 1–31.

Ikoma, A., Steinhoff, M., Ständer, S., Yosipovitch, G., & Schmelz, M. (2006). The neurobiology of itch. *Nature Reviews Neuroscience*, *7*(7), 535–547.

Indo, Y. (2001). Molecular Basis of Congenital Insensitivity to Pain With Anhidrosis (CIPA): Mutations and Polymorphisms in TRKA (NTRK1) Gene Encoding the Receptor Tyrosine Kinase for Nerve Growth Factor. In *INDO HUMAN MUTATION* (Vol. 18).

Indo, Y. (2018). NGF-dependent neurons and neurobiology of emotions and feelings: Lessons from congenital insensitivity to pain with anhidrosis. In *Neuroscience and Biobehavioral Reviews* (Vol. 87, pp. 1–16). Elsevier Ltd.

Indo, Y., Tsuruta, M., Hayashida, Y. et al. (1996). Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. *Nat Genet*, *13*, *485–48*.

Itoh, Y; Yagishita, S. Nakajima, T. N. (1986). Congenitallnsensitivity to Pain with Anhidrosis: Morphological and Morphometrical Studies on the Skin and Peripheral Nerves. *Neuropediatrics*, *1980*.

Jayaraj, N. D., Miller, R. J., Menichella, D. M., Bhattacharyya, B. J., Belmadani, A. A., Ren, D., Rathwell, C. A., Hackelberg, S., Hopkins, B. E., Gupta, H. R., Miller, R. J., & Menichella, D. M. (2018). Reducing CXCR4-mediated nociceptor hyperexcitability reverses painful diabetic neuropathy. The Journal of Clinical Investigation, *128*(6), 2205–2225.

Johnson, E. M., Taniuchi, M., & DiStefano, P. S. (1988). Expression and possible function of nerve growth factor receptors on Schwann cells. *Trends in Neurosciences*, *11*(7), 299–304.

Johnston, M. V., Rutkowski, J. L., Wainer, B. H., Long, J. B., & Mobley, W. C. (1987). NGF effects on developing forebrain cholinergic neurons are regionally specific. *Neurochemical Research*, *12*(11), 985–994.

Jones, M. G., Munson, J. B., & Thompson, S. W. N. (1999). A role for nerve growth factor in sympathetic sprouting in rat dorsal root ganglia. *Pain*, *79*(1), 21–29.

Julius, D. (2013). TRP channels and pain. In *Annual Review of Cell and Developmental Biology* (Vol. 29).

Kaplan, D. R., Martin-Zanca, D., & Parada, L. F. (1991). Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. *Nature*, *350*(6314), 158–160.

Kaplan DR and Miller FD. (2000). NGF-TrkA signaling-Kaplan2000. *Current Opinion in Neurobiology*, *10:381–391*.

Kirouac, G. J. (2021). The Paraventricular Nucleus of the Thalamus as an Integrating and Relay Node in the Brain Anxiety Network. *Frontiers in Behavioral Neuroscience*, *15*(February), 1–14.

Klifto, K. M., & Dellon, A. L. (2021). Silas Weir Mitchell, MD, LLD, FRC: Neurological Evaluation and Rehabilitation of the Injured Upper Extremity. *Hand*, *16*(1), 128–133.

Kobayashi, K., Fukuoka, T., Obata, K., Yamanaka, H., Dai, Y., Tokunaga, A., & Noguchi, K. (2005). Distinct expression of TRPM8, TRPA1, and TRPV1 mRNAs in rat primary afferent neurons with Aδ/C-fibers and colocalization with Trk receptors. *Journal of Comparative Neurology*, *493*(4), 596–606.

Koltzenburg, M. (1999). Neutralization of endogenous NGF prevents the sensitization of nociceptors supplying inflamed skin. *European Journal of Neuroscience*, *11*(5), 1698–1704.

Kohoutová, L., Atlas, L. Y., Büchel, C., Buhle, J. T., Geuter, S., Jepma, M., Koban, L., Krishnan, A., Lee, D. H., Lee, S., Roy, M., Schafer, S. M., Schmidt, L., Wager, T. D., & Woo, C. W. (2022). Individual variability in brain representations of pain. *Nature Neuroscience*, *25*(6), 749–759.

Kramer, I., Sigrist, M., De Nooij, J. C., Taniuchi, I., Jessell, T. M., & Arber, S. (2006). A role for Runx transcription factor signaling in dorsal root ganglion sensory neuron diversification. *Neuron*, *49*(3), 379–393.

Kuruvilla, R., Zweifel, L. S., Glebova, N. O., Lonze, B. E., Valdez, G., Ye, H., & Ginty, D. D. (2004). A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. *Cell*, *118*(2), 243–255

LaBranche, T. P., Bendele, A. M., Omura, B. C., Gropp, K. E., Hurst, S. I., Bagi, C. M., Cummings, T. R., Grantham, L. E., Shelton, D. L., & Zorbas, M. A. (2017). Nerve growth factor inhibition with tanezumab influences weight-bearing and subsequent cartilage damage in the rat medial meniscal tear model. *Annals of the Rheumatic Diseases*, *76*(1), 295–302

Laird, J. M. A., & Cervero, F. A. (1996). Review article Mechanisms of touch-evoked pain (allodynia): a new model. *Pain*, *68*, 13–23.

Lallemend, F., & Ernfors, P. (2012). Molecular interactions underlying the specification of sensory neurons. *Trends in Neurosciences*, *35*(6), 373–381

Lane, N. E., & Corr, M. (2017). Osteoarthritis in 2016: Anti-NGF treatments for pain — two steps forward, one step back? *Nature Publishing Group*

Lane, N. E., Schnitzer, T. J., Birbara, C. A., Mokhtarani, M., Shelton, D. L., Smith, M. D., & Brown, M. T. (2010). Tanezumab for the Treatment of Pain from Osteoarthritis of the Knee. *New England Journal of Medicine*, *363*(16), 1521–1531.

Langer, J., Goebel, H. H., & Veit, S. (1981). Eccrine sweat glands are not innervated in hereditary sensory neuropathy type IV - An electron-microscopic study. *Acta Neuropathologica*, *54*(3), 199–202.

Langford, D. J., Crager, S. E., Shehzad, Z., Smith, S. B., Sotocinal, S. G., Levenstadt, J. S., Chanda, M. L., Levitin, D. J., & Mogil, J. S. (2006). Social modulation of pain as evidence for empathy in mice. *Science*, *312*(5782), 1967–1970.

Larrea, L. G., & Peyron, R. (2013). *Pain matrices and neuropathic pain matrices : A review . To cite this version : Pain matrices and neuropathic pain matrices : a review*. *154*(Suppl 1).

Latina, V., Caioli, S., Zona, C., Ciotti, M. T., Amadoro, G., & Calissano, P. (2017). Impaired NGF/TrKA signaling causes early AD-linked presynaptic dysfunction in cholinergic primary neurons. *Frontiers in Cellular Neuroscience*, *11*.

Lee, F. S., Kim, A. H., Khursigara, G., & Chao, M. V. (2001). The uniqueness of being a neurotrophin receptor. *Current Opinion in Neurobiology 11,281-286*.

Lee, K. F., Davies, A. M., & Jaenisch, R. (1994). p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. *Development*, *120*(4), 1027–1033.

Legat, F. J. (2021). Itch in Atopic Dermatitis – What Is New? *Frontiers in Medicine*, *8*(May), 1–11.

Leipold, E., Liebmann, L., Korenke, G. C., Heinrich, T., Gießelmann, S., Baets, J., Ebbinghaus, M., Goral, R. O., Stödberg, T., Hennings, J. C., Bergmann, M., Altmüller, J., Thiele, H., Wetzel, A., Nürnberg, P., Timmerman, V., De Jonghe, P., Blum, R., Schaible, H. G., … Kurth, I. (2013). A de novo gain-of-function mutation in SCN11A causes loss of pain perception. *Nature Genetics* 45, 11, 1399–1407

Levanon, D., Bettoun, D., Harris-Cerruti, C., Woolf, E., Negreanu, V., Eilam, R., Bernstein, Y., Goldenberg, D., Xiao, C., Fliegauf, M., Kremer, E., Otto, F., Brenner, O., Lev-Tov, A., & Groner, Y. (2002). The Runx3 transcription factor regulates development and survival of TrkC dorsal root ganglia neurons. *EMBO Journal*, *21*(13), 3454–3463.

Levi-Montalcini, R. (1987). *Years Later*. *237*,5.

Levi-Montalcini, R., & Angeletti, P. U. (1968). Nerve growth factor. *Physiological Reviews*, *48*(8), 1–4.

Levy Erez, D., Levy, J., Friger, M., Aharoni-Mayer, Y., Cohen-Iluz, M., & Goldstein, E. (2010). Assessment of cognitive and adaptive behaviour among individuals with congenital insensitivity to pain and anhidrosis. *Developmental Medicine and Child Neurology*, *52*(6), 559–562.

Lewin, G. R., Ritter, A. M., & Mendell, L. M. (1993). Nerve Growth Factor-induced Hyperalgesia in the Neonatal and Adult Rat. In *The Journal of Neuroscience* 13,5

Li, N., Guo, S., Wang, Q., Duan, G., Sun, J., Liu, Y., Zhang, J., Wang, C., Zhu, C., Liu, J., & Zhang, X. (2019). Heterogeneity of clinical features and mutation analysis of NTRK1 in Han Chinese patients with congenital insensitivity to pain with anhidrosis. *Journal of Pain Research*, *12*, 453–465.

Lingueglia, E., De Weille, J. R., Bassilana, F., Heurteaux, C., Sakaif, H., Waldmann, R., & Lazdunskig, M. (1997). A modulatory subunit of acid sensing ion channels in brain and dorsal root ganglion cells. *Journal of Biological Chemistry*, *272*(47), 29778–29783

Liu, Z., Liu, J., Liu, G., Cao, W., Liu, S., Chen, Y., Zuo, Y., Chen, W., Chen, J., Zhang, Y., Huang, S., Qiu, G., Giampietro, P. F., Zhang, F., Wu, Z., & Wu, N. (2018). Phenotypic heterogeneity of intellectual disability in patients with congenital insensitivity to pain with anhidrosis: A case report and literature review. *Journal of International Medical Research*, *46*(6), 2445–2457.

Liu, Q., Tang, Z., Surdenikova, L., Kim, S., Patel, K. N., Kim, A., Ru, F., Guan, Y., Weng, H. J., Geng, Y., Undem, B. J., Kollarik, M., Chen, Z. F., Anderson, D. J., & Dong, X. (2009). Sensory Neuron-Specific GPCR Mrgprs Are Itch Receptors Mediating Chloroquine-Induced Pruritus. *Cell*, *139*(7), 1353–1365.

Liu, Y., & Ma, Q. (2011). Generation of somatic sensory neuron diversity and implications on sensory coding. *Current Opinion in Neurobiology*, *21*(1), 52–60.

Loeser, J. D., & Treede, R. D. (2008). The Kyoto protocol of IASP Basic Pain Terminology. *Pain*, *137*(3), 473–477.

Loewenthal, N., Levy, J., Schreiber, R., Pinsk, V., Perry, Z., Shorer, Z., & Hershkovitz, E. (2005). Nerve growth factor-tyrosine kinase A pathway is involved in thermoregulation and adaptation to stress: Studies on patients with hereditary sensory and autonomic neuropathy type IV. *Pediatric Research*, *57*(4), 587–590.

Lolignier, S., Eijkelkamp, N., & Wood, J. N. (2015). Mechanical allodynia. *Pflugers Archiv European Journal of Physiology*, *467*(1), 133–139

Louis, E. D., & York, G. K. (2006). Weir Mitchell's observations on sensory localization and their influence on Jacksonian neurology. *Neurology*, *66*(8), 1241–1244.

Luo, W., Wickramasinghe, S. R., Savitt, J. M., Griffin, J. W., Dawson, T. M., & Ginty, D. D. (2007). A Hierarchical NGF Signaling Cascade Controls Ret-Dependent and Ret-Independent Events during Development of Nonpeptidergic DRG Neurons. *Neuron*, *54*(5), 739–754.

Ma, Q. (2010). Labeled lines meet and talk: Population coding of somatic sensations. *Journal of Clinical Investigation*, *120*(11), 3773–3778

Machado, N. L. S., Bandaru, S. S., Abbott, S. B. G., & Saper, C. B. (2020). Ep3R-expressing glutamatergic preoptic neurons mediate inflammatory fever. *Journal of Neuroscience*, *40*(12), 2573–2588.

MacInnis, B. L., & Campenot, R. B. (2002). Retrograde support of neuronal survival without retrograde transport of nerve growth factor. *Science*, *295*(5559), 1536–1539

Makkerh, J. P. S., Ceni, C., Auld, D. S., Vaillancourt, F., Dorval, G., & Barker, P. A. (2005). p75 neurotrophin receptor reduces ligand-induced Trk receptor ubiquitination and delays Trk receptor internalization and degradation. *EMBO Reports*, *6*(10), 936–941

Malmberg, A. B., Brandon, E. P., Idzerda, R. L., Liu, H., McKnight, G. S., & Basbaum, A. I. (1997). Diminished inflammation and nociceptive pain with preservation of neuropathic pain in mice with a targeted mutation of the type I regulatory subunit of cAMP-dependent protein kinase. *Journal of Neuroscience*, *17*(19), 7462–7470.

Mamet, J., Baron, A., Lazdunski, M., & Voilley, N. (2002). Proinflammatory mediators, stimulators of sensory neuron excitability via the expression of acid-sensing ion channels. *Journal of Neuroscience*, *22*(24), 10662–10670.

Mantyh, P. W., Koltzenburg, M., Mendell, L. M., Tive, L., & Shelton, D. L. (2011). Antagonism of Nerve Growth Factor-TrkA Signaling and the Relief of Pain.

Mardy, S., Miura, Y., Endo, F., Matsuda, I., Sztriha, L., Frossard, P., Moosa, A., Ismail, E. A. R., Macaya, A., Andria, G., Toscano, E., Gibson, W., Graham, G. E., & Indo, Y. (1999). Congenital Insensitivity to Pain with Anhidrosis: Novel Mutations in the TRKA (NTRK1) Gene Encoding A High-Affinity Receptor for Nerve Growth Factor. In *Am. J. Hum. Genet* (Vol. 64).

Markus, A., Patel, T. D., & Snider, W. D. (2002). Neurotrophic factors and axonal growth. *Curr Opin Neurobiol, 3*, 523–531.

Marmigère, F., & Ernfors, P. (2007). Specification and connectivity of neuronal subtypes in the sensory lineage. *Nature Reviews Neuroscience*, *8*(2), 114–127

Marrone, M. C., Morabito, A., Giustizieri, M., Chiurchiù, V., Leuti, A., Mattioli, M., Marinelli, S., Riganti, L., Lombardi, M., Murana, E., Totaro, A., Piomelli, D., Ragozzino, D., Oddi, S., Maccarrone, M., Verderio, C., & Marinelli, S. (2017). TRPV1 channels are critical brain inflammation detectors and neuropathic pain biomarkers in mice. *Nature Communications*, *8*.

Martínez-Navarro, M., Maldonado, R., & Baños, J. E. (2019). Why mu-opioid agonists have less analgesic efficacy in neuropathic pain? *European Journal of Pain (United Kingdom)*, *23*(3), 435–454.

McMahon, S. B. (1996). NGF as a mediator of inflammatory pain. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *351*(1338), 431–440.

McMahon, S. B., & Koltzenburg, M. (1992). Itching for an explanation. *Trends in Neurosciences*, *15*(12), 497–501.

McNeil, B. D., Pundir, P., Meeker, S., Han, L., Undem, B. J., Kulka, M., & Dong, X. (2015). Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions. *Nature*, *519*(7542), 237–241.

Melzack, R., & Wall, P. D. (1962). On the nature of cutaneous sensory mechanisms. *Brain*, *85*(2), 331–356.

Melzack, Ronald. (1990). L 88 ~) 1990,. *Science*, *13*(3), 88–92.

Melzack, Ronald, & Casey, K. (1968). Sensory, motivational, and central control determinants of pain. *The Skin Senses*, *1*(January 1968), 423–443.

Melzack, Ronald, & Wall, P. D. (1965). GateControl-Pain mechanisms - a new theory. *Science* 150, 971–979.

Minde, J., Svensson, O., Holmberg, M., Solders, G., & Toolanen, G. (2006). Orthopedic aspects of familial insensitivity to pain due to a novel nerve growth factor beta mutation. *Acta Orthopaedica*, *77*(2), 198–202.

Minde, J., Toolanen, G., Andersson, T., Nennesmo, I., Remahl, I. N., Svensson, O., & Solders, G. (2004). Familial insensitivity to pain (HSAN V) and a mutation in the NGFB gene. A neurophysiological and pathological study. *Muscle and Nerve*, *30*(6), 752–760.

Miranda, C., Zanotti, G., Pagliardini, S., Ponzetto, C., Pierotti, M. A., & Greco, A. (2002). Gain of function mutations of RTK conserved residues display differential effects on NTRK1 kinase activity. *Oncogene*, *21*, 8334–8339.

Mishra, S. K., & Hoon, M. A. (2010). Ablation of TrpV1 neurons reveals their selective role in thermal pain sensation. *Molecular and Cellular Neuroscience*, *43*(1), 157–163.

Mitchell, S. W., Morehouse, G. R., & Keen, W. W. (2007). Gunshot wounds and other injuries of nerves. 1864. *Clinical Orthopaedics and Related Research*, *458*, 35–39.

Moayedi, M., & Davis, K. D. (2013). Theories of pain: From specificity to gate control. *Journal of Neurophysiology*, *109*(1), 5–12.

Mobley, W. C., Rutkowski, J. L., Tennekoon, G. I., Gemski, J., Buchanan, K., & Johnston, M. V. (1986). Nerve growth factor increases choline acetyltransferase activity in developing basal forebrain neurons. *Molecular brain research*, 53–62.

Moffitt, J. R., Bambah-Mukku, D., Eichhorn, S. W., Vaughn, E., Shekhar, K., Perez, J. D., Rubinstein, N. D., Hao, J., Regev, A., Dulac, C., & Zhuang, X. (2018). Molecular, spatial, and functional single-cell profiling of the hypothalamic preoptic region. *Science*, *362*(6416).

Molliver, D. C., Wright, D. E., Leitner, M. L., Parsadanian, A. S., Doster, K., Wen, D., Yan, Q., & Snider, W. D. (1997). IB4-Binding DRG Neurons Switch from NGF to GDNF Dependence in Early Postnatal Life ferent members of the neurotrophin family have actions that are specific for subsets of neurons that mediate. *Silos-Santiago* 19.

Monteiro C., Cardoso-Cruz H., Galhardo V., (2019). Animal models of congenital hypoalgesia: Untapped potential for assessing pain-related plasticity. *Neuroscience Letters*, 702, 51-60.

Montelius, A., Marmigère, F., Baudet, C., Aquino, J. B., Enerbäck, S., & Ernfors, P. (2007). Emergence of the sensory nervous system as defined by Foxs1 expression. *Differentiation*, *75*(5), 404–417.

Morrison, S. F. (2016). Central neural control of thermoregulation and brown adipose tissue. *Autonomic Neuroscience: Basic and Clinical* 196, 14–24.

Morrison, S. F., & Nakamura, K. (2011). *Central neural pathways for thermoregulation*. Front Biosci. 16, 74–104.

Moshourab, R. A., Wetzel, C., Martinez-Salgado, C., & Lewin, G. R. (2013). Stomatin-domain protein interactions with acid-sensing ion channels modulate nociceptor mechanosensitivity. *Journal of Physiology*, *591*(22), 5555–5574

Murthy, S. E., Loud, M. C., Daou, I., Marshall, K. L., Schwaller, F., Kühnemund, J., Francisco, A. G., Keenan, W. T., Dubin, A. E., Lewin, G. R., & Patapoutian, A. (2018). The mechanosensitive ion channel Piezo2 mediates sensitivity to mechanical pain in mice. *Science Translational Medicine*, *10*(462).

Miyamoto, K., Kume, K., & Ohsawa, M. (2017). Role of microglia in mechanical allodynia in the anterior cingulate cortex. *Journal of Pharmacological Sciences*, *134*(3), 158–165.

Nafe, J. P. (1929). A quantitative theory of feeling. *Journal of General Psychology*, *2*(2–3), 199–211.

Nassar M.A., Levato A., Stirling L.C., Wood J.N. (2005). Neuropathic Pain Develops Normally in Mice Lacking both Nav1.7 and Nav1.8. *Molecular Pain*. 1.

Nakamura, K., & Morrison, S. F. (2007). Central efferent pathways mediating skin cooling-evoked sympathetic thermogenesis in brown adipose tissue. *Am J Physiol Regul Integr Comp Physiol*, *292(1)*, R127–R136.

Nélaton, A. (1852). Affection singulière des os du pied. Gazette des hôpitaux civils et militaires. 4,13

Nencini, S., Morgan, M., Thai, J., Jobling, A. I., Mazzone, S. B., & Ivanusic, J. J. (2021). Piezo2 Knockdown Inhibits Noxious Mechanical Stimulation and NGF-Induced Sensitization in A-Delta Bone Afferent Neurons. Frontiers in Physiology, 12(July), 1–13.

Nencini, S., Ringuet, M., Kim, D. H., Chen, Y. J., Greenhill, C., & Ivanusic, J. J. (2017). Mechanisms of nerve growth factor signaling in bone nociceptors and in an animal model of inflammatory bone pain. *Molecular Pain*, *13*, 1–19.

Nicholson, G. A., Dawkins, J. L., Blair, I. P., Kennerson, M. L., Gordon, M. J., Cherryson, A. K., & J Nash, T. B. (1996). The gene for hereditary sensory neuropathy type I (HSN-I) maps to chromosome 9q22.1-q22.3. *Nature Genetics*, *13*(1), 101–104.

Nocchi, L., Roy, N., D'Attilia, M., Dhandapani, R., Maffei, M., Traista, A., Castaldi, L., Perlas, E., Chadick, C. H., & Heppenstall, P. A. (2019). Interleukin-31-mediated photoablation of pruritogenic epidermal neurons reduces itch-associated behaviours in mice. *Nature Biomedical Engineering*, *3*(2), 114–125.

Nolano, M., Crisci, C., Santoro, L., Barbieri, F., Casale, R., Kennedy, W. R., Wendelschafer-Crabb, G., Provitera, V., Di Lorenzo, N., & Caruso, G. (2000). Absent innervation of skin and sweat glands in congenital insensitivity to pain with anhidrosis. *Clinical Neurophysiology*, *111*(9), 1596–1601.

Nykjaer, A., Lee, R., Teng, K. K., Jansen, P., Madsen, P., Nielsen, M. S., Jacobsen, C., Kliemannel, M., Schwarz, E., Willnow, T. E., Hempstead, B. L., & Petersen, C. M. (2004). Sortilin is essential for proNGF-induced neuronal cell death. *Nature*, *427*(6977), 843–848.

Obata, K., Katsura, H., Mizushima, T., Yamanaka, H., & Kobayashi, K. (2005). TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury.

Ohtsubo, K., Marth, J.D. (2006) Glycosylation in cellular mechanisms of health and disease. *Cell*. 126(5), 855-67.

Ojeda, S. R., Hill, D. F., & Katz, K. H. (1991). The genes encoding nerve growth factor and its receptor are expressed in the developing female rat hypothalamus. *Molecular Brain Research*, *9*(1–2), 47–55.

Omerbašić, D., Smith, E. S. J., Moroni, M., Homfeld, J., Eigenbrod, O., Bennett, N. C., Reznick, J., Faulkes, C. G., Selbach, M., & Lewin, G. R. (2016). Hypofunctional TrkA Accounts for the Absence of Pain Sensitization in the African Naked Mole-Rat. *Cell Reports*, *17*(3), 748–758.

Ossipov, M. H., Dussor, G. O., & Porreca, F. (2010). Review series Central modulation of pain. *The Journal of Clinical Investigation*, *120*(11), 3779–3787

Paoletti, F., Malerba, F., Ercole, B.B., Lamba, D., Cattaneo, A. (2015) A comparative analysis of the structural, functional and biological differences between Mouse and Human Nerve Growth Factor. *Biochimica et biophysica acta*, 1854:187-197.

Patel, T. D., Jackman, A., Rice, F. L., Kucera, J., & Snider, W. D. (2000). Development of Sensory Neurons in the Absence of NGF/TrkA Signaling In Vivo. *Neuron* 25.

Pavlenko, D., Funahashi, H., Sakai, K., Lozada, T., Yosipovitch, G., & Akiyama, T. (2020). IL-23 modulates histamine-evoked itch and responses of pruriceptors in mice. *Experimental Dermatology*, *29*, 1209–1215.

Peirs, C., Williams, S. P. G., Zhao, X., Walsh, C. E., Gedeon, J. Y., Cagle, N. E., Goldring, A. C., Hioki, H., Liu, Z., Marell, P. S., & Seal, R. P. (2015). Dorsal Horn Circuits for Persistent Mechanical Pain. *Neuron*, *87*(4), 797–812.

Perez-Pinera, P., García-Suarez, O., Germanà, A., Díaz-Esnal, B., de Carlos, F., Silos-Santiago, I., del Valle, M. E., Cobo, J., & Vega, J. A. (2008). Characterization of sensory deficits in TrkB knockout mice. *Neuroscience Letters*, *433*(1), 43–47.

Perini, I., Tavakoli, M., Marshall, A., Minde, J., & Morrison, I. (2016). Rare human nerve growth factor-β mutation reveals relationship between C-afferent density and acute pain evaluation. *Journal of Neurophysiology*, *116*(2), 425–430.

Perl, E. R. (2007). Perl 2007 - Ideas about pain. Nature Neuroscience Review, *8*, 71–80.

Phan, T. X., Ton, H. T., Gulyás, H., Pórszász, R., Tóth, A., Russo, R., Kay, M. W., Sahibzada, N., & Ahern, G. P. (2020). TRPV1 expressed throughout the arterial circulation regulates vasoconstriction and blood pressure. *Journal of Physiology*, *598*(24), 5639–5659.

Pizzorusso, T., Fagiolini, M., Gianfranceschi, L., Porciatti, V., & Maffei, L. (2000). Role of neurotrophins in the development and plasticity of the visual system: Experiments on dark rearing. *International Journal of Psychophysiology*, *35*(2–3), 189–196.

Prescott, S. A., Ma, Q., & De Koninck, Y. (2014). Normal and abnormal coding of somatosensory stimuli causing pain. *Nature Neuroscience*, *17*(2), 183–191

Price, M. P., Lewin, G. R., Mcilwrath, S. L., Cheng, C., Xie, J., Heppenstall, P. A., Stucky, C. L., Mannsfeldt, A. G., Brennan, T. J., Drummond, H. A., Qiao, J., Benson, C. J., Tarr, D. E., Hrstka, R. F., Yang, B., Williamson, R. A., & Welsh, M. J. (2000). *Price Bnc1 2000*. *171*(1996), 1007–1012.

Price, M. P., McIlwrath, S. L., Xie, J., Cheng, C., Qiao, J., Tarr, D. E., Sluka, K. A., Brennan, T. J., Lewin, G. R., & Welsh, M. J. (2001). The DRASIC cation channel contributes to the detection of cutaneous touch and acid stimuli in mice. *Neuron*, *35*(2), 407.

Priest, B. T., Murphy, B. A., Lindia, J. A., Diaz, C., Abbadie, C., Ritter, A. M., Liberator, P., Iyer, L. M., Kash, S. F., Kohler, M. G., Kaczorowski, G. J., MacIntyre, D. E., & Martin, W. J. (2005). Contribution of the tetrodotoxin-resistant voltage-gated sodium channel Nav1.9 to sensory transmission and nociceptive behavior. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(26), 9382–9387.

Priestley, J. V., Michael, G. J., Averill, S., Liu, M., & Willmott, N. (2002). Regulation of nociceptive neurons by nerve growth factor and glial cell line derived neurotrophic factor. *Canadian Journal of Physiology and Pharmacology*, *80*(5), 495–505.

Procacci, P., & Maresca, M. (1994). *Guest Editorial Descartes ' physiology of pain*. *58*, 3959.

Proske, U. (2005). What is the role of muscle receptors in proprioception? *Muscle and Nerve*, *31*(6), 780–787.

Ran, C., Hoon, M. A., & Chen, X. (2016). The coding of cutaneous temperature in the spinal cord. *Nature Neuroscience*, *19*(9), 1201–1209

Ranade, S. S., Woo, S. H., Dubin, A. E., Moshourab, R. A., Wetzel, C., Petrus, M., Mathur, J., Bégay, V., Coste, B., Mainquist, J., Wilson, A. J., Francisco, A. G., Reddy, K., Qiu, Z., Wood, J. N., Lewin, G. R., & Patapoutian, A. (2014). Piezo2 is the major transducer of mechanical forces for touch sensation in mice. *Nature*, *516*(729), 121–125.

Rizzi, C., Tiberi, A., Giustizieri, M., Marrone, M. C., Gobbo, F., Carucci, N. M., Meli, G., Arisi, I., D'Onofrio, M., Marinelli, S., Capsoni, S., & Cattaneo, A. (2018). NGF steers microglia toward a neuroprotective phenotype. *GLIA*, *66*(7), 1395–1416.

Ro, L., Chen, S., Tang, L., & Jacobs, J. M. (1996). Effect of NGF and anti-NGF on neuropathic pain in rats following chronic constriction injury of the sciatic nerve. *Neuroscience Letters*, *218*, 87–90.

Rosemberg, S., Marie, S. K. N., & Kiiemann, S. (1994). *Congenital Insensitivity to Pain With Anhidrosis (Hereditary and Autonomic Neur type IV)*.

Rosenthal, A., Goeddel, D. V., Nguyen, T., Lewis, M., Shih, A., Laramee, G. R., Nikolics, K., & Winslow, J. W. (1990). Primary structure and biological activity of a novel human neurotrophic factor. *Neuron*, *4*(5), 767–773.

Rotthier, A., Auer-Grumbach, M., Janssens, K., Baets, J., Penno, A., Almeida-Souza, L., Van Hoof, K., Jacobs, A., De Vriendt, E., Schlotter-Weigel, B., Löscher, W., Vondráček, P., Seeman, P., De Jonghe, P., Van Dijck, P., Jordanova, A., Hornemann, T., & Timmerman, V. (2010). Mutations in the SPTLC2 subunit of serine palmitoyltransferase cause hereditary sensory and autonomic neuropathy type i. *American Journal of Human Genetics*, *87*(4), 513–522.

Ruit, K. G., Osborne, P. A., Schmidt, R. E., Johnson, E. M., & Snider, W. D. (1990). Nerve growth factor regulates sympathetic ganglion cell morphology and survival in the adult mouse. *Journal of Neuroscience*, *10*(7), 2412–2419.

Salomons, T. V., Iannetti, G. D., Liang, M., & Wood, J. N. (2016). The "pain matrix" in pain-free individuals. *JAMA Neurology*, *73*(6), 755–756.

Salter, M. W., & Stevens, B. (2017). Microglia emerge as central players in brain disease. *Nature Medicine*, *23*(9), 1018–1027.

Schuelert, N., Just, S., Corradini, L., Kuelzer, R., Bernloehr, C., & Doods, H. (2015). The bradykinin B1 receptor antagonist BI113823 reverses inflammatory hyperalgesia by desensitization of peripheral and spinal neurons. *European Journal of Pain (United Kingdom)*, *19*(1), 132–142.

Schwartzlow, C., & Kazamel, M. (2019). Hereditary Sensory and Autonomic Neuropathies: Adding More to the Classification. In *Current Neurology and Neuroscience Reports* 19, 8.

Scott-Solomon, E., & Kuruvilla, R. (2018). Mechanisms of neurotrophin trafficking via Trk receptors. *Molecular and Cellular Neuroscience*, *91*(1), 25–33.

Seal, R. P., Wang, X., Guan, Y., Raja, S. N., Woodbury, C. J., Basbaum, A. I., & Edwards, R. H. (2009). Injury-induced mechanical hypersensitivity requires C-low threshold mechanoreceptors. *Nature*, *462*(7273), 651–655.

Shaikh, S. S., Chen, Y. C., Halsall, S. A., Nahorski, M. S., Omoto, K., Young, G. T., Phelan, A., & Woods, C. G. (2017). A Comprehensive Functional Analysis of NTRK1 Missense Mutations Causing Hereditary Sensory and Autonomic Neuropathy Type IV (HSAN IV). *Human Mutation*, *38*(1), 55–63.

Shao, Y., Akmentin, W., Toledo-Aral, J. J., Rosenbaum, J., Valdez, G., Cabot, J. B., Hilbush, B. S., & Halegoua, S. (2002). Pincher, a pinocytic chaperone for nerve growth factor/TrkA signaling endosomes. *Journal of Cell Biology*, *157*(4), 679–691.

Shelton, D. L., Zeller, J., Ho, W. H., Pons, J., & Rosenthal, A. (2005). Nerve growth factor mediates hyperalgesia and cachexia in auto-immune arthritis. *Pain*, *116*(1–2), 8–16.

Sherrington CS. (1906). The Integrative Action of the Nervous System. In *Scribner, New York* (Vol. 27, Issue 701, pp. 885–889).

Sinclair, D. C. (1955). Cutaneous sensation and the doctrine of specific energy. *Brain*, *78*(4), 584–614.

Slaugenhaupt, S. A., Blumenfeld, A., Gill, S. P., Leyne, M., Mull, J., Cuajungco, M. P., Liebert, C. B., Chadwick, B., Idelson, M., Reznik, L., Robbins, C. M., Makalowska, I., Brownstein, M. J., Slaugenhaupt, S. A., Scheidereit, C., Maayan, C., Axelrod, F. B., & Gusella, J. F. (2001). Tissue-specific expression of a splicing mutation in the IKBKAP gene causes familial dysautonomia. *American Journal of Human Genetics*, *68*(3), 598–605.

Smeyne, R. J., Klein, R., Schnappt, A., Long, L. K., Bryant, S., Lewin, A., Lira, S. A., & Barbacid, M. (1994). Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. *368*(5), 17–20.

Smith, M. L., Asada, N., & Malenka, R. C. (2021). Anterior cingulate inputs to nucleus accumbens control the social transfer of pain and analgesia. *Science*, *371*(6525), 153–159.

Smith, E. S. J., Park, T. J., & Lewin, G. R. (2020). Independent evolution of pain insensitivity in African mole-rats: origins and mechanisms. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* 206, 313–325.

Smith, E. S. J., & Lewin, G. R. (2009). Nociceptors: a phylogenetic view. *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology*, *195*(12), 1089–1106.

Sneddon, L. U. (2020). *Comparative Physiology of Nociception and Pain*. Review Physiology, 63–73.

Snider, W. D., & Wright, D. E. (1996). Neurotrophins cause a new sensation. *Neuron*, *16*(2), 229–232.

Soltoff, S. P., Rabin, S. L., Cantley, L. C., & Kaplan, D. R. (1992). Nerve growth factor promotes the activation of phosphatidylinositol 3- kinase and its association with the trk tyrosine kinase. *Journal of Biological Chemistry*, *267*(24), 17472–17477.

Song, K., Wang, H., Kamm, G. B., Pohle, J., de Castro Reis, F., Heppenstall, P., Wende, H., & Siemens, J. (2016). The TRPM2 channel is a hypothalamic heat sensor that limits fever and can drive hypothermia. *Science*, *353*(6306), 1387–1393.

Story, G. M., Peier, A. M., Reeve, A. J., Eid, S. R., Mosbacher, J., Hricik, T. R., Earley, T. J., Hergarden, A. C., Andersson, D. A., Hwang, S. W., McIntyre, P., Jegla, T., Bevan, S., & Patapoutian, A. (2003). ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell*, *112*(6), 819–829.

Sun, Y. G., & Chen, Z. F. (2007). A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord. *Nature*, *448*(7154), 700–703.

Sung, K., Ferrari, L. F., Yang, W., Chung, C., Zhao, X., Gu, Y., Lin, S., Zhang, K., Cui, B., Pearn, M. L., Maloney, M. T., Mobley, W. C., Levine, J. D., & Wu, C. (2018). Swedish nerve growth factor mutation (NGFR100W) defines a role for TrkA and p75NTR in nociception. *Journal of Neuroscience*, *38*(14), 3394–3413.

Swanson, A. G. (1963). Congenital insensitivity to pain with anhidrosis. *Annals of Neurology*, *78*(3), 500.

Swanson, A. G., Buchan, G. C., & Alvord, E. C. (1965). Anatomic Changes in Congenital Insensitivity to Pain. *American Neurological Association*, *12*.

Szöke, G., Rényi-Vámos, A., & Bider, M. A. (1996). Osteoarticular manifestations of congenital insensitivity to pain with anhidrosis. *International Orthopaedics*, *20*(2), 107–110.

Takano, N., Sakurai, T., & Kurachi, M. (2005). Effects of anti-nerve growth factor antibody on symptoms in the NC/Nga mouse, an atopic dermatitis model. *Journal of Pharmacological Sciences*, *99*(3), 277–286.

Takazawa, T., & MacDermott, A. B. (2010). *Synaptic pathways and inhibitory gates in the spinal cord dorsal horn*.

Tan, C. L., & Knight, Z. A. (2018). Regulation of Body Temperature by the Nervous System. *Neuron*, *98*(1), 31–48.

Tanaka, A., & Matsuda, H. (2005). Expression of nerve growth factor in itchy skins of atopic NC/NgaTnd mice. *Journal of Veterinary Medical Science*, *67*(9), 915–919.

Tanaka, Y., Niwa, S., Dong, M., Farkhondeh, A., Wang, L., Zhou, R., & Hirokawa, N. (2016). The Molecular Motor KIF1A Transports the TrkA Neurotrophin Receptor and Is Essential for Sensory Neuron Survival and Function. *Neuron*, *90*(6), 1215–1229.

Tansley, S., Gu, N., Guzmán, A. U., Cai, W., Wong, C., Lister, K., Muñoz-Pino, E., Yousefpour, N., Roome, R. B., Heal, J., Wu, N., Castonguay, A., Lean, G., Muir, E. M., Kania, A., Prager-Khoutorsky, M., Zhang, J., Gkogkas, C. G., Fawcett, J. W., … Khoutorsky, A. (2022). Microglia-mediated degradation of perineuronal nets promotes pain. *Science*

Testa, G., Calvello, M., Cattaneo, A., & Capsoni, S. (2019a). Cholinergic striatal neurons are increased in HSAN V homozygous mice despite reduced NGF bioavailability. Biochemical and Biophysical Research Communications, 509:763–766.

Testa, G., Mainardi, M., Morelli, C., Olimpico, F., Pancrazi, L., Petrella, C., Severini, C., Florio, R., Malerba, F., Stefanov, A., Strettoi, E., Brandi, R., Arisi, I., Heppenstall, P., Costa, M., Capsoni, S., & Cattaneo, A. (2019b). The NGFR100W mutation specifically impairs nociception without affecting cognitive performance in a mouse model of hereditary sensory and autonomic neuropathy type V. *Journal of Neuroscience*, 39:9702–9715.

Testa, G., Cattaneo, A., & Capsoni, S. (2021). Understanding pain perception through genetic painlessness diseases: the role of NGF and proNGF. *Pharmacological Research*, 169, 105662.

Theriault, F. M., Roy, P., & Stifani, S. (2004). AML1/Runx1 is important for the development of hindbrain cholinergic branchiovisceral motor neurons and selected cranial sensory neurons. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(28),

Todd, A. J. (2017). Identifying functional populations among the interneurons in laminae I-III of the spinal dorsal horn. *Molecular Pain* 13.

Tominaga, M., & Tominaga, T. (2005). Structure and function of TRPV1. *Pflugers Archiv European Journal of Physiology*, *451*(1), 143–150.

Tomlinson, R. E., Li, Z., Li, Z., Minichiello, L., Riddle, R. C., Venkatesan, A., & Clemens, T. L. (2017). NGF-TrkA signaling in sensory nerves is required for skeletal adaptation to mechanical loads in mice. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(18), E3632–E3641.

Toscano, E., Casa, R. della, Mardy, S., Gaetaniello, L., Sadile, F., Indo, Y., Pignata, C., & Andria, G. (2000). Multisystem Involvement in Congenital Insensitivity to Pain with Anhidrosis (CIPA), a Nerve Growth Factor Receptor(Trk A)-Related Disorder. *Neuropediatrics*, *31*(1), 39–41.

Toyoda, M., Nakamura, M., Makino, T., Hino, T., Kagoura, M., & Morohashi, M. (2002). Nerve growth factor and substance P are useful plasma markers of disease activity in atopic dermatitis. *British Journal of Dermatology*, *147*(1), 71–79

Treede, R. D., & Baron, R. (2008). How to detect a sensory abnormality. *European Journal of Pain*, *12*(4), 395–396.

Treede, R. D., Meyer, R. A., Raja, S. N., & Campbell, J. N. (1992). Peripheral and central mechanisms of cutaneous hyperalgesia. *Progress in Neurobiology*, *38*(4), 397–421.

Ugolini, G., Marinelli, S., Covaceuszach, S., Cattaneo, A., & Pavone, F. (2007). The function neutralizing anti-TrkA antibody MNAC13 reduces inflammatory and neuropathic pain. *PNAS*

Ullrich, A., & Schlessingert, J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Sexual Plant Reproduction*, *61*(1), 203–212.

Ultsch, M. H., Wiesmann, C., Simmons, L. C., Henrich, J., Yang, M., Reilly, D., Bass, S. H., & De Vos, A. M. (1999). Crystal structures of the neurotrophin-binding domain of TrkA, TrkB and TrkC. *Journal of Molecular Biology*, *290*(1), 149–159.

Usoskin, D., Furlan, A., Islam, S., Abdo H., Lonnerberg P., Lou D., Hjerling-Leffler J., Haeggstrom J., Kharchenko O., Kharchenko P.V., Linnarsson, S., Ernfors P. (2015). *Nat Neurosci*., 18, 145–153.

Vaegter, C. B., Jansen, P., Fjorback, A. W., Glerup, S., Skeldal, S., Kjolby, M., Richner, M., Erdmann, B., Nyengaard, J. R., Tessarollo, L., Lewin, G. R., Willnow, T. E., Chao, M. V., & Nykjaer, A. (2011). Sortilin associates with Trk receptors to enhance anterograde transport and neurotrophin signaling. *Nature Neuroscience*, *14*(1), 54–63.

Verhoeven, K., De Jonghe, P., Coen, K., Verpoorten, N., Auer-Grumbach, M., Kwon, J. M., FitzPatrick, D., Schmedding, E., De Vriendt, E., Jacobs, A., Van Gerwen, V., Wagner, K., Hartung, H. P., & Timmerman, V. (2003). Mutations in the small GTP-ase late endosomal protein RAB7 cause Charcot-Marie-Tooth type 2B neuropathy. *American Journal of Human Genetics*, *72*(3), 722–727.

Watson, F.L., Porcionatto, M.A., Bhattacharyya, A., Stiles, C.D., Segal, R.A. (1998). TrkA glycosylation regulates receptor localization and activity. *J Neurobiol*. 39(2), 323-36.

Wercberger, R., & Basbaum, A. I. (2019). Spinal cord projection neurons: a superficial, and also deep analysis. *Current Opinion in Physiology*, *11*, 109–115.

Wheeler D.W., Lee M.C., Harrison E.K., Menon D.K., Woods C.G. (2014). Case Report: Neuropathic pain in a patient with congenital insensitivity to pain. *F1000Res*. 26, 135.

White, F. A., Silos-Santiago, I., Molliver, D. C., Nishimura, M., Phillips, H., Barbacid, M., & Snider, W. D. (1996). Synchronous onset of NGF and TrkA survival dependence in developing dorsal root ganglia. *Journal of Neuroscience*, *16*(15), 4662–4672.

Willnow, T. E., Petersen, C. M., & Nykjaer, A. (2008). VPS10P-domain receptors - Regulators of neuronal viability and function. *Nature Reviews Neuroscience*, *9*(12), 899–909.

Wise, B. L., Seidel, M. F., & Lane, N. E. (2021). The evolution of nerve growth factor inhibition in clinical medicine. *Nature Reviews Rheumatology*, *17*(1), 34–46.

Woo, S. H., Lukacs, V., De Nooij, J. C., Zaytseva, D., Criddle, C. R., Francisco, A., Jessell, T. M., Wilkinson, K. A., & Patapoutian, A. (2015). Piezo2 is the principal mechanotransduction channel for proprioception. *Nature Neuroscience*, *18*(12), 1756–1762.

Woo, S. H., Ranade, S., Weyer, A. D., Dubin, A. E., Baba, Y., Qiu, Z., Petrus, M., Miyamoto, T., Reddy, K., Lumpkin, E. A., Stucky, C. L., & Patapoutian, A. (2014). Piezo2 is required for Merkel-cell mechanotransduction. *Nature*, *509*(7502), 622–626.

Woolf, C J, & Mannion, R. J. (1999). Neuropathic pain: aetiology, s y m p t o m s , m e c h a n i s m s , a n d m a n a g e m e n t. *The Lancet*, *353*(1), 1959–64.

Woolf, C J, Safieh-Garabedian, B., Ma, Q.-P., Crillyi, P., & Winter4, J. (1994). Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. *Neuroscience* 62,2.

Woolf, Clifford J., & Ma, Q. (2007). Nociceptors-Noxious Stimulus Detectors. *Neuron* 55, 353–364.

Wu, H., Petitpré, C., Fontanet, P., Sharma, A., Bellardita, C., Quadros, R. M., Jannig, P. R., Wang, Y., Heimel, J. A., Cheung, K. K. Y., Wanderoy, S., Xuan, Y., Meletis, K., Ruas, J., Gurumurthy, C. B., Kiehn, O., Hadjab, S., & Lallemend, F. (2021). Distinct subtypes of proprioceptive dorsal root ganglion neurons regulate adaptive proprioception in mice. *Nature Communications*, *12*(1),

Xu, Z. Z., Kim, Y. H., Bang, S., Zhang, Y., Berta, T., Wang, F., Oh, S. B., & Ji, R. R. (2015). Inhibition of mechanical allodynia in neuropathic pain by TLR5-mediated A-fiber blockade. *Nature Medicine*, *21*(11), 1326–1331.

Yamashita, T., Tucker, K. L., & Barde, Y. A. (1999). Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. *Neuron*, *24*(3), 585–593.

Yarmolinsky, D. A., Peng, Y., Pogorzala, L. A., Rutlin, M., Hoon, M. A., & Zuker, C. S. (2016). Coding and Plasticity in the Mammalian Thermosensory System. *Neuron*, *92*(5), 1079–1092.

Ye, H., Kuruvilla, R., Zweifel, L. S., & Ginty, D. D. (2003). Evidence in support of signaling endosome-based retrograde survival of sympathetic neurons. *Neuron*, *39*(1), 57–68.

Ye, M., Lehigh, K. M., & Ginty, D. D. (2018). Multivesicular bodies mediate long-range retrograde NGF-TrkA signaling. *ELife*, *7*, 1–29.

Yu, T., Calvo, L., Anta, B., López-Benito, S., Southon, E., Chao, M. V., Tessarollo, L., & Arévalo, J. C. (2011). Regulation of Trafficking of Activated TrkA Is Critical for NGF-Mediated Functions. *Traffic*, *12*(4), 521–534.

Yue, Sze W., Y., Yuan, L., Braz, J. M., Basbaum, A. I., & Julius, D. (2022). TRPV1 drugs alter core body temperature via central projections of primary afferent sensory neurons. *ELife*, *11*(e), 80139.

Zhang, K., Ben Kenan, R. F., Osakada, Y., Xu, W., Sinit, R. S., Chen, L., Zhao, X., Chen, J. Y., Cui, B., & Wu, C. (2013). Defective axonal transport of Rab7 GTPase results in dysregulated trophic signaling. *Journal of Neuroscience*, *33*(17), 7451–7462.

Zhang, X., Huang, J., & McNaughton, P. A. (2005a). NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *EMBO Journal*, *24*(24), 4211–4223.

Zhang, X., Huang, J., & McNaughton, P. A. (2005b). NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *EMBO Journal*, *24*(24), 4211–4223.

Zhou, X. F., Rush, R. A., & McLachlan, E. M. (1996). Differential expression of the p75 nerve growth factor receptor in glia and neurons of the rat dorsal root ganglia after peripheral nerve transection. *Journal of Neuroscience*, *16*(9), 2901–2911.

Zhu, W., Galoyan, S. M., Petruska, J. C., Oxford, G. S., & Mendell, L. M. (2004). A developmental switch in acute sensitization of small dorsal root ganglion (DRG) neurons to capsaicin or noxious heating by NGF. *Journal of Neurophysiology*, *92*(5), 3148–3152.

Zimmerman, A., Bai, L., & Ginty, D. D. (2014). The gentle touch receptors of mammalian skin. *Science* 346, 950–954.

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