From Department of Neuroscience
Karolinska Institutet, Stockholm, Sweden

CALCIUM-BINDING PROTEINS AS MARKERS AND FUNCTIONAL DETERMINANTS OF NEURONS IN PAIN NETWORKS

Mingdong Zhang

Karolinska
Institutet

Stockholm 2017

Cover illustration: Immunohistochemical double labeling of ATF3 (green) and GFAP (red) in mouse dorsal root ganglion after 2 weeks of axotomy.

All previously published papers were reproduced with permission from the publisher.
Published by Karolinska Institutet.
Printed by E-Print AB
© Mingdong Zhang, 2017
ISBN 978-91-7676-610-1

Calcium-binding proteins as markers and functional determinants of neurons in pain networks

THESIS FOR DOCTORAL DEGREE (Ph.D.)

ACADEMIC DISSERTATION

for the degree of PhD at Karolinska Institutet

The thesis will be defended in public in the Hillarp Lecture Hall, Retzius väg 8, Karolinska Institutet, Stockholm, Sweden

Friday the 24th of March 2017, 09.00

By

Mingdong Zhang

Principal Supervisor:
Prof. Tibor Harkany
Karolinska Institutet
Department of Neuroscience

Co-supervisors:
Prof. Tomas Hökfelt
Karolinska Institutet
Department of Neuroscience

Dr. Jan Mulder
Karolinska Institutet
Department of Neuroscience
Laboratory for Life Science

Opponent:
Prof. Marco Celio
Department of Medicine
University of Fribourg

Examination Board:
Prof. Håkan Aldskogius
Uppsala University
Department of Neuroscience

Prof. Anita Aperia
Karolinska Institutet
Department of Women's and Children's Health

Prof. Lou Brundin
Karolinska Institutet
Department of Clinical Neuroscience

To my family

ABSTRACT

The thesis focuses on the anatomical and cellular distribution of three EF-hand calcium binding proteins, secretagogin, neuronal calcium-binding protein 1 (NECAB1) and NECAB2 in dorsal root ganglia (DRGs) and spinal cord of three species, mouse, rat and human, and their possible roles in pathophysiological pain.

In **Paper I** and **Paper IV**, we report that the expression of secretagogin is limited to a small subpopulation of peptidergic neurons in mouse and human DRGs expressing calcitonin gene-related peptide (CGRP). Secretagogin is present both in the cell bodies in the DRGs and in the central branches in lamina I of the dorsal horn and in peripheral branches together with CGRP; it thus centrifugally transported. The loss of secretagogin (a knockout mouse) does not affect the development of pain hypersensitivity after nerve injury or experimentally induced inflammation.

In **Paper II**, we demonstrate a wide expression of NECAB1/2 in many cell bodies in mouse DRGs and in cell bodies/nerve terminals with a wide distribution in different laminae in the spinal cord. NECAB2 is expressed in excitatory neurons in the spinal cord, showing a punctate staining and often co-localization with vesicular glutamate transporter 2 (VGLUT2) and synaptophysin. NECAB2 in DRGs is distinctly down regulated, at both mRNA and protein levels, by peripheral nerve injury.

In **Paper III**, we show a conserved excitatory property and laminar distribution of NECAB2 in mouse, rat and human spinal cord, while NECAB1 exhibits species diversity with regards to neurochemical properties in mouse and rat spinal cord. NECAB1 is present in oligodendrocytes surrounding axons in the white matter of the human spinal cord. We also reveal a differential expression of NECAB2, calbindin-D28k and calretinin in ependymal cells surrounding/within (human) the spinal central canal when comparing rodents and human.

In **Paper IV**, we characterize a NECAB2 population in mouse DRGs using a new NECAB2 antibody validated with help of a *Necab2* knockout mouse. These NECAB2 neurons cover previously defined the C-low threshold mechanoreceptors (LTMRs) and Aδ D-hair LTMRs. Genetically induced loss of NECAB2 attenuates inflammatory but not neuropathic pain. This may, tentatively, be mediated by modulation of brain-derived neurotrophic factor (BDNF) expressed in DRGs, and through the interaction with its receptor tyrosine receptor kinase B (TrkB) in the spinal cord to modulate spinal glutamatergic neurotransmission.

LIST OF SCIENTIFIC PAPERS

I. Shi TJ*, Xiang Q*, **Zhang MD**, Tortoriello G, Hammarberg H, Mulder J, Fried K, Wagner L, Josephson A, Uhlén M, Harkany T, Hökfelt T
Secretagogin is expressed in sensory CGRP neurons and in spinal cord of mouse and complements other calcium-binding proteins, with a note on rat and human
Mol Pain. 2012 Oct 29;8:80

II. **Zhang MD**, Tortoriello G, Hsueh B, Tomer R, Ye L, Mitsios N, Borgius L, Grant G, Kiehn O, Watanabe M, Uhlén M, Mulder J, Deisseroth K, Harkany T, Hökfelt T
Neuronal calcium-binding proteins 1/2 localize to dorsal root ganglia and excitatory spinal neurons and are regulated by nerve injury
Proc Natl Acad Sci U S A. 2014 Mar 25;111(12):E1149-58

III. **Zhang MD**, Barde S, Szodorai E, Josephson A, Mitsios N, Watanabe M, Attems J, Lubec G, Kovács GG, Uhlén M, Mulder J, Harkany T, Hökfelt T
Comparative anatomical distribution of neuronal calcium-binding protein (NECAB) 1 and -2 in rodent and human spinal cord
Brain Struct Funct. 2016 Sep;221(7):3803-23

IV. **Zhang MD**, Su J, Adori C, Cinquina C, Malenczyk K, Girach F, Peng CG, Ernfors P, Löw P, Borgius L, Kiehn O, Uhlén M, Mulder J, Harkany T, Hökfelt T
A role for the calcum binding protein NECAB2 in inflammatory pain in mouse
Manuscript

* shared first authorship

PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Romanov RA*, Zeisel A*, Bakker J, Girach F, Hellysaz A, Tomer R, Alpár A, Mulder J, Clotman F, Keimpema E, Hsueh B, Crow AK, Martens H, Schwindling C, Calvigioni D, Bains JS, Máté Z, Szabó G, Yanagawa Y, **Zhang MD**, Rendeiro A, Farlik M, Uhlén M, Wulff P, Bock C, Broberger C, Deisseroth K, Hökfelt T, Linnarsson S, Horvath TL, Harkany T
Molecular interrogation of hypothalamic organization reveals distinct dopamine neuronal subtype
Nat Neurosci. 2017 Feb;20(2):176-188

II. **Zhang MD**, Barde S, Yang T, Lei B, Eriksson LI, Mathew JP, Andreska T, Akassoglou K, Harkany T, Hökfelt TG, Terrando N
Orthopedic surgery modulates neuropeptides and BDNF expression at the spinal and hippocampal levels
Proc Natl Acad Sci U S A. 2016 Oct 25;113(43):E6686-E6695
Selected as the highlight in 'This Week in PNAS'

III. Wang P, Li H, Barde S, **Zhang MD**, Sun J, Wang T, Zhang P, Luo H, Wang Y, Yang Y, Wang C, Svenningsson P, Theodorsson E, Hökfelt TG, Xu ZQ
Depression-like behavior in rat: Involvement of galanin receptor subtype 1 in the ventral periaqueductal gray
Proc Natl Acad Sci U S A. 2016 Aug 9;113(32):E4726-35

IV. Tesoriero C*, Codita A*, **Zhang MD***, Cherninsky A, Karlsson H, Grassi-Zucconi G, Bertini G, Harkany T, Ljungberg K, Liljeström P, Hökfelt TG, Bentivoglio M, Kristensson K
H1N1 influenza virus induces narcolepsy-like sleep disruption and targets sleep-wake regulatory neurons in mice
Proc Natl Acad Sci U S A. 2016 Jan 19;113(3):E368-77

V. Romanov RA*, Alpár A*, **Zhang MD**, Zeisel A, Calas A, Landry M, Fuszard M, Shirran SL, Schnell R, Dobolyi Á, Oláh M, Spence L, Mulder J, Martens H, Palkovits M, Uhlen M, Sitte HH, Botting CH, Wagner L, Linnarsson S, Hökfelt T, Harkany T
A secretagogin locus of the mammalian hypothalamus controls stress hormone release
EMBO J. 2015 Jan 2;34(1):36-54

VI. Shi TJ, Xiang Q, **Zhang MD**, Barde S, Kai-Larsen Y, Fried K, Josephson A, Glück L, Deyev SM, Zvyagin AV, Schulz S, Hökfelt T
Somatostatin and its 2A receptor in dorsal root ganglia and dorsal horn of mouse and human: expression, trafficking and possible role in pain
Mol Pain. 2014 Feb 13;10:12.

VII. Shi TJ*, **Zhang MD***, Zeberg H, Nilsson J, Grünler J, Liu SX, Xiang Q, Persson J, Fried KJ, Catrina SB, Watanabe M, Arhem P, Brismar K, Hökfelt TG
Coenzyme Q10 prevents peripheral neuropathy and attenuates neuron loss in the db-/db- mouse, a type 2 diabetes model
Proc Natl Acad Sci U S A. 2013 Jan 8;110(2):690-5

* shared first authorship

CONTENTS

1 Introduction 1
1.1 Peripheral somatosensory modalities 1
1.2 Neuronal circuits in spinal dorsal horn 5
1.3 Pain and chronic pain 6
1.4 EF-hand calcium-binding proteins and pain 8
1.5 Candidate EF-hand calcium-binding proteins 10
2 Aims of thesis 13
3 Materials and Methods 15
3.1 Animals and human tissues 15
3.2 Animal models 15
3.3 Behavioral analyses 16
3.4 Quantitative real time-PCR (RT-qPCR) 16
3.5 Western blotting 16
3.6 Tissues and immunohistochemistry 18
3.7 In situ hybridization and immunohistochemistry 20
3.8 Microscopy, quantification and image processing 20
3.9 CLARITY volume imaging 21
3.10 iDISCO+ volume imaging 21
4 Results and Discussion 23
4.1 Secretagogin expression in DRGs and spinal cord from rodents and human, and its potential functions in pain sensation 23
4.2 Expression of neuronal calcium-binding proteins 1/2 in DRGs and spinal cord, and effects of nerve injury 26
4.3 The excitatory property of spinal NECAB2-IR interneurons is conserved in rodents and human 28
4.4 A role for NECAB2 in inflammatory pain 29
5 Concluldling remarks 35
6 Acknowledgements 37
7 References 39

LIST OF ABBREVIATIONS

ATF3	Activating transcription factor 3
BDNF	Brain-derived neurotrophic factor
CaBP	Calcium-binding protein
CGRP	Calcitonin gene-related peptide
DRGs	Dorsal root ganglia
IB4	Isolectin B4
Iba1	Ionized calcium binding adaptor molecule 1
i.p.	Intraperitoneal
IR	Immunoreactive
KO	Knockout
LI	Like immunoreactivity
LTMRs	Low-threshold mechanoreceptors
NECAB2	Neuronal calcium-binding protein 2
NF200	Neurofilament 200
NPs	Neuronal profiles
PKCγ	Protein kinase C gamma
PV	Parvalbumin
S.C.	Spinal cord
SNI	Spared nerve injury
SP	Substance P
TH	Tyrosine hydroxylase
TrkA	Tyrosine receptor kinase A
TrkB	Tyrosine receptor kinase B
VGLUT1	Vesicular glutamate transporter 1
VGLUT2	Vesicular glutamate transporter 2
WT	Wild type

1 INTRODUCTION

1.1 PERIPHERAL SOMATOSENSORY MODALITIES

Organisms, from nematodes to vertebrates, have developed different sensory modalities to perceive and react to their environment. In mammals, eyes, ears, snouts, tongue and skin are sensory organs with specialized cellular structures and receptors for specific stimuli. Cutaneous skin, our largest sensory organ, not only transduces touch, pressure and temperature information into the central nervous system but also pain sensations, involving a wide variety of sensory neuron subtypes innervating skin (and internal organs) (Basbaum et al., 2009; Zimmerman et al., 2014).

Dorsal root ganglia (DRGs) are specialized structures composed of cell bodies of primary somatosensory neurons and surrounding satellite glia cells organized in two ‘columns’ along the spinal cord (Figure 1*A*) (Bunge et al., 1967; Ramón y Cajal and Azoulay, 1952; Ramón y Cajal et al., 1995). An adult sensory neuron is a pseudo-unipolar cell with a peripheral branch innervating the skin (or internal organs) and a central branch projecting to the spinal cord (Figure 1*B*,*C*). Such peripheral sensory neurons conduct impulses from peripheral nerve endings towards the cell bodies and then, via the dorsal roots, to the spinal cord. However, impulses also propagate directly from peripheral nerves to central roots and spinal cord, that is ‘bypassing’ the cell somata (Ramón y Cajal and Azoulay, 1952; Willis, 2007).

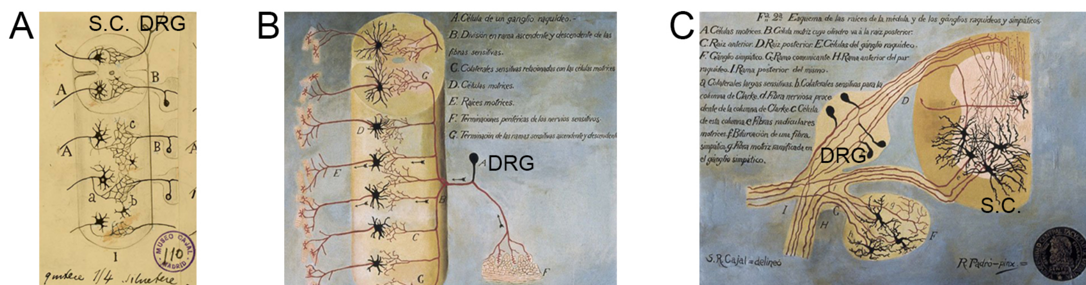

Figure 1. Illustrations of dorsal root ganglia and spinal cord drawn by Ramón y Cajal. *A*) Cell bodies of DRG neurons are organized (only right side shown) of the spinal cord. The orientation of the spinal cord is ventral horn to the left and dorsal horn to the right. The spinal ganglia are bilateral structures dorso-lateral to the spinal dorsal horn. *B,C*) Pseudo-unipolar DRG neurons relay the sensory information from the periphery (skin) to the spinal cord. A sympathetic ganglion below spinal ganglion is also included in panel *C*. (modified from Ramón y Cajal’s drawings)

DRG neurons were originally classified into two main categories: large pale and small dark neurons, according to the size of their perikarya and their morphology based on the distribution of Nissl bodies (Lawson, 1979). Using immunohistochemistry, further categories have been identified. Thus, it could be shown that those light neurons (large pale) but not

small dark neurons were specifically labeled by a monoclonal antibody against the phosphorylated 200 kDa neurofilament subunit (NF200) (Dahl et al., 1988; Lawson et al., 1984). The small dark neurons were further categorized on their transmitter substance, in particular neuropeptides, first substance P (SP, peptidergic neurons) (Hokfelt et al., 1975) and subsequently non-peptidergic neurons. DRG neurons could also be classified as Aα/β-fiber, Aδ-fiber or C-fiber neurons according to their conduction velocity (linear relationship between the conduction velocity and the diameter of nerve axons, Figure 2), which correlated to the staining with NF200 but not simply to their cell size (Lawson and Waddell, 1991; Lee et al., 1986). Aβ-fibers are heavily myelinated and have rapid conduction velocity (>30 m/s), Aδ-fibers are thinly myelinated and exhibiting intermediate (5-30 m/s) conduction velocity, whereas C-fibers are unmyelinated and have the slowest conduction velocity (0.2-2 m/s) (Figure 2) (Horch et al., 1977; Li et al., 2011; Perl, 2007). Conduction velocities can vary considerably across species. The rat DRG neuronal conduction velocities are: Aα-fiber, 30-55 m/s; Aβ-fiber, 14-30 m/s; Aδ-fiber, 2.2-8 m/s and C-fiber, less than 1.4 m/s (Harper and Lawson, 1985).

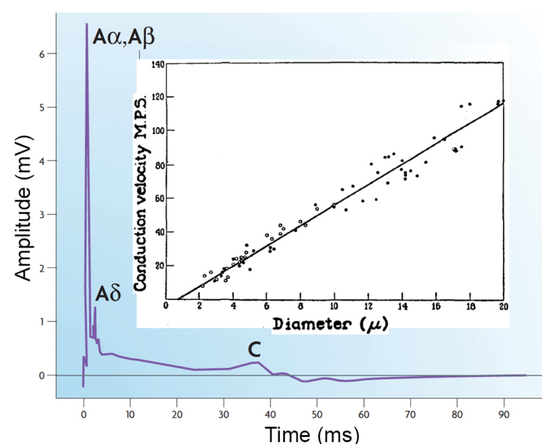

Figure 2. The compound action potential of a cat cutaneous nerve. The outer graph shows the compound action potential of the cat saphenous nerve including the Aα/β-, Aδ- and C-fibers. The inset shows the linear relationship between the velocities of conduction and the diameter of nerves. (modified from Gasser 1941 and Perl 2007)

From a functional perspective, DRG neurons are composed of different populations like *nociceptors* (pain); *pruriceptors* (itch); *thermoreceptors* (temperature); and *low-threshold mechanoreceptors* (LTMRs, non-painful mechanical stimuli/touch) (Zimmerman et al., 2014).

Nociceptors Noxious stimuli (e.g. intense thermal, mechanical or chemical stimuli) are detected and delivered by a heterogeneous population of DRG neurons named nociceptors

(Basbaum et al., 2009), which represent a first line of defense against any potentially threatening or damaging inputs from the environment or internal organs (Woolf and Ma, 2007). The term 'nociceptor' was coined by Charles Sherrington to define the neural apparatus responsible for detecting noxious stimuli at the dawn of the 20th century. He also defined the noxious stimulus as "one with an intensity and quality sufficient to trigger reflex withdrawal, autonomic responses, and pain" (Sherrington, 1906; Woolf and Ma, 2007). Nociceptors are heterogeneous, composed of medium-sized, thinly myelinated Aδ-fibers and small-sized unmyelinated C-fibers. The former mediate acute and fast pain, which is different from the large-sized myelinated Aβ-fibers responding to innocuous mechanical stimuli. The unmyelinated fibers convey slow pain (Basbaum et al., 2009). The Aδ nociceptors are further divided into two types based on electrophysiological analysis: high–threshold mechanical nociceptors with relatively high heat threshold (>50°C, type I) and nociceptors with much lower heat threshold (type II) (Basbaum et al., 2009). The unmyelinated C fibers are also heterogeneous, since they are polymodal, including the major population (about 45%) responding to both heat and mechanical stimuli (Basbaum et al., 2009; Perl, 2007; Schmidt et al., 1995). Another interesting population of unmyelinated C fibers (silent nociceptors) is sensitized to heat and/or mechanical stimuli after topical application of mustard oil or capsaicin (Schmidt et al., 1995). The heterogeneity of unmyelinated C fibers could further be divided into peptidergic and non-peptidergic nociceptors according to the expression of neuropeptides and different receptors for growth factors (Basbaum et al., 2009; Hokfelt et al., 1980; Hökfelt et al., 2013; McMahon and Priestley, 2005). Peptidergic neurons express neuropeptides like SP, somatostatin, calcitonin-gene related peptide (CGRP) and tyrosine receptor kinase A (TrkA), whereas non-peptidergic neurons express neurotrophic receptor c-Ret, Mass related G-protein coupled receptors (MRGPRs) family, purinergic receptor P2X ligand-gated ion channel 3 (P2X3) and also bind the isolectin B4 (IB4) (Lallemend and Ernfors, 2012; Ma, 2012; Marmigere and Ernfors, 2007). Most recently, molecular criteria are used to classify nociceptors and also other DRG neuron populations, showing consistency with prior knowledge of sensory neuron function and also broadening our knowledge of diversity and complexity of sensory types of neurons based on single cell RNA-sequencing (Chiu et al., 2014; Li et al., 2016; Usoskin et al., 2015).

Pruriceptors Itch, clinically known as pruritus, is an unpleasant sensation and emotional experience that produces a desire to scratch (Davidson and Giesler, 2010). Chronic itch is a complex sensation with many similarities to pain, which are also mediated by unmyelinated C-fibers (Ikoma et al., 2006; Sun and Chen, 2007). Instead of having a clear set of separate primary sensory neurons for itch and pain, most mediators have prominent roles in both in

general (Ikoma et al., 2006). Vesicular glutamate transporter type 2 (VGLUT2), one of three markers for glutamatergic neurons (Herzog et al., 2001; Herzog et al., 2004), which is expressed in the majority of Aδ- and C-fibers, is involved in the modulation of both itch and pain (Brumovsky et al., 2007; Lagerstrom et al., 2010; Liu et al., 2010; Scherrer et al., 2010). However, a recent study shows that Mass-related G-protein coupled receptor A3 (MrgprA3) defines a subpopulation of nociceptors specially linked to itch without altering pain-like behavior (Han et al., 2013).

Thermoreceptors Temperatures exceed normal range (>43°C or <15°C) not only evoke a thermal sensation, but also pain (LaMotte and Campbell, 1978; Tominaga and Caterina, 2004). A subpopulation of nociceptors called C-fiber mechano-heat responsive nociceptors (CMH) evokes the subjective sensation of burning pain (LaMotte and Campbell, 1978). Here the discovery of the transient receptor potential (TRP) ion channel family has been of major importance (Julius, 2013). The receptor for capsaicin, transient receptor potential subfamily 5 vanilloid 1 (a cation channel) TRPV1, is responsible for noxious heat (Caterina et al., 1997; Mishra et al., 2011), whereas TRPM8 is activated by cool temperature (McKemy et al., 2002; Peier et al., 2002). The extreme heat and cold is sensed by TRPV2 and TRPA1, respectively (Caterina et al., 1999; Story et al., 2003).

Low-threshold mechanoreceptors LTMRs are activated by innocuous mechanical stimulus although some of them can also be activated by thermal stimulus (Abraira and Ginty, 2013). LTMRs represent a diverse group of somatosensory neurons including Aβ-, Aδ-fibers and C-LTMRs (tyrosine hydroxylase, TH population) based on conduction velocity (Brumovsky et al., 2006; Horch et al., 1977; Li et al., 2011). According to the rates of adaption to sustained mechanical stimulus, LTMRs are classified as slowly, intermediately, or rapidly adapting-LTMRs (Johnson and Hsiao, 1992). They can also be distinguished by the cutaneous end organs that they innervate and their preferred stimuli. Recent studies support a model of mechanosensation, where the activities of Aβ-, Aδ- and C-LTMRs are integrated within LTMR columns in the dorsal horn and processed into outputs underlying the perception of touch sensation (Abraira and Ginty, 2013; Zimmerman et al., 2014). Furthermore, the deep dorsal horn (LTMR-recipient zone, the cellular and synaptic architecture) is implicated in processing LTMR information encoding tactile perception through interneurons with high degree of neuronal diversity (Abraira et al., 2017).

1.2 NEURONAL CIRCUITS IN SPINAL DORSAL HORN

Spinal dorsal horn neurons, including interneurons and projection neurons, receive sensory inputs from peripheral nerve endings (of DRGs) innervating the skin and deeper tissues of the body, which respond to a wide range of stimuli (mechanical, heat and chemical) from the environment (Todd, 2006, 2010). The central projections of DRG neurons terminate in the dorsal horn with an organized distribution pattern, which is determined by the property sensory modality (touch, itch and pain) and the region that they innervate (Abraira et al., 2017; Rexed, 1952; Sun and Chen, 2007; Todd, 2010). The incoming information integrated in DRG neurons is further processed by secondary neuronal circuits (first synapses) involving both excitatory and inhibitory interneurons, and is relayed to projection neurons ascending to different brain areas like brainstem, thalamus and sensory cortex (Todd et al., 2000). Descending projections from brainstem is also involved in the modulation of the spinal pain circuits (Basbaum and Fields, 1984). In addition, nociceptive information is conveyed directly and/or indirectly to motor neurons in the ventral horn, which contributes to nocifensive reflexes at spinal level (Arcourt et al., 2017; Steffens and Schomburg, 1993; Wiesenfeld-Hallin et al., 1988). Hence, three components including central projections of primary afferents (DRG neurons), neurons in the spinal dorsal horn (second-order neurons) and descending fibers from supraspinal level are involved in the organization of neural circuits in the spinal dorsal horn (Willis and Coggeshall, 2004).

The gray matter of spinal cord can be divided into 10 laminae (I-X) layers based on neuronal size and distribution density (cyto-architecture) (Rexed, 1952), which is similar in several species (Molander et al., 1984). Central projections from Aδ and C fibers (peptidergic and non-peptidergic) arborize mainly in the superficial layers (composed of laminae I and II) of the spinal cord, while Aβ fibers terminate in the deeper layers (laminae III–V) of the spinal cord. Peptidergic nociceptors ($CGRP^+$) project mainly to lamina I and the outer layer of lamina II (IIo), whereas the non-peptidergic ones ($IB4^+$) project to the central portion of lamina II (Sakamoto et al., 1999). Lamina II, characterized by a translucent appearance in unstained sections due to the lack of myelination, corresponds to the region previously named the substantia gelatinosa.

Projection neurons (glutamatergic), often expressing neurokinin 1 receptor (NK1r) (Mantyh et al., 1995), are densely distributed in lamina I and scattered through the deeper layers (III–VI) (Littlewood et al., 1995). Axons of these projection neurons cross the midline, ascend in the anterolateral tract (ALT) and target supraspinal areas including lateral parabrachial area, several medullary nuclei, periaqueductal grey matter (PAG) and thalamus

(Al-Khater and Todd, 2009; Gauriau and Bernard, 2004).

Interneurons are the great majority type of the neurons in laminae I–III of spinal cord, whose axons arborize locally. They can be divided into inhibitory (GABAergic/glycinergic) and excitatory (glutamatergic) interneurons. The inhibitory interneurons account for ~30% of all neuronal profiles in laminae I and II, and ~40% in lamina III (Polgar et al., 2013a). All these inhibitory interneurons are GABAergic, but glycine is used as a co-transmitter with GABA (Todd and Sullivan, 1990). Excitatory interneurons account for 60-70% of all neuronal profiles in laminae I-III, and they can be labeled with vesicular glutamate transporter 2 (VGLUT2) and some neurochemical markers like calcium-binding proteins (calbindin-D28k) and protein kinase C γ (PKC γ) (Antal et al., 1991; Polgar et al., 1999; Todd et al., 2003).

1.3 PAIN AND CHRONIC PAIN

Pain is defined as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" by the International Association for the Study of Pain (IASP). In the seventeenth century, Rene Descartes proposed the concept of a pain pathway, where the environmental stimulus could be delivered to the brain through a 'thread' which connecting the environment and the brain (Melzack and Wall, 1965). This is, even if highly simplified, similar to the modern view of the anatomical organization of the pain pathway, where the stimulus activates peripheral nerve endings in the skin, and then via the DRG are integrated in the spinal cord, and ascend to the brain stem, thalamus and finally cortex (Costigan et al., 2009). The application of modern neurobiological techniques to the pain field has rapidly increased our understanding of pain and its pathophysiology, which in turn may provide targets for the development of new therapies.

There are three main manifestations of pain, *nociceptive pain*, *inflammatory pain* and *neuropathic pain* (Basbaum and Woolf, 1999; Scholz and Woolf, 2002). Nociceptive pain is activated only by dangerous, noxious stimuli acting on specialized high-threshold sensory neurons (nociceptors) and continues only if noxious stimuli persist. Once the tissue has been injured or by infection, plasma extravasation and infiltration of immune cells occur, and then multiple chemical mediators are released from damaged and infiltrated inflammatory cells. Those released inflammatory mediators can sensitize (mutual communications between immune cells and nociceptors) the peripheral sensory nerves surrounding the injured part (with further central sensitization), which is characteristic for *inflammatory pain*. This will

minimize movement of, or contact with, the injured tissue until healing has occurred (Basbaum and Woolf, 1999; Costigan et al., 2009; Ji et al., 2002; McMahon, 1996). Pain is one of the cardinal features (together with redness, heat, swelling and loss of function) of inflammation. Acute inflammatory pain retains some resemblance to nociceptive pain, where the protective role can be readily appreciated (Woolf, 1989). However, chronic inflammation leading to a host of diseases like rheumatoid arthritis and fibromyalgia is often detrimental. It is still unclear whether chronic inflammation is essential for driving chronic pain as the acute inflammation does acute pain. The interaction between inflammation progress and pain processing are bidirectional. Non-neuronal cells from the immune system like monocytes, macrophages, T lymphocytes, keratinocytes and glia cells can communicate with nociceptive neurons in the peripheral and second-order neurons in the central nervous system by "listening and talking" to them (Ji et al., 2016).

Neuropathic pain is "caused by a lesion or disease of the somatosensory system" and will in several instances not resolve, thus seemingly lacking any beneficial functions. (Basbaum and Woolf, 1999; Jensen et al., 2011). Diabetes mellitus, infection, nerve compression, nerve trauma, channelopathies, autoimmune disease and bone cancer may cause neuropathic pain (Campbell and Meyer, 2006; Mantyh, 2014). The estimated population prevalence of neuropathic pain is between 6.9%-10% (van Hecke et al., 2014). This definitely affects the quality of patients' life and causes economic burden not only for the individual but also the society. Non-steroidal anti-inflammatory drugs do not help patients suffering from neuropathic pain, other available drugs like tricyclic antidepressants, serotonin- and noradrenaline-uptake inhibitors, the anticonvulsants gabapentin and pregabalin, or opioids that all have limited efficacy and undesirable side effects (Finnerup et al., 2015). More than two-thirds of neuropathic patients suffer because of the insufficient pain relief. This poor response is possibly caused by the heterogeneous etiology, pathophysiology and clinical symptoms for individual patients (Jensen et al., 2011). An alternative approach, with the ultimate aim of obtaining a better treatment outcome, involves examining and classifying patients aiming for mechanism-based, individual therapy (Baron, 2006).

Major progress has been achieved in the past decades in understanding the underlying mechanisms of peripheral neuropathic pain (*peripheral and central sensitization*). But much less is understood with regards to central neuropathic pain (Campbell and Meyer, 2006; Costigan et al., 2009). Multiple sites along the neural axis for sensory sensation are altered after peripheral nerve injury. Peripheral sensitization manifests as spontaneous activity and ectopic sensitivity to mechanical, thermal or chemical stimuli originating from the injured

nociceptors and neighboring sensitized intact nociceptors (Campbell and Meyer, 2006; LaMotte et al., 1982). Central sensitization refers to the augmented response of central signaling neurons (Woolf, 1983). Synaptic facilitation contributes to dynamic tactile allodynia and secondary hypersensitivity. Disinhibition through inhibitory interneurons (like PV interneurons in laminae II/III) and descending modulatory pathways facilitate dorsal horn sensitization (Petitjean et al., 2015; Todd, 2010, 2015). Also the immune system, both peripherally and centrally, plays a critical role in neuropathic pain (Campbell and Meyer, 2006; Costigan et al., 2009; McMahon et al., 2015).

1.4 EF-HAND CALCIUM-BINDING PROTEINS AND PAIN

The calcium ion (Ca^{2+}) is an essential element for numerous biological functions (Chin and Means, 2000). In the nervous system, calcium-triggered phenomena, ranging from synaptic neurotransmitter release to modulation of gene expression, is reflected by the existence of a cohort of different calcium-binding proteins (CaBPs) many of which belong to the EF-hand super-family (Baimbridge et al., 1992; Burgoyne, 2007; Mikhaylova et al., 2011). EF-hand CaBPs can be divided into *calcium buffer* and *calcium sensor* proteins according to the affinity for Ca^{2+}. The former have a very high affinity for Ca^{2+} and do not exhibit conformational changes upon the binding of Ca^{2+} (thought to chelate Ca^{2+}), whereas *calcium sensor* proteins have a lower affinity for Ca^{2+} and show considerable conformational changes upon Ca^{2+}-binding, which usually triggers a protein interaction and downstream signaling pathway (Burgoyne, 2007; Mikhaylova et al., 2011).

Calmodulin, an ancestral *calcium sensor* with four EF-hand calcium-binding motifs, is the best studied and ubiquitously expressed in all eukaryotic cells (Chin and Means, 2000). Some other EF-hand CaBPs related to calmodulin (like neuronal calcium sensor protein family, NCS) are only enriched and expressed in the nervous system, where they have distinct roles in the modulation of neuronal functions, including in the somatosensory system. Neuronal calcium sensor-1 (NCS-1), the most widely expressed NCS protein, is present in most DRG neurons and spinal dorsal horn (Averill et al., 2004). *Ex vivo* study shows that NCS-1 participates in the regulation of neurite outgrowth and growth cone morphology of DRG neurons (Iketani et al., 2009). Calsenilin, another member of the NCS family, was discovered as the mediator for processing of amyloid precursor protein through the interaction with presenilin (Buxbaum et al., 1998; Leissring et al., 2000). Calsenilin is also named KChIP3 (Kv channel-interacting protein 3), since it can modulate A-type potassium channels through interaction with the cytoplasmic amino terminus of Kv4 alpha-subunits, and DREAM

(downstream regulatory element antagonist modulator). Here, it acts as a repressor of transcription for specific genes containing the downstream regulatory element (DRE) (An et al., 2000; Carrion et al., 1999). DREAM/Calsenilin/KChIP3 has been reported to modulate neuropathic and inflammatory pain as a transcriptional repressor of prodynorphin in the spinal dorsal horn (Cheng et al., 2002; Costigan and Woolf, 2002).

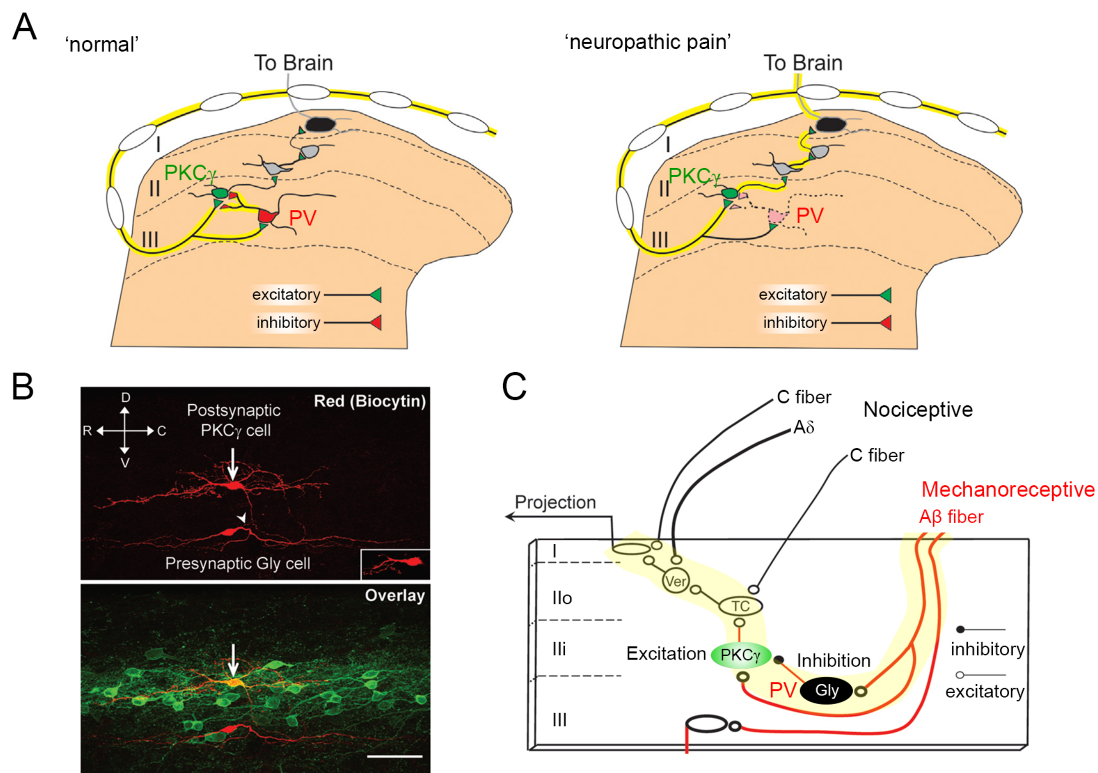

Figure 3 PV interneurons mediate tactile allodynia after peripheral nerve injury. *A*) A VGLUT1^{+} myelinated Aβ fiber (highlighted in yellow) projects to a PKCγ (excitatory) and a PV (inhibitory) interneuron. Under normal conditions, touch sensation will not engage the PKCγ nociceptive circuit. After peripheral nerve injury, the dendrites of PV interneurons detach from PKCγ interneurons, where the touch signal will be delivered through the local circuits to projection neurons and produce pain. *B,C*) Glycinergic interneurons in lamina III of spinal cord have been shown to be involved in a feed-forward inhibitory circuit that prevents the Aβ input from activating the PKCγ nociceptive pathway. Arrowhead indicates the presynaptic glycinergic interneuron, whereas the arrow indicates postsynaptic PKCγ interneuron in (*B*). Green labeled interneurons are PKCγ in (*B*). Both transient central (TC) and vertical interneurons in (*C*) are excitatory. (modified from Lu 2013 and Petitjean 2015)

Parvalbumin (PV), the anatomical marker for GABAergic neurons in the brain (Celio, 1986; Celio and Heizmann, 1981), is expressed in DRGs (proprioceptors) and spinal cord (Antal et al., 1990; Celio, 1990; Usoskin et al., 2015). In spinal dorsal horn, PV positive

neurons are mainly located in the inner layer of lamina II and lamina III, whereas the PV-immunoreactive (IR) fibers sharply delineate the inner layer of lamina II (Antal et al., 1990; Celio, 1990). PV, together with galanin, neuropeptide Y and neuronal nitric oxide synthase, define populations of neurochemically non-overlapping inhibitory interneurons in rat spinal dorsal horn (Laing et al., 1994; Polgar et al., 2013b). PV interneurons in laminae II/III have been shown to be the gatekeepers of innocuous touch-induced mechanical hypersensitivity after peripheral nerve injury (Figure 3*A,B*) (Petitjean et al., 2015). Those PV interneurons normally prevent touch sensation inputs from engaging PKCγ-mediated nociceptive circuits through the direct innervation on PKCγ neurons (Neumann et al., 2008). PKCγ labels a specific population of excitatory interneurons in the inner layer of lamina II and has been shown to be involved in the development of neuropathic pain (Malmberg et al., 1997; Polgar et al., 1999). After nerve injury, the innervation of PV interneurons on PKCγ neurons is detached, which leads to activation of the touch stimulus of PKCγ interneurons, transient central neurons (excitatory), vertical neurons (excitatory) and finally to the projection neurons (glutamatergic) in lamina I to produce pain feeling of pain in the brain (Fig. 3*A-D*) (Lu et al., 2013; Petitjean et al., 2015). Here, PV is the anatomical marker for glycinergic interneurons (also GABAergic, as said, glycine is as co-transmitter with GABA in lamina I-III) for the theory that a feed-forward glycinergic neural circuit gates mechanical allodynia (Lu et al., 2013; Petitjean et al., 2015; Todd, 2010).

1.5 CANDIDATE EF-HAND CALCIUM-BINDING PROTEINS

Although EF-hand CaBPs have been extensively studied over the past decades, analysis of select members of a >150- member protein superfamily inevitably fails to resolve the many essential cellular contributions of these proteins, often leading to a role as mere, but still very useful, "markers" of particular cell types (Girard et al., 2015; Schwaller, 2009). Significant caveats of our present understanding are due to the lack of "deorphanization" efforts to identify new CaBPs containing EF-hand, and their rigid classification into *buffer* and *sensor* categories. During our systematic analysis of the human proteome (Mulder et al., 2009a), we have established the distribution of ~60 CaBPs, including >15 with entirely unknown distribution or functions. By using predictive structure-function analysis, it was found that most of these proteins exhibit unconventional, dual *buffer*/*sensor* activities, suggesting that these proteins can modulate cellular fate decision and synaptic responsiveness, if they are integrated into specific interactome networks in particular cell types. Here, we are interested in several of those CaBPs including secretagogin and Neuronal calcium-binding protein 1/2

(NECAB1/2), whose anatomical distribution and possible roles in modulation of pain signaling at the spinal level up till now have been unknown.

Secretagogin was discovered as a CaBPs of the EF-hand family, cloned from human β cells of pancreatic island of Langerhans and endocrine cells of the gastrointestinal gland (Wagner et al., 2000). Human secretagogin, composed of six EF-hand motifs, contains 276 amino acids, with a calculated molecular weight of 32 kDa (Alpar et al., 2012). Expression of secretagogin precedes many other CaBPs in post-mitotic, migratory neurons in the nervous system at embryonic stages (Alpar et al., 2012; Mulder et al., 2010; Mulder et al., 2009b). Secretagogin expression persists during neurogenesis in the adult brain, and also confined to subsets of differentiated neurons in the central and peripheral nervous system, as well as in the neuroendocrine systems (Mulder et al., 2010; Mulder et al., 2009b). Secretagogin has been involved in the control of neuronal turnover and differentiation, such as in neoplastic brain and endocrine tumors (Birkenkamp-Demtroder et al., 2005). Furthermore, secretagogin could bind to the SNARE protein complex, where it might function as a *calcium sensor* modulating release of neurotransmitters, neuropeptides and/or hormones (Bauer et al., 2011; Rogstam et al., 2007). Very recent results have demonstrated a novel role of secretagogin in a hierarchical hormonal cascade along the hypothalamic-pituitary-adrenal axis orchestrating bodily response to stress (Romanov et al., 2015).

NECAB1/2 are CaBPs with two N-terminal EF-hand motifs and a putative antibiotic biosynthesis monooxygenase (ABM) domain in the C-terminal, which are linked by a NECAB homogeneous region (Canela et al., 2007; Sugita et al., 2002). NECAB1 was discovered as an interacting target for the presynaptic calcium-sensor synaptotagmin I, whereas NECAB2 was found as a down-stream target of Pax6 participated in the mammalian retinal primordium development (Bernier et al., 2001; Sugita et al., 2002; Sugita and Sudhof, 2000). NECAB1-like immunoreactivity (LI) is expressed in many mouse brain areas mainly restricted to the cerebral cortex, striatum, hippocampus, as well as a subset of thalamic, especially midline nuclei. *Necab1* mRNA was also found in the temporal lobe of human brain (Wu et al., 2007). The recent in situ hybridization (and immunohistochemistry) study in mouse shows that *Necab1* mRNA^{+} neurons are scattered the hippocampus, whereas the *Necab2* mRNA is strongly expressed in pyramidal neurons of CA2 (Zimmermann et al., 2013). Single-cell RNA sequencing has shown that both *Necab1* and *Necab2* transcripts are existed in mouse DRG neurons. Furthermore, *Necab2* is identified as a marker for the subpopulation of thinly myelinated DRG neurons, that is the tyrosine receptor kinase B labeled population (Usoskin et al., 2015). Finally, in situ hybridization data for the two *Necab*

transcripts in mouse brain and spinal cord is available in the Allen Brain Atlas (Henry and Hohmann, 2012; Lein et al., 2007).

Against this background it appears a relevant and interesting task to map and characterize secretagogin and NECAB1/2 at the spinal level, that is in DRGs and spinal cord, and explore possible functions in spinal pain circuits.

2 AIMS OF THESIS

The overall aim of this thesis was to characterize the anatomical distribution of secretagogin and NECAB1/2 in DRGs and spinal cord, and explore the potential functions involved in pain sensation under pathological conditions.

1. To characterize the anatomical distribution pattern of secretagogin in DRGs and spinal cord from rodents and human, and study possible effects of loss of secretagogin on the development of pain hypersensitivity (Paper I and IV).

2. To map and characterize the expression of NECAB1/2 in DRGs and spinal cord, and to study the effect of nerve injury on their expression and distribution (Paper II); and to compare NECAB1/2 expression in spinal cord between rodents and human (Paper III).

3. To explore the functional role of NECAB2 in pain circuits with nerve injury and inflammation models using a *Necab2* KO mouse (Paper IV).

3 MATERIALS AND METHODS

3.1 ANIMALS AND HUMAN TISSUES

WT male C57BL/6N mice (adult, ~16 wk of age), *secretagogin* KO mice, *Necab2* KO mice, *Hoxb8*::*Flp*, *Vglut2*::EGFP, *Vglut2::Cre*, *Gad67*$^{+/gfp}$ mice and *Arc::CreERT2/ZsGreen* mice were included in this thesis work. A *Necab2* floxed mouse was generated by the insertion of a trapping cassette composed of an *FRT* (flippase recognition target) flanked *lacZ*/neomycin sequence followed by a *loxP* site between exon 3 and exon 4, and an additional *loxP* site was inserted downstream of exon 6. The transcription of *Necab2* was disturbed by the trapping cassette, and therefore we used this strain as *Necab2* KO mice through the thesis. This also applied to *secretagogin* KO mice. The experiments were conducted in accordance with Swedish policy for the use of research animals and human samples, and were approved by a local ethical committee (Stockholms Norra djurförsöksetiska nämnd, N98/035, N71/09, N172/11, N134/12, N101/14 and N16/15). Efforts were made to minimize the number of mice used for the studies and their suffering throughout the thesis work. Human spinal cord, dorsal root ganglia and hippocampus tissues were also included in paper I and paper III.

3.2 ANIMAL MODELS

Complete transection of the sciatic nerve (axotomy) at mid-thigh level of the left hind leg was performed as previously described (Wall et al., 1979). Briefly, mice were anesthetized with 1.5–1.8% (vol/vol) isoflurane (Baxter); the left sciatic nerve was tightly ligated and transected at the mid-thigh level, and a 5-mm portion of the distal part was removed to prevent regeneration, then the muscle and skin were closed with 5-0 silk sutures.

Spared nerve injury (SNI) surgical procedures were performed under anesthesia with isoflurane as previously described (Decosterd and Woolf, 2000; Pertin et al., 2012). Briefly, the skin of the mid-thigh from left lateral surface was incised, and a separation was made directly through the biceps femoris muscle exposing three terminal branches of the sciatic nerve: common peroneal, tibial and sural nerves. The common peroneal and tibial nerves were tightly ligated with 6-0 silk (Ethicon), transected together distally to the ligation and a piece of 1-2 mm of the nerve was removed from the distal stump avoiding any contact with or stretching of the intact sural nerve during the surgery process. Finally, muscle and skin were closed in two layers with 5-0 silk stitches.

For carrageenan model, 20 μl 1% lambda carrageenan (Sigma) was injected into intra-plantar of the hind paw with a 28-G needle and syringe (Morris, 2003; Ren and Dubner, 1999).

3.3 BEHAVIORAL ANALYSES

Withdrawal threshold was tested in transparent plastic domes on a metal mesh floor and measured by a logarithmically incremental stiffness of 0.04, 0.07, 0.16, 0.40, 0.60, 1.0, and 2.0 (g) von Frey Filament (Stoelting) combined with an up–down method to assess tactile allodynia (Bas et al., 2012; Chaplan et al., 1994; Decosterd and Woolf, 2000). For cutoff, the 2.0 hair was selected as the upper limit for testing. For mechanical hyperalgesia a safety pin was used, and the duration of paw withdrawal was recorded (Decosterd and Woolf, 2000). Cold allodynia was tested with a drop of acetone, and the duration of the withdrawal response was recorded (Decosterd and Woolf, 2000).

3.4 QUANTITATIVE REAL TIME-PCR (RT-QPCR)

Total RNA was isolated from mouse lumbar (L4-6) DRGs and corresponding spinal cord segments using TRI Reagent (Sigma), and cDNA was generated from 500 ng RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. RT-qPCR was performed using Maxima SYBR Green Master Mix with ROX (Thermo Scientific) on QuantStudio5 System (Applied Biosystems), and the specific primers used in this study were listed in Paper IV. The amplification conditions included an initial stage at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. All assays were performed in duplicate, and the levels of transcripts were analyzed by comparative CT ($2^{-\Delta\Delta CT}$) method relative to *Gapdh*.

3.5 WESTERN BLOTTING

Total proteins were extracted from freshly dissected DRGs and spinal cord from different animal models using radioimmunoprecipitation assay lysis buffer [RIPA buffer, 50 mM Tris·HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1 mM EDTA] containing protease inhibitor mixture (P8340; Sigma). After sonication (on ice), protein lysates were centrifuged at 12,000 g for 22 min at 4°C. The supernatants were collected, and measured with Bradford Protein Assay for protein concentration (Bio-Rad Laboratories). Loading samples containing 20~30 μg of total protein lysate in Laemmli sample buffer (1×, final) were loaded and separated on 10% SDS/PAGE gels, transferred onto 0.45 μm PVDF membranes pretreated with methanol (Millipore), blocked with 5% nonfat dry milk in TBS containing 0.1% Tween-20 (TBST) for 1 h at room temperature (RT),

and incubated with primary antibody at 4°C for 2 ~ 3 days. The membranes were incubated with HRP-conjugated secondary antibodies for 1 ~ 2 h at RT (1:5,000 ~ 1:10,000; DAKO), washed in TBST buffer (20 min × 3), developed with ECL solution for 5 min (GE Healthcare), and scanned on a ChemiDOC+ Imaging system (Bio-Rad Laboratories). The PVDF membranes were stripped with low pH stripping buffer, blocked with 5% nonfat milk and re-probed with anti-GAPDH antibody (anti-mouse, 1:10,000 ~ 20,000 in TBST containing 5% BSA; Ambion), used as the loading control. Non-saturated images were quantified with Image Lab software (Bio-Rad Laboratories).

Table 1. Antibodies used for the thesis studies

Antibody	Host	Supplier/Catalog number	Dilution
BDNF	Rabbit /polyclonal	Amgen Inc	1:2,000
calbindin-D28k	Rabbit /polyclonal	Swant /CB 38	1:400 (normal)
calbindin-D28k	Mouse /monoclonal	Swant /CB 300	1:500 (normal)
calretinin	Rabbit /polyclonal	Swant /7699/3H	1:400 (normal)
c-Fos	Rabbit /polyclonal	Calbiochem /PC38	1:2,000
CGRP	Rabbit /polyclonal	Terenius L. (Stockholm) and Christensson I. (Uppsala)	1:20,000 (normal)
Cre	Rabbit /polyclonal	Schütz G. (Heidelberg, Germany)	1:4,000
GAD67	Mouse /monoclonal	Millipore /MAB406	1:500 (normal)
GFP	Chicken /polyclonal	Abcam /ab13970	1:4,000
Iba1	Rabbit /polyclonal	WAKO /019-19741	1.4,000
NECAB1	Rabbit /polyclonal	Atlas Antibodies AB /HPA023629	1:1,000
NECAB2	Rabbit /polyclonal	Atlas Antibodies AB /HPA014144	1:1,000
NECAB2	Rabbit /polyclonal	Atlas Antibodies AB /HPA013998	1:10,000
NF200	Mouse /monoclonal	Sigma /N0142	1:500 (normal)
parvalbumin	Rabbit /polyclonal	Swant /PV 25	1:400 (normal)
PKCγ	Rabbit /polyclonal	Santa Cruz /sc-211	1:2,000
PKCγ	Mouse /monoclonal	Luiten PG. (Groningen, The Netherlands)	1:100
secretagogin	Rabbit /polyclonal	Atlas Antibodies AB /HPA006641	1:2,000
SST2A	Rabbit monoclonal	Schulz S. (Magdeburg, Germany)/UMB-1	1:100
synaptophysin	Mouse /monoclonal	Millipore /MAB5258	1:1,000 (normal)
VGLUT1	Goat /polyclonal	Watanabe M. (Sapporo, Japan)	1:300 (normal)
VGLUT2	Guinea pig /polyclonal	Watanabe M. (Sapporo, Japan)	1:500 (normal)
TH	Rabbit /polyclonal	Goldstein M. (New York)	1:4,000

3.6 TISSUES AND IMMUNOHISTOCHEMISTRY

Mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p. administration; APL) and perfused with 4% paraformaldehyde (PFA) as previously described (Shi et al., 2012). The lumbar (L4 and L5) DRGs, lumbar spinal cord, sciatic nerve, and the hind leg paws were dissected out and postfixed in 4% PFA for 90 min on ice, and rinsed with 10% (wt/vol) sucrose in 0.10 M phosphate buffer containing 0.01% sodium azide (Merck) and 0.02% bacitracin (Sigma). Tissues were kept in 10% sucrose for 2 d at 4°C. Human DRGs and spinal cord tissues were immersion fixed with 4% PFA on ice for 2 h, followed by rinsing with 10% sucrose for 2 d as described above for animal tissue.

All trimmed tissues from different groups were arranged together and embedded with optimal cutting temperature compound (HistoLab AB), frozen with liquid carbon dioxide, and sectioned on a cryostat (Thermo) for DRGs (12 μm thick) and spinal cord (20 μm thick). The sections were mounted onto Superfrost Plus microscope slides (VWR International) with heating and stored at −20°C. DRG and spinal cord sections were dried at RT for 30 min and then incubated with different primary antibodies (listed in Table 1) diluted in PBS containing 0.2% (wt/vol) BSA (Sigma) and 0.3% Triton X-100 (Sigma) in a humid chamber for 48 h in the cold room. Immunoreactivities were visualized using the TSA Plus kit (PerkinElmer) as previously described (Shi et al., 2012). For double labeling, slides were selected after TSA labeling, rinsed in PBS for 20 min and then incubated with primary antibodies for 48 ~ 72 h at 4°C. The slides were first washed in PBS for 30 min and then incubated with Cy3- (Cy5-) conjugated secondary antibody IgG (1:100 ~ 300; Jackson Immunoresearch Laboratories) at RT for 2 h; after rinsing, the sections were mounted with 1,4-Diazabicyclo[2.2.2]octane (DABCO, Sigma) medium. For quantification, the counterstaining with PI or DAPI (Sigma) was in some cases added before mounting the cover glass.

It should be noted that the immunostaining signals varied considerably with different antibodies depending on the degree of fixation. For example, the staining of spinal cord from two different animals with ATF3, Iba1 and BDNF 2 weeks after SNI injury varied considerably: mouse #1 was well perfused, mouse #2 less well. The difference between these two perfusions is probably due to a differential distribution of the fixative: in mouse #2 (bad fixation) we observed that the fixative via the lung circulation penetrated though the nostrils /mouth, that is less fixative reached the spinal cord than in mouse #1. Although ATF3 and BDNF antibodies produced a good staining in mouse #1, the best Iba1 staining was seen after ‘bad’ fixation (mouse #2) (Figure 4*A-F*). Similarly, the NF200 antibody shows non-specific

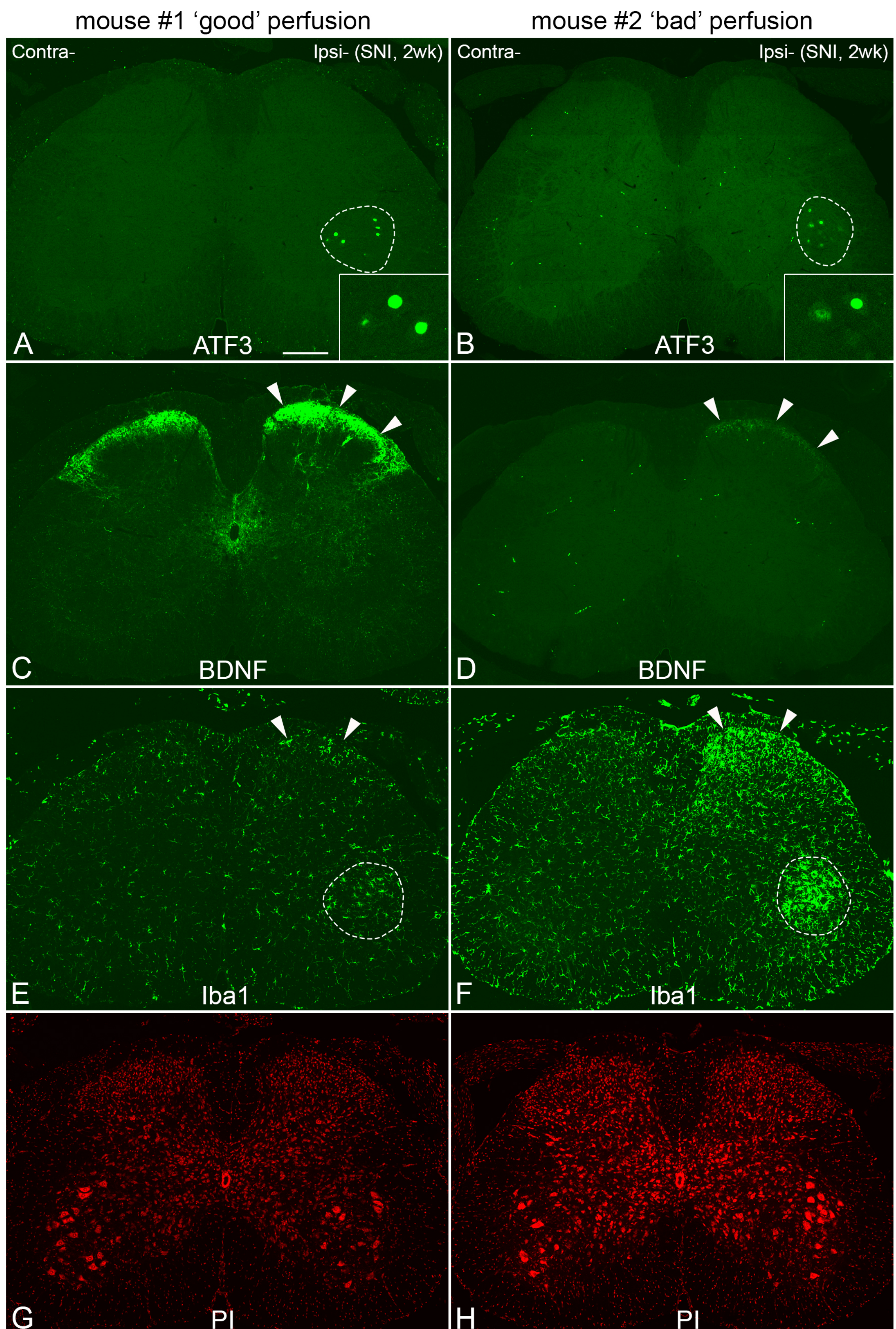

Figure 4 Differential staining patterns of ATF3, BDNF and Iba1 in the spinal cord after 2 wk of SNI injury due to different qualities of fixation. In the well-perfused mouse (*A,C,E,G*), ATF3 induction in the nucleus of injured motor neurons can clearly be seen (*A*), and the accumulation of BDNF-LI in the spinal dorsal horn is also distinctly shown ipsilaterally (*C*). However, the expected activation of

microglia visualized with Iba1 antibody is not obvious (*E*). In the less well-perfused mouse (*B,D,F*) the reversed situation is encountered. Both ATF3 (*B, cf. A*) and BDNF (*D, cf. C*) antisera show a very weak signal. In contrast, Iba1 staining of microglia is strong in both dorsal and ventral horns (*F, cf. E*). The counterstaining with PI is also affected by fixation, whereby the less well-fixed spinal cord shows a stronger staining (*H, cf. A*). Scale bar: 200 µm in *A-H*.

staining in the satellite glial cells and neuronal membranes in well-fixed DRGs, but gives apparently excellent results after less successful fixation. Therefore, it is important to individually analyze each antibody with regard to sensitivity to fixation. In this way more reliable results may be obtained when, for example, studying the experimental effects of peripheral nerve injury on the expression of a specific target like activation of Iba1 expression as shown in Figure 4*E* and *F*.

3.7 IN SITU HYBRIDIZATION AND IMMUNOHISTOCHEMISTRY

In situ hybridization for *Necab1* and *Necab2* in spinal cord with radioactive ^{35}S-labeled probes was performed as described with minor modifications (Le Maitre et al., 2013).

For combined in situ hybridization and immunohistochemistry, spinal cord cryosections (20 µm) prepared as mentioned above for immunohistochemistry were processed, and hybridizations were performed as previously described with minor modifications (Peng et al., 2012). Digoxigenin-labeled *Necab2* RNA probe was used for in situ and combined with PKCγ immunohistochemistry (Zhang et al., 2016).

3.8 MICROSCOPY, QUANTIFICATION AND IMAGE PROCESSING

Representative confocal images were acquired on an LSM700 confocal laser-scanning microscope (Zeiss). Emission spectra for each dye were used as follows: DAPI (<480 nm), FITC/Alexa Fluro488/Cy2 (505–540 nm), Cy3/PI (560–610 nm), and Cy5 (>640 nm). For projection images, orthogonal z-stacks were acquired with a depth interval of 1 µm (water objective, 40×). Images were processed using ZEN2012 software (Zeiss). Multi-panel figures were assembled in Adobe Photoshop CS6 software (Adobe Systems).

For the quantification of neuron profiles (NPs) in DRGs, three to five sections were selected from different levels and stained for specific markers. Sections were tile-scanned with an LSM700 laser-scanning microscope equipped with a Plan-Apochromat M27 objective (20× and N.A. of 0.80) and quantified by using Adobe Photoshop CS6 or ImageJ v.1.46 (National Institutes of Health) software. The cross-sectional area and intensity (mean gray value) were also collected using ImageJ. Size distribution was performed according to

the criteria: small (<300 μm^2), medium (300-700 μm^2) and large (>700 μm^2) described by Scherrer et al. (Scherrer et al., 2010). For the intensity analysis in spinal cord, sections were tile-scanned with an LSM700 laser-scanning microscope equipped with an EC Plan-Neofluar objective with a magnification of 10× and N.A. of 0.30. The intensity (mean gray value) from different spinal dorsal horn layers was collected with ImageJ.

3.9 CLARITY VOLUME IMAGING

This procedure was carried out in Prof. Deisseroth laboratory at Stanford University as described previously (Zhang et al., 2014).

3.10 IDISCO+ VOLUME IMAGING

iDISCO+ volume immunostaining and clearing process were performed as described earlier (Renier et al., 2016). Briefly, spinal cord blocks were washed in 0.01 M PBS 3 times in 5 ml Eppendorf tubes and then were dehydrated with methanol/water series 1 h each. The samples were bleached with 5% hydrogen peroxide in 100% methanol overnight at 4°C. Then, they were rehydrated, incubated in permeabilization solution for 2 d and kept in blocking solution for 2 d, both at 37°C (0.2% Triton-X100 / 20% DMSO / 0.3 M glycine in 0.01 M PBS + 0.02% sodium azide, 0.2% Triton-X100 / 10% DMSO / 6% normal donkey serum in 0.01 M PBS + 0.02% sodium azide, respectively). The samples were then incubated with primary antibody [NECAB2, rabbit polyclonal (HPA014144), 1:200] solution for 4 d at 37°C (antibody diluent: 0.2% Tween-20 / 10 μg/ml heparin / 5% DMSO / 3% normal donkey serum in 0.01 M PBS + 0.02% sodium azide). After extensive washing, the blocks were incubated in secondary antibody (1:200; goat anti-rabbit, conjugated with Alexa Fluor 647; Molecular Probes) solution (0.2% Tween-20 / 10 μg/ml heparin / 3% normal donkey serum in 0.01 M PBS + 0.02% sodium azide). Then, the blocks were dehydrated in methanol/water series, incubated in 66% dichloromethane / 33% methanol for 3 h, in 100% dichloromethane for 2 × 15 min, and then the blocks were removed to tubes filled with 100% dibenzyl ether and stored in this solution for long term.

For imaging, a light sheet microscope (Ultramicroscope II, Lavision Biotec) was used with the following parameters. Laser power: 72%; exposure time: 100ms; light sheet N.A.: 0.056; magnification: 3.2×; step size: 2 μm; 25-step dynamic focus with 'contrast filtered' merging algorithm. Altogether, 5.2 mm and 2 mm length of samples (medulla-cervical spinal cord and lumbar spinal cord, respectively) were acquired. The serials of images then were converted to IMS file and the 3D vision of acquisitions was reconstructed in the Imaris™ 8.4.0 software.

Light brightness/contrast adjustment, gamma correction and background subtraction were applied to obtain the best quality of 3D image.

4 RESULTS AND DISCUSSION

4.1 SECRETAGOGIN EXPRESSION IN DRGS AND SPINAL CORD FROM RODENTS AND HUMAN, AND ITS POTENTIAL FUNCTIONS IN PAIN SENSATION

Secretagogin was expressed in a subpopulation of mouse DRG neurons (~7%), mainly small- to medium-sized ones, and was co-localized with CGRP (a marker for peptidergic nociceptors, Figure 5*A*) but not with IB4 (a marker for non-peptidergic nociceptors, Figure 5*D*). In rat DRGs, secretagogin was expressed only in ~3% DRG neurons and only in small-sized ones. They co-localized neither with CGRP nor IB4 (Figure 5*B,E*). However, the NP population expressing secretagogin in human DRGs was larger, ~10%, and also belonged to peptidergic group, and also co-expressed CGRP (Figure 5*C,F*). Therefore, this neurochemical property of secretagogin population observed in mouse was conserved in human DRGs, but not in rat.

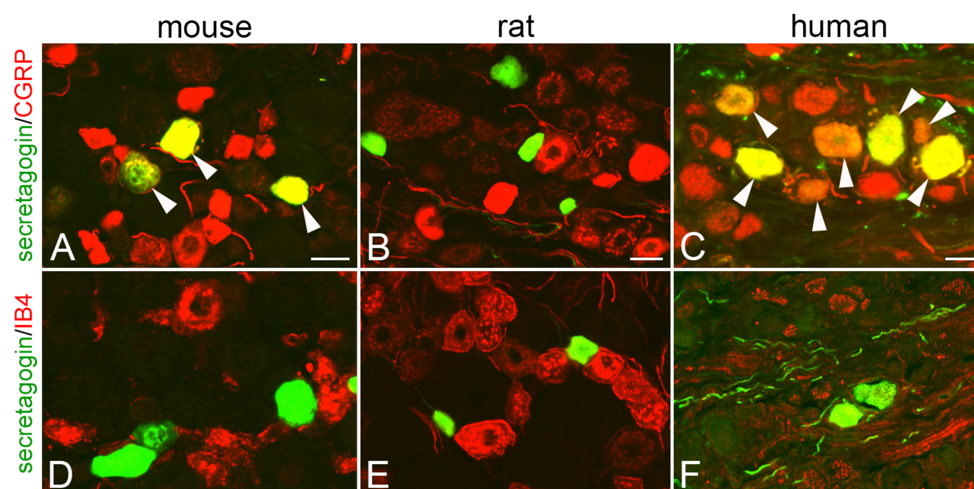

Figure 5 Co-localization of secretagogin with CGRP or IB4 in DRGs from mouse, rat and human. *A-C*) Double labeling of secretagogin and CGRP in DRGs. *D-F*) Double labeling of secretagogin and IB4 in DRGs. Arrowheads indicate co-localization of secretagogin and CGRP in mouse and human DRGs. Scale bars: 20 µm in *A,D* and *B,E*, 50 µm in *C,F* (modified from **Paper I**)

Secretagogin, synthesized in mouse DRG neurons, was transported both peripherally and centrally, and co-localized with CGRP. In **Paper I** we observed, in the mouse spinal cord, a specific layer in the central lamina II composed of neuronal cell bodies and processes stained with the secretagogin antibody and overlapping with IB4. Secretagogin-LI was also found in fibers in lamina I, which overlapped with CGRP and had been anterogradely transported from the cell bodies in the DRG. The secretagogin-LI in lamina I disappeared after dorsal root rhizotomy, in parallel with CGRP. However, the dorsal root rhizotomy did not affect the secretagogin staining in lamina II. Seretagogin was also expressed in motor neurons in the

spinal ventral horn. Axotomy did apparently not affect the expression of secretagogin in DRGs or spinal cord.

More recently we received a *secretagogin* KO mouse strain that we had ordered from the UCDAVIS Knockout Mouse Project (KOMP) Repository. We thus now have had the opportunity to validate the secretagogin antibody specificity with KO tissues and to re-examine the secretagogin staining at the spinal level. The results showed that secretagogin staining in WT DRGs, its central projections in lamina I and in motor neurons disappeared in KO tissue (Figure 6*A-D*), but that the secretagogin-LI in cell bodies and processes in the central part of lamina II remained, as indicated with arrowheads in Fig. 7. Therefore, the 'true' expression of secretagogin is limited to a small subpopulation of peptidergic neurons in DRGs, which projects to lamina I of the lumbar spinal dorsal horn, and in motor neurons.

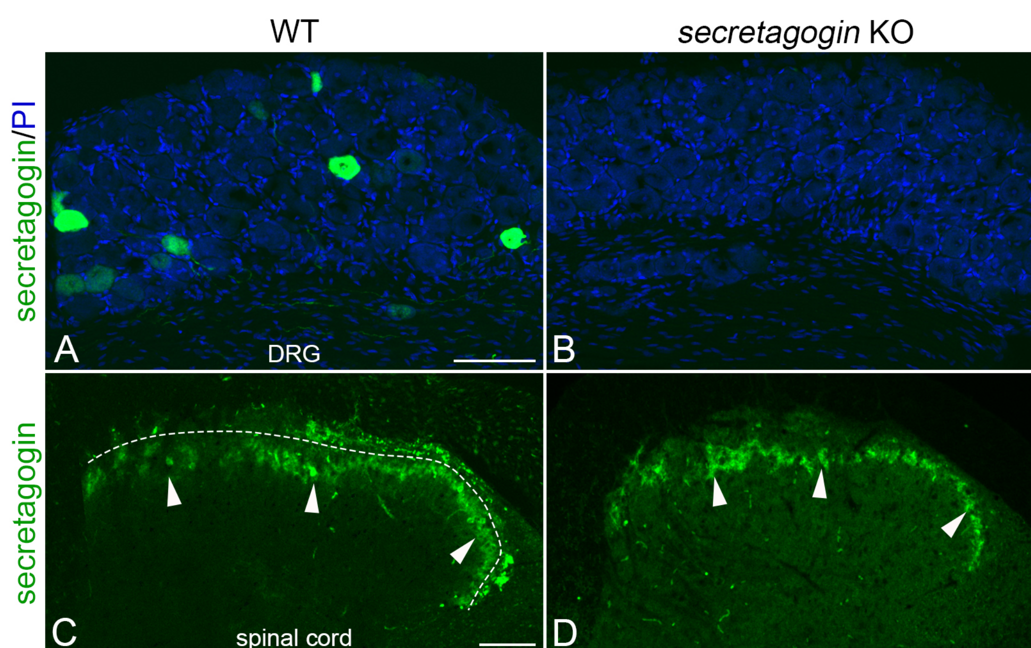

Figure 6 Validation of secretagogin antibody in DRGs and spinal cord with *secretagogin* KO tissues. *A,B*) Secretagogin staining in DRGs from WT and KO mice, counterstained with PI. *C,D*) Secretagogin staining in the spinal dorsal horn from both WT and KO mice. Arrowheads indicate the non-specific labeling of secretagogin in lamina II. The dashed line in (*C*) separates the central projections of secretagogin from DRGs in lamina I and the non-specific labeling in lamina II. Scale bars: 100 µm in *A,B* and 100 µm in *C,D* (modified from **Paper IV**)

We have shown that secretagogin is co-localized with CGRP, a 37-amino acid peptide, which interacts with the co-localized and likely co-released neuropeptide substance P (Wiesenfeld-Hallin et al., 1984), and is modulated by peripheral nerve injury and inflammation (Kuraishi et al., 1989; Verge et al., 1995; Woolf and Wiesenfeld-Hallin, 1986;

Zhang et al., 2001). The availablilty of *secretagogin* KO mouse also made it possible to explore a possible functional role of secretagogin in the DRG system (**Paper IV**). We thus tested such mice and wild types in the SNI model and after peripheral inflammation. The *secretagogin* KO mice developed tactile and cold allodynia as well as mechanical hypersensitivity in the SNI model (Figure 7*A-C*) and tactile allodynia for inflammatory pain after carrageenan (Figure 7D) to the same extent as seen in WT mice. Thus, this CaBP does likely not modulate carrageenan-induced inflammatory- or SNI-induced neuropathic pain-like behaviors.

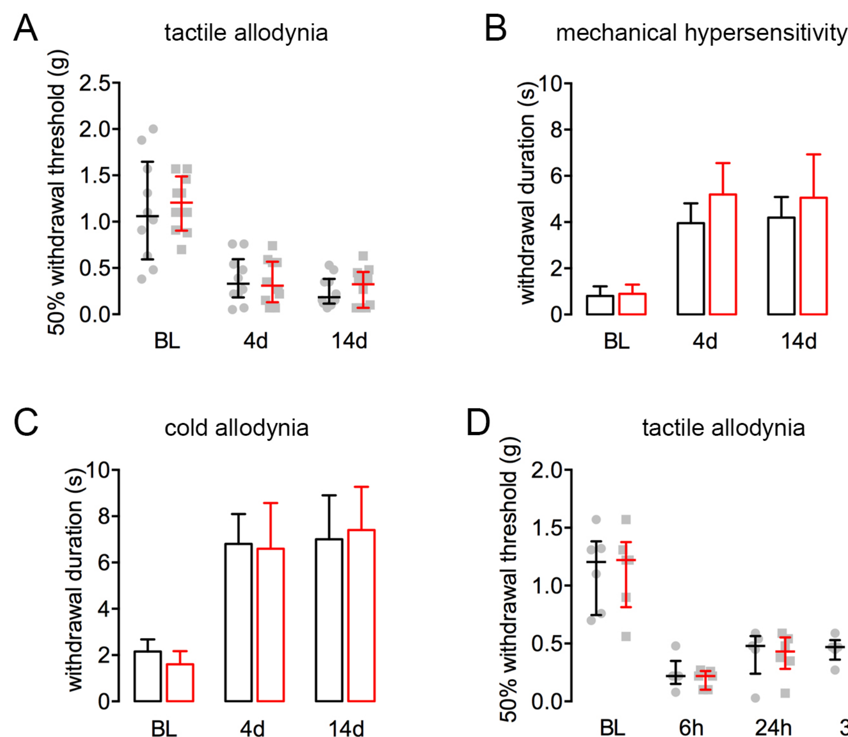

Figure 7 Pain-like behaviors comparing WT and *secretagogin* KO mice in the SNI and inflammation models. *A-C*) Pain-like behaviors developed similarly in WT and KO mice after SNI injury. Tactile allodynia was performed with von Frey filaments test, mechanical hypersensitivity was tested with pinprik and the cold allodynia was induced with acetone stimulus. *D*) Tactile allodynia was developed in both WT and KO mice after peripheral carrageenan stimulation. Black lines stand for WT mice, whereas red lines stand for KO mice. (modified from **Paper IV**)

CGRP plays an important role in neurogenic inflammation (Kilo et al., 1997) and facilitates central sensitization, possibly through the inhibition of SP degradation in the spinal dorsal horn (Le Greves et al., 1985; Safieh-Garabedian et al., 1995). Here we did not observe a difference of pain-like behaviors between WT and KO mice in the carrageenan model, which however does not have the neurogenic inflammation component (Louis et al., 1989). It would be interesting to study the effect of secretagogin in the complete Freund's adjuvant-

induced arthritis model in future work, a model where CGRP is up-regulated and which includes neurogenic inflammation (Kuraishi et al., 1989).

Our group has also analysed secretagogin in the hypothalamus, with special focus on neurons expressing corticotrophin-releasing hormone (CRH) in the paraventricular nucleus of hypothalamus projecting to the median eminence (Romanov et al., 2015). This is the final common pathway in the brain for the control of the hypothalamic-pituitary-adrenal gland (HPA axis), that is the stress axis (Aguilera, 1994; Vale et al., 1983). In Romanov et al. (2015) evidence was presented that secretagogin modulated CRH release in the median eminence in response to acute stress, possibly through controlling vesicular trafficking and the CRH releasing machinery (Romanov et al., 2015). This suggests that secretagogin may be of importance in stress, one of the central bodily functions. Since secretagogin in DRG neurons is colocalized with another neuropeptide, CGRP, it would be interesting to know if this CaBP has a similar role in CGRP release.

4.2 EXPRESSION OF NEURONAL CALCIUM-BINDING PROTEINS 1/2 IN DRGS AND SPINAL CORD, AND EFFECTS OF NERVE INJURY

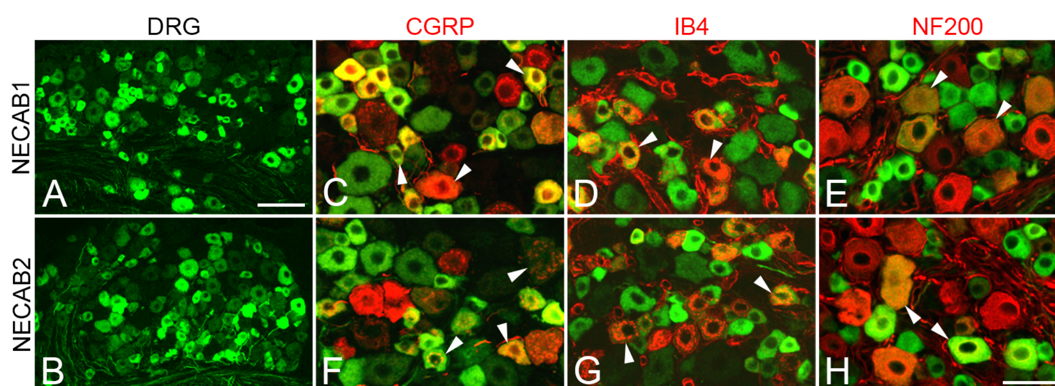

Figure 8 Expression of NECAB1 and NECAB2 in mouse DRGs. *A,B*) NECAB1 and NECAB2 are abundant in DRG neurons. *C-E*) Double labeling of NECAB1 with the peptidergic marker CGRP, the non-peptidergic marker IB4 or NF200, a marker for myelinated fibers in DRGs. *F-H*) Double labeling of NECAB2 with peptidergic marker CGRP, non-peptidergic marker IB4 or NF200 marker for myelinated fibers in DRGs. Arrowheads indicate co-localization, with different intensity, of the respective markers. Scale bars: 100 µm in *A,B*, 50 µm in *C-H* (modified from **Paper II**)

In **Paper II,** we reported that both NECAB1 and NECAB2 were abundant and expressed in ~65% and ~73% of mouse DRGs, respectively (Figure 8*A,B*). And they showed a similar distribution pattern, mainly in small- and medium-sized neurons (both peptidergic and non-peptidergic) but also in some large-sized neurons (Figure 8*C-H*). This contrasts to the distributions of the classic EF-hand CaBPs like parvalbumin (proprioceptor), calbindin-D28k,

calretinin in DRGs or secretagogin, which only labels a limited subpopulation of peptidergic neurons as described in Paper I (Arber et al., 2000; Carr et al., 1989; Celio, 1990; Ren et al., 1993; Shi et al., 2012; Usoskin et al., 2015). But note that we in **Paper IV** 'corrected' this percentage of NECAB2 using a new, specific antibody validated by *Necab2* KO tissue, which showed a much lower NPs of NECAB2 in DRGs. The staining of NECAB2 in spinal cord was correct as explained in detail in **Paper IV**.

In **Paper II**, we found a complementary distribution pattern in the spinal dorsal horn for NECAB1 and NECAB2, with only a narrow band of overlap in lamina IIi. NECAB1 was enriched in the superficial layers of the spinal cord, where the neuronal cell bodies and proximal dendrites were labeled (Figure 9*A-D*). NECAB1-IR neurons were in addition scattered throughout the deep layers, including the area surrounding the central canal and also motor neurons in the ventral horn were positive. A specific group of NECAB1-IR commissural interneurons in the medial spinal ventral horn (laminae VII/VIII) could be identified thanks to the use of CLARITY, a method that allows immunostaining of large samples, like the spinal cord, after making the tissue transparent (Chung et al., 2013).

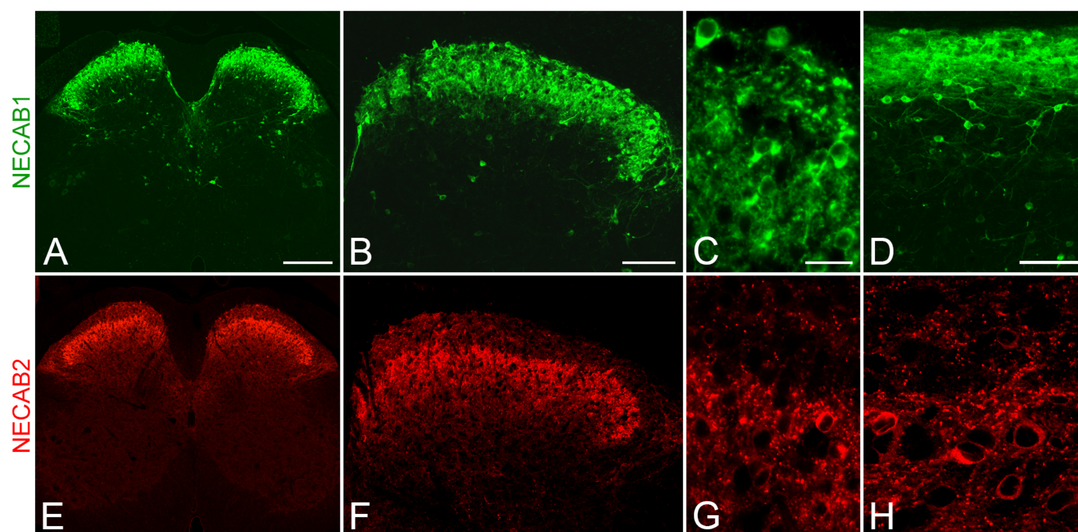

Figure 9 Expression of NECAB1 and NECAB2 in mouse spinal cord. *A-D*) Expression of NECAB1 in the spinal cord showing an overview in (*A*), the dorsal horn in (*B*), a high magnification of the superficial layers in (*C*) and a longitudinal view in (*D*). *E-H*) Expression of NECAB2 in the spinal cord from the similar levels as shown for NECAB1, except (*H*), which is a high magnification in the longitudinal direction. Scale bars: 200 μm in *A,E*, 100 μm in *B,F*, 20 μm in *C,G,H* and 100 μm in *D* (modified from **Paper II**)

NECAB2-LI was widely distributed across the spinal cord, with the highest intensity in lamina IIi, extending to the deep layers (III and IV), with a much weaker staining in the ventral horn (Figure 9*E*). NECAB2 was characterized by a punctate staining, even the

neuronal cell body staining was often punctate, extending from the cytoplasm into the proximal dendrites (Figure 9*F-H*). The punctate staining of NECAB2 was often co-localized with VGLUT2 and synaptophysin (Figure 10). Although both NECAB1 and NECAB2 were mainly found in excitatory interneurons (VGLUT2 positive) in the dorsal horn, the NECAB2-IR interneurons in lamina IIi overlapped with PKCγ excitatory interneurons, but this was not the case for NECAB1.

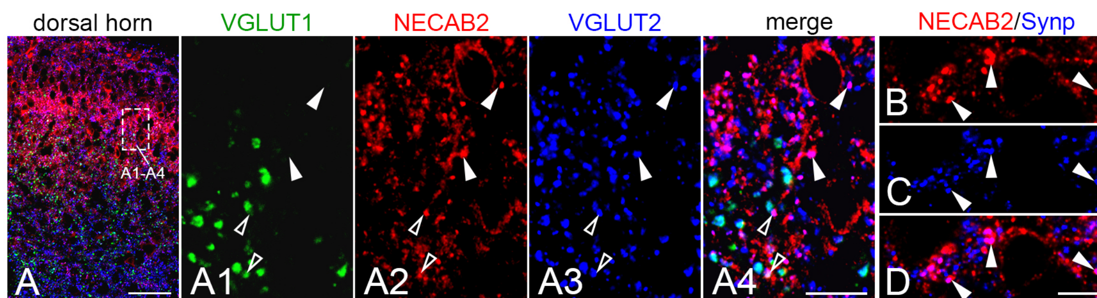

Figure 10 Co-existence of NECAB2 with VLGUT1/VGLUT2 or synaptophysin in the dorsal horn. *A*) Triple labeling of NECAB2 with VGLUT1 and VGLUT2. *B-D*) Double labeling of NECAB2 and synaptophysin (Synp). Arrowheads indicate co-localization of NECAB2 with VGLUT2 but not VGLUT1 or synaptophysin, whereas the open arrowheads indicate the co-localization of NECAB2 with VGLUT1 and VLGUT2. Scale bars: 50 µm in *A*, 10 µm in *A1-A4* and 5 µm in *B-D* (modified from **Paper II**)

Peripheral never injury (axotomy) down-regulated NECAB2 expression in DRGs at both mRNA and protein levels, but for NECAB1 NPs only a very small drop was observed. We considered the modulation of the NECAB2 in DRGs after nerve injury to represent an adaptive action to reduce the glutamatergic signaling, shifting the balance towards inhibition in the spinal pain circuits. Other studies have shown that modulation of the balance between excitation and inhibition in the spinal circuits could attenuate neuropathic pain (Braz et al., 2012; Foster et al., 2015). Potential effects of NECAB2 could potentially also have an effect on the glutamatergic neurotransmission by its expression in PKCγ excitatory interneurons, since this molecule has been shown to be involved in the development of neuropathic pain (Malmberg et al., 1997).

4.3 THE EXCITATORY PROPERTY OF SPINAL NECAB2-IR INTERNEURONS IS CONSERVED IN RODENTS AND HUMAN

In **Paper III**, we compared the distribution of NECAB1 and NECAB2 in spinal cord from mouse, rat and human at both mRNA and protein levels. For *Necab1* mRNA, the distribution pattern was similar from superficial layers to the area surrounding central canal in mouse and rat, but expression in motor neurons was only found in mouse. This overlap was true also at

the protein level; even for the commissural interneurons demonstrated in mouse in the medial ventral spinal cord, which also showed up in rat. Compared to rodents, in human the *Necab1* RNA probe only gave a signal in a few neurons scattered in the deep layers of the spinal dorsal horn and in some neurons surrounding the central canal. The immunostaining of NECAB1 in the white matter of the human spinal cord was apparently surrounding axons, that is likely expressed in oligodendrocytes. Furthermore, mouse and rat show distinct differences with regard to the neurochemical heterogeneity of NECAB1^{+} neurons in the superficial dorsal horn.

The *Necab2* mRNA was observed in neurons throughout the gray matter but not in motor neurons of mouse spinal cord, with most intense signal (and highest neuronal density) for interneurons in lamina IIi, and extending into lamina III. The distribution of *Necab2* mRNA was more homogeneous in the rat. The human spinal cord showed a similar distribution of *Necab2* mRNA in the dorsal horn, although the signal was much weaker compared to mouse. In the area around the central canal an interesting species difference was observed with regard to the ependymal cells: regular pseudostratified, ciliated epithelium was seen in rodents, whereas human ependymal cells were not well 'organized' and occupied the lumen. The protein expression of NECAB2 was similar, especially when comparing the high expression level in lamina IIi. The punctate staining character was conserved, although some differences appeared, such as the NECAB2-LI in the human spinal ventral horn and NECAB2-IR neurons in the ependymal cells of the central canal. The excitatory character of NECAB2-IR interneurons was conserved regarding the co-localization with PKCγ and the co-localization with VGLUT2 and synaptophysin.

Our results revealed that the association of NECAB2 to excitatory neuronal circuits in the spinal cord was evolutionarily conserved across the mammalian species investigated so far. In contrast, NECAB1 expression was more heterogeneous. Thus, our study suggests that the phenotypic segregation of NECAB1 and -2 to respective excitatory and inhibitory spinal systems can underpin functional modalities in determining the fidelity of synaptic neurotransmission and neuronal responsiveness, and might bear translational relevance to humans.

4.4 A ROLE FOR NECAB2 IN INFLAMMATORY PAIN

We early on initiated the generation of a *Necab2* KO mouse, which recently has become available and now could be used in the experiments reported in **Paper IV**. The NECAB2 antibody (HPA013998) used in our previous study (**Paper II**) (Zhang et al., 2014) still

showed staining in DRGs from *Necab2* KO mice, however not in the spinal cord (Figure 11*A-D*). The new NECAB2 antibody (HPA014144), recognizing the NECAB2 C-terminal and used throughout **Paper IV**, did not show any off target staining in DRGs or spinal cord (Figure 11*E-H*). However, both antibodies showed specific bands for NECAB2 with western blotting. The difference in staining in DRGs could be caused either by a truncated NECAB2 in *Necab2* KO mice encoded by its exons 1-3 (the *lacZ* cassette was inserted between exon 3 and 4 of the *Necab2* gene), or by cross-reactivity to NECAB1, in view of the high similarity between the human NECAB2 PrEST used for immunization and mouse NECAB1 (68%). We prefer the latter alternative, since the HPA013998 NECAB2 antibody did not show any NECAB2 staining in the spinal cord from *Necab2* KO mice (Figure 11*D*).

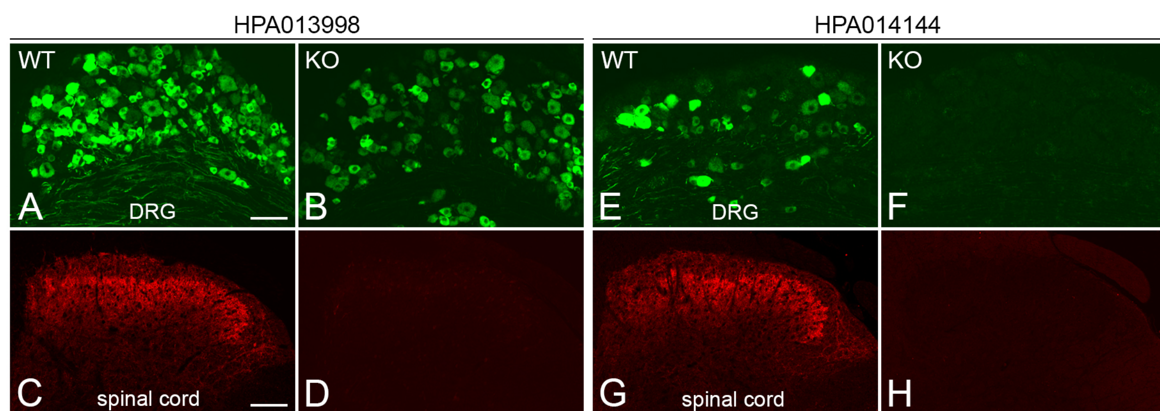

Figure 11 Validation of NECAB2 antibodies with tissues from *Necab2* KO mice. *A-D*) Staining of NECAB2 antibody (HPA013998, against the N-terminal of NECAB2) in DRGs and spinal cord from both WT and KO mice. *E-H*) Staining of NECAB2 antibody (HPA014144, against of the C-terminal of NECAB2) in DRGs and spinal cord from both WT and KO mice. Scale bars: 100 μm in *A,B,E* and *F*, 200 μm in *C,D,G* and *H* (modified from **Paper IV**)

The decrease of NECAB2$^+$ NPs in DRGs after peripheral nerve injury (axotomy) was also confirmed with the 'new' HPA014144 antibody (from 34% to 13%), which is in agreement with the decrease in NECAB2$^+$ NPs seen after staining with HPA013998 antibody in our previous study (from 74% to 54%, **Paper II**) (Zhang et al., 2014). Taken together, it is our view that the 'new' NECAB2 antibody gives the correct staining in DRGs, as well as in the spinal cord. These results also show that an antibody may show correct staining in the spinal cord but not in DRGs of the same mouse (or vise versa with regard to the secretagogin antibody). These results underline the importance of testing antibody specificity for each tissue/organ/system analysed; and that KO animals represent an important tool for establishing antibody specificity (Uhlen et al., 2016).

The HPA014144 NECAB2 antibody defined a very specific population of sensory neurons in DRGs. It labeled ~30% of DRG neurons, mainly small- and medium-sized neurons, which were neither peptidergic nor nonpeptidergic (Figure 12*A,B*). Instead, the NECAB2-IR neurons covered both the C-low threshold mechanoreceptor (tyrosine hydroxylase (TH)$^{+}$, the TH population; Figure 12*C*) and Aδ D-hair low threshold mechanoreceptors (tyrosine receptor kinase B, the TrkB population; Figure 12*D*). The small-sized NECAB2 DRG neurons were VGLUT2^{+}, but the medium-sized ones were neither VGLUT2^{+} nor VGLUT1^{+} (Figure 12*E*). A direct comparison between the NECAB2 population (defined by immunohistochemistry) with the DRG single cell RNA-sequencing by Usoskin et al. (Usoskin et al., 2015), is summarized in Figure 12*F* and *G*, and shows a high degree of similarity.

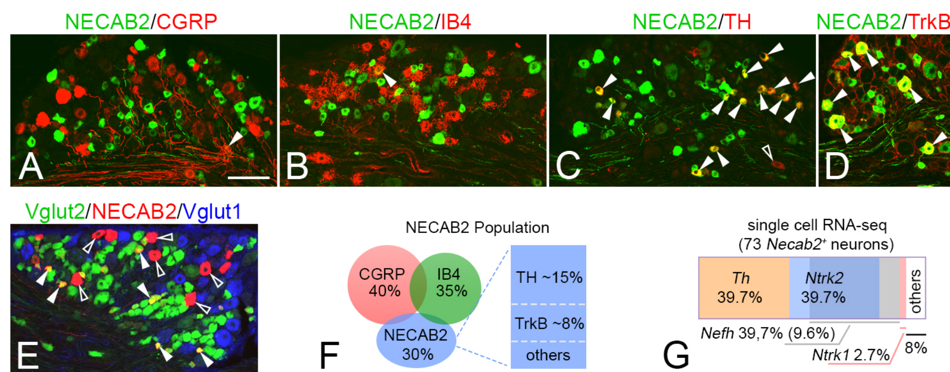

Figure 12 *A,B)* Co-localization of NECAB2 with the peptidergic marker CGRP (*A*) or the non-peptidergic marker IB4 (*B*), both for nociceptors. *C,D)* Co-localization of NECAB2 with tyrosine hydroxylase (TH, C-Low threshold mechanoreceptor, C-LTMR, *C*) and tyrosine receptor kinase B (TrkB, Aδ D-hair LTMR, *D*). *E)* Co-localization of NECAB2 with VGLUT1 and VGLUT2 (*Vglut2::EGFP* reporter mice). *F)* Summarized distribution and composition of the NECAB2 population in DRGs. *G)* Data analysis of *Necab2* population components in DRGs at mRNA level from single cell RNA-sequencing (Usoskin et al., 2015; open resource). Arrowheads indicate co-localization, whereas the open arrowheads indicate lack of co-localization. Scale bar: 100 µm in *A-E* (Modified from **Paper IV**)

Acute pain sensation (noxious, mechanical stimulus) was intact in *Necab2* KO mice with no sex difference. After SNI, the *Necab2* KO mice developed tactile allodynia, mechanical hypersensitivity and cold allodynia to the same degree as the WT mice (Figure 13*A-C*). For λ carrageenan induced inflammation, there was no difference in the amount or time course of the edema, comparing *Necab2* KO and WT mice (Figure 13*D*). Analysis of tactile allodynia showed that acute inflammatory pain had a similar time course in *Necab2* KO and WT mice (Figure 13*E*). However, the inflammatory pain in *Necab2* KO mice was attenuated already

after 24 h, reaching basal threshold at d 3, whereas the inflammatory pain (tactile allodynia) was maintained in WT mice until d 7, that is as long as recordings were made (Figure 13*E-G*).

Increased expression of BDNF in DRGs is an accepted marker for peripheral inflammation (Cho et al., 1997). This growth factor is expressed in rat peptidergic nociceptors, stored in large dense core vesicles and released in spinal superficial layers (Michael et al., 1997). Peripheral inflammation (formalin and carrageenan) up-regulates BDNF expression in DRGs (both C- and A-fibers) and increases its release in the spinal cord, which leads to central sensitization by binding to TrkB receptors and subsequent NMDA receptor and c-Fos activation (Garraway et al., 2003; Salio et al., 2005; Thompson et al., 1999). Blocking BDNF in the spinal cord could attenuate tactile-induced progressive inflammatory pain hypersensitivity and heat hypersensitivity, but not acute tactile allodynia (Groth and Aanonsen, 2002; Kerr et al., 1999; Mannion et al., 1999).

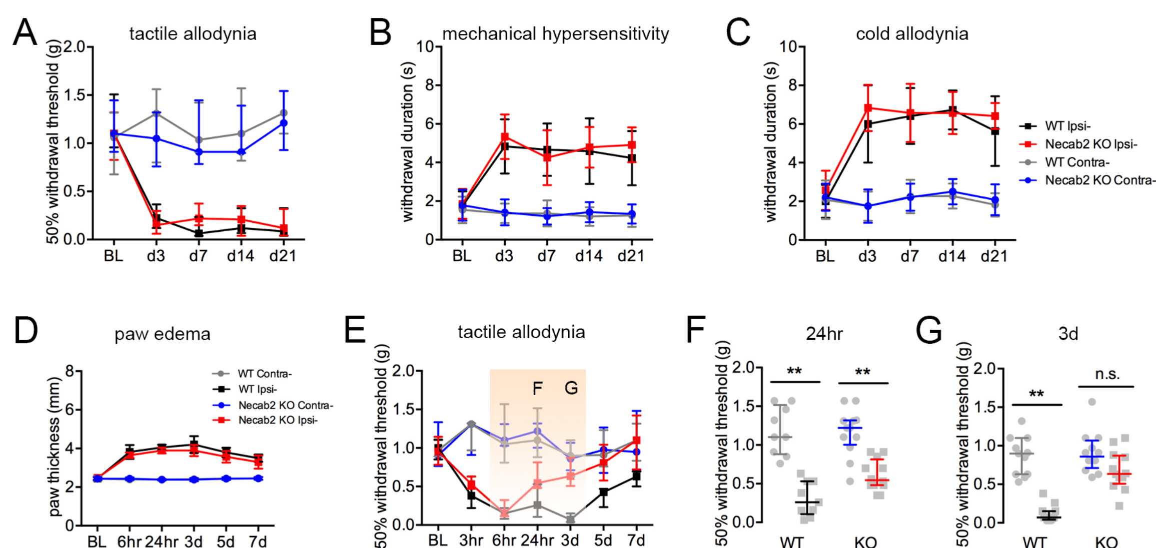

Figure 13 Involvement of NECAB2 in inflammatory but not neuropathic pain. *A-C*) The *Necab2* KO mice developed tactile allodynia (von Frey filaments, innocuous stimulus), mechanical hyper-sensitivity (noxious mechanical stimulus, pinprick) and cold allodynia (acetone stimulus) in SNI model as WT mice. *D*) Time course of paw edema, the typical symptom of inflammation, in the hind paw from WT and *Necab2* KO mice after intra-plantar injection of λ carrageenan. *E-G)* Development of tactile allodynia in WT and KO mice after peripheral carrageenan stimulus. * $p < 0.05$, ** $p < 0.01$. (Modified from **Paper IV**)

In **Paper IV**, we showed an increase of BDNF^{+} NPs in WT DRGs after peripheral inflammation. This increase in percentage was not seen in *Necab2* KO DRGs, but their was a significant increase in BDNF-LI intensity of individual cell bodies (small and medium-sized),

suggesting that also in KO mice there is increased signaling through BDNF. However, the analysis of adjacent segments (L2/3) in these mice shows no increase in BDNF-LI. This suggests that, in general, BDNF signaling at the spinal level is attenuated in KO mice, which could contribute to a more rapid recovery from inflammatory pain. Interestingly, peripheral noxious stimulation (heat) of foot also triggers c-Fos activation in the spinal cord beyond L4 and L5, as shown both in transverse sections (Menetrey et al., 1989) and with the iDISCO volume imaging method (Renier et al., 2014). However, how the loss of NECAB2 leads to attenuated BDNF modulation after inflammation is still unclear. The TrkB population in DRGs could be a potential target, since ~11% BDNF-IR neurons are TrkB^{+} (which is covered by NECAB2) in mouse DRGs (Salio and Ferrini, 2016; Salio et al., 2005).

5 CONCLULDING REMARKS

Great progress in the understanding of neuropathic and inflammatory pain, both at cellular and molecular levels, has been achieved since Melzack and Wall proposed the "Gate Control of Pain" theory in 1965. Much attention has been paid to molecule families like neuropeptides, receptors, ion channels, synaptic vesicle proteins and cytokines. However, the study of a possible role of EF-hand CaBPs (one of the largest protein families encoded by the genome) in the primary somatosensory system has been 'ignored'. This thesis focused on three EF-hand CaBPs: secretagogin, NECAB1 and NECAB2 in DRGs and spinal cord in three species, mouse, rat and human, and possible roles in pathophysiological pain.

Secretagogin labeled a small subpopulation of peptidergic neurons in DRGs and was anterogradely transported, together with CGRP, both to the periphery and centrally to the spinal cord. Knockout of secretagogin did not affect the development of pain hypersensitivity in peripheral nerve injury or carrageenan induced inflammation models. However, we believe that a role of secretagogin should be explored; it could, for example, modulate the release of CGRP, since we have shown such a role in the control of release of another peptide: the stress hormone CRH in the hypothalamus.

NECAB1 was expressed in a large population of DRGs neurons, contrasting the classic CaBPs. NECAB2, the other family member, specifically labeled a population covering both the C-LTMRs and Aδ D-hair LTMRs. Knockout of *Necab2* attenuated inflammatory pain, possibly through the modulation of BDNF in DRG neurons and interaction with its receptor TrkB to regulate spinal glutamatergic neurotransmission.

Taken together, our studies have initiated the exploration of three further members of the large family of CaBPs, and have defined, in three species, their cellular distribution at the spinal level and certain of their properties in some pain models. We believe/hope that this may be followed by further projects aiming a deeper understanding of the role of CaBPs in sensory signaling.

6 ACKNOWLEDGEMENTS

I would like to thank all the people who have helped me and contributed to the completion of this thesis in various ways:

Tibor – Thank you for your scientific guidance, patience and constant support. I am truly grateful that you were always encouraging me to broad my knowledge, learn new techniques, allowing me to grow up as an independent scientist. And you never leave me behind.

Tomas – Thank you for spending enormous time to teach me with patience and let me grow up under your wings in the past six years. And thank you for trusting me and supporting me to participate in so many interesting projects with different background.

Jan – Thanks for great support with different techniques, antibodies, and my favorite micro rongeurs.

Robert Schnell, my mentor, thanks for asking when we met in the campus from time to time.
Kaj, thanks for supporting me to start my study in Karolinska and your efforts to help me, and fika for Friday meetings.
Sten, thanks for introducing me to the pain research field and professional training for working with tiny spinal ganglia.
Ole, for nice discussions when you passed by the corridor and thanks for generous supports for different reporter mice.
Patrik, thanks for your comments for the half time review.
Niccolò, for collaboration with the exciting project.
Krister, very happy to run the influenza project with you and inspiring discussions.
Gunnar, thanks for the inputs about the anatomical structure of spinal cord.
Masahiko Watanabe, thanks for generous supplying of so many reliable antibodies.

THö group past and present:
Csaba, thanks for your help to show me how to dip the in situ slides and iDISCO volume imaging, and interesting talks about science and philosophy. **Swapnali**, for the contribution of so many amazing in situ results and helpful tips for thesis. **Joanne**, for your efforts to keep the lab in order. **Blanca**, thanks for your contribution to keep the lab well organized. **Karin**, thanks for your help with the administrative documents and other different letters in Swedish, which makes our life much easier in Stockholm. **Nick**, for helping with the atlas antibodies and the *Necab2* KO mice together with **Tony**. **David**, for mooncakes and cookies from China. **Ida**, for being such a pleasant person. **Kang**, for sharing experience with study and

research. **Tommy**, thanks for preparing the general solutions/buffers in the lab. **Ulla**, for taking care of the lab when Blanca was on vacation. **Pablo**, thanks for your excellent work and thesis book which helped a lot for my thesis work.

THa group members in Vienna:
Roman, thanks for sharing your experience, having coffee together downstairs in MolNeuro and the joke about the PhD student and monkey which I can understand better now ☺. **Daniela**, still remember we did the presentations for PhD registration together four years ago and now finish the studies at the same time. **Vale**, **Kasia**, **Erik**, **Edit**, **Fatima**, **Harald**, **Jonas**, **Johannes**, **Alessa**, **Patrick**, **Jan** (Igor's group), **Romana** (Prof. Klausberger's group), **Ingrid** and **Orsi**: you are all great persons that I have met and helped me a lot when I was in Vienna. **Igor**, thanks for recommending coffee and cakes.

Sandra, for accepting us to Dept. of Neuroscience from MBB.

Slav and **Dimitrii**, good to have you guys to populate our corridor and bring great coffee machine. **Nina**, for encouraging words and recommendations for tooth treatment. **Håkan,** for sharing your experience with telephone operators. **Natalie,** for helping with mice. **Peter,** for printing posters, **Lotta**, for the introduction course for the confocal, **Xinming Wang**, **Yang Xuan**, **Jianren Song**, **Yu Qian**, **Shanzheng Yang**, **Songbai Zhang**, **Dawei Zhang** for helpful chats and discussions.

Alessandra, for helping with administrative documents and booking flight tickets.

Lili, for nice discussions about science and career.

Changgeng, for binging me so close to miRNA and pain, and extensive discussions.

Jilai Liu, very lucky to start the bachelor project with you! Thanks for your patience, sharing knowledge and attitudes towards science.

Thanks for the continuous support from my parents and parents-in-law.

Jie, thanks for supporting me all the way, especially the hard times in the dark winters of Stockholm.

7 REFERENCES

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

Abraira, V.E., Kuehn, E.D., Chirila, A.M., Springel, M.W., Toliver, A.A., Zimmerman, A.L., Orefice, L.L., Boyle, K.A., Bai, L., Song, B.J.*, et al.* (2017). The Cellular and Synaptic Architecture of the Mechanosensory Dorsal Horn. Cell *168*, 295-310 e219.

Aguilera, G. (1994). Regulation of pituitary ACTH secretion during chronic stress. Front Neuroendocrinol *15*, 321-350.

Al-Khater, K.M., and Todd, A.J. (2009). Collateral projections of neurons in laminae I, III, and IV of rat spinal cord to thalamus, periaqueductal gray matter, and lateral parabrachial area. J Comp Neurol *515*, 629-646.

Alpar, A., Attems, J., Mulder, J., Hokfelt, T., and Harkany, T. (2012). The renaissance of Ca2+-binding proteins in the nervous system: secretagogin takes center stage. Cellular signalling *24*, 378-387.

An, W.F., Bowlby, M.R., Betty, M., Cao, J., Ling, H.P., Mendoza, G., Hinson, J.W., Mattsson, K.I., Strassle, B.W., Trimmer, J.S.*, et al.* (2000). Modulation of A-type potassium channels by a family of calcium sensors. Nature *403*, 553-556.

Antal, M., Freund, T.F., and Polgar, E. (1990). Calcium-binding proteins, parvalbumin- and calbindin-D 28k-immunoreactive neurons in the rat spinal cord and dorsal root ganglia: a light and electron microscopic study. The Journal of comparative neurology *295*, 467-484.

Antal, M., Polgar, E., Chalmers, J., Minson, J.B., Llewellyn-Smith, I., Heizmann, C.W., and Somogyi, P. (1991). Different populations of parvalbumin- and calbindin-D28k-immunoreactive neurons contain GABA and accumulate 3H-D-aspartate in the dorsal horn of the rat spinal cord. J Comp Neurol *314*, 114-124.

Arber, S., Ladle, D.R., Lin, J.H., Frank, E., and Jessell, T.M. (2000). ETS gene Er81 controls the formation of functional connections between group Ia sensory afferents and motor neurons. Cell *101*, 485-498.

Arcourt, A., Gorham, L., Dhandapani, R., Prato, V., Taberner, F.J., Wende, H., Gangadharan, V., Birchmeier, C., Heppenstall, P.A., and Lechner, S.G. (2017). Touch Receptor-Derived Sensory Information Alleviates Acute Pain Signaling and Fine-Tunes Nociceptive Reflex Coordination. Neuron *93*, 179-193.

Averill, S., Robson, L.G., Jeromin, A., and Priestley, J.V. (2004). Neuronal calcium sensor-1 is expressed by dorsal root ganglion cells, is axonally transported to central and peripheral terminals, and is concentrated at nodes. Neuroscience *123*, 419-427.

Baimbridge, K.G., Celio, M.R., and Rogers, J.H. (1992). Calcium-binding proteins in the nervous system. Trends Neurosci *15*, 303-308.

Baron, R. (2006). Mechanisms of disease: neuropathic pain--a clinical perspective. Nature clinical practice Neurology *2*, 95-106.

Bas, D.B., Su, J., Sandor, K., Agalave, N.M., Lundberg, J., Codeluppi, S., Baharpoor, A., Nandakumar, K.S., Holmdahl, R., and Svensson, C.I. (2012). Collagen antibody-induced

arthritis evokes persistent pain with spinal glial involvement and transient prostaglandin dependency. Arthritis Rheum *64*, 3886-3896.

Basbaum, A.I., Bautista, D.M., Scherrer, G., and Julius, D. (2009). Cellular and molecular mechanisms of pain. Cell *139*, 267-284.

Basbaum, A.I., and Fields, H.L. (1984). Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. Annu Rev Neurosci *7*, 309-338.

Basbaum, A.I., and Woolf, C.J. (1999). Pain. Current biology : CB *9*, R429-431.

Bauer, M.C., O'Connell, D.J., Maj, M., Wagner, L., Cahill, D.J., and Linse, S. (2011). Identification of a high-affinity network of secretagogin-binding proteins involved in vesicle secretion. Molecular bioSystems *7*, 2196-2204.

Bernier, G., Vukovich, W., Neidhardt, L., Herrmann, B.G., and Gruss, P. (2001). Isolation and characterization of a downstream target of Pax6 in the mammalian retinal primordium. Development *128*, 3987-3994.

Birkenkamp-Demtroder, K., Wagner, L., Brandt Sorensen, F., Bording Astrup, L., Gartner, W., Scherubl, H., Heine, B., Christiansen, P., and Orntoft, T.F. (2005). Secretagogin is a novel marker for neuroendocrine differentiation. Neuroendocrinology *82*, 121-138.

Braz, J.M., Sharif-Naeini, R., Vogt, D., Kriegstein, A., Alvarez-Buylla, A., Rubenstein, J.L., and Basbaum, A.I. (2012). Forebrain GABAergic neuron precursors integrate into adult spinal cord and reduce injury-induced neuropathic pain. Neuron *74*, 663-675.

Brumovsky, P., Villar, M.J., and Hokfelt, T. (2006). Tyrosine hydroxylase is expressed in a subpopulation of small dorsal root ganglion neurons in the adult mouse. Exp Neurol *200*, 153-165.

Brumovsky, P., Watanabe, M., and Hokfelt, T. (2007). Expression of the vesicular glutamate transporters-1 and -2 in adult mouse dorsal root ganglia and spinal cord and their regulation by nerve injury. Neuroscience *147*, 469-490.

Bunge, M.B., Bunge, R.P., Peterson, E.R., and Murray, M.R. (1967). A light and electron microscope study of long-term organized cultures of rat dorsal root ganglia. The Journal of cell biology *32*, 439-466.

Burgoyne, R.D. (2007). Neuronal calcium sensor proteins: generating diversity in neuronal Ca2+ signalling. Nature reviews Neuroscience *8*, 182-193.

Buxbaum, J.D., Choi, E.K., Luo, Y., Lilliehook, C., Crowley, A.C., Merriam, D.E., and Wasco, W. (1998). Calsenilin: a calcium-binding protein that interacts with the presenilins and regulates the levels of a presenilin fragment. Nature medicine *4*, 1177-1181.

Campbell, J.N., and Meyer, R.A. (2006). Mechanisms of neuropathic pain. Neuron *52*, 77-92.

Canela, L., Lujan, R., Lluis, C., Burgueno, J., Mallol, J., Canela, E.I., Franco, R., and Ciruela, F. (2007). The neuronal Ca(2+) -binding protein 2 (NECAB2) interacts with the adenosine A(2A) receptor and modulates the cell surface expression and function of the receptor. Molecular and cellular neurosciences *36*, 1-12.

Carr, P.A., Yamamoto, T., Karmy, G., Baimbridge, K.G., and Nagy, J.I. (1989). Parvalbumin is highly colocalized with calbindin D28k and rarely with calcitonin gene-related peptide in dorsal root ganglia neurons of rat. Brain research *497*, 163-170.

Carrion, A.M., Link, W.A., Ledo, F., Mellstrom, B., and Naranjo, J.R. (1999). DREAM is a Ca2+-regulated transcriptional repressor. Nature *398*, 80-84.

Caterina, M.J., Rosen, T.A., Tominaga, M., Brake, A.J., and Julius, D. (1999). A capsaicin-receptor homologue with a high threshold for noxious heat. Nature *398*, 436-441.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., and Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature *389*, 816-824.

Celio, M.R. (1986). Parvalbumin in most gamma-aminobutyric acid-containing neurons of the rat cerebral cortex. Science *231*, 995-997.

Celio, M.R. (1990). Calbindin D-28k and parvalbumin in the rat nervous system. Neuroscience *35*, 375-475.

Celio, M.R., and Heizmann, C.W. (1981). Calcium-binding protein parvalbumin as a neuronal marker. Nature *293*, 300-302.

Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M., and Yaksh, T.L. (1994). Quantitative assessment of tactile allodynia in the rat paw. Journal of neuroscience methods *53*, 55-63.

Cheng, H.Y., Pitcher, G.M., Laviolette, S.R., Whishaw, I.Q., Tong, K.I., Kockeritz, L.K., Wada, T., Joza, N.A., Crackower, M., Goncalves, J.*, et al.* (2002). DREAM is a critical transcriptional repressor for pain modulation. Cell *108*, 31-43.

Chin, D., and Means, A.R. (2000). Calmodulin: a prototypical calcium sensor. Trends in cell biology *10*, 322-328.

Chiu, I.M., Barrett, L.B., Williams, E.K., Strochlic, D.E., Lee, S., Weyer, A.D., Lou, S., Bryman, G.S., Roberson, D.P., Ghasemlou, N.*, et al.* (2014). Transcriptional profiling at whole population and single cell levels reveals somatosensory neuron molecular diversity. Elife *3*.

Cho, H.J., Kim, S.Y., Park, M.J., Kim, D.S., Kim, J.K., and Chu, M.Y. (1997). Expression of mRNA for brain-derived neurotrophic factor in the dorsal root ganglion following peripheral inflammation. Brain research *749*, 358-362.

Chung, K., Wallace, J., Kim, S.Y., Kalyanasundaram, S., Andalman, A.S., Davidson, T.J., Mirzabekov, J.J., Zalocusky, K.A., Mattis, J., Denisin, A.K.*, et al.* (2013). Structural and molecular interrogation of intact biological systems. Nature *497*, 332-337.

Costigan, M., Scholz, J., and Woolf, C.J. (2009). Neuropathic pain: a maladaptive response of the nervous system to damage. Annual review of neuroscience *32*, 1-32.

Costigan, M., and Woolf, C.J. (2002). No DREAM, No pain. Closing the spinal gate. Cell *108*, 297-300.

Dahl, D., Labkovsky, B., and Bignami, A. (1988). Neurofilament phosphorylation in axons and perikarya: immunofluorescence study of the rat spinal cord and dorsal root ganglia with monoclonal antibodies. The Journal of comparative neurology *271*, 445-450.

Davidson, S., and Giesler, G.J. (2010). The multiple pathways for itch and their interactions with pain. Trends in neurosciences *33*, 550-558.

Decosterd, I., and Woolf, C.J. (2000). Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain *87*, 149-158.

Finnerup, N.B., Attal, N., Haroutounian, S., McNicol, E., Baron, R., Dworkin, R.H., Gilron, I., Haanpaa, M., Hansson, P., Jensen, T.S.*, et al.* (2015). Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. Lancet neurology *14*, 162-173.

Foster, E., Wildner, H., Tudeau, L., Haueter, S., Ralvenius, W.T., Jegen, M., Johannssen, H., Hosli, L., Haenraets, K., Ghanem, A.*, et al.* (2015). Targeted ablation, silencing, and activation establish glycinergic dorsal horn neurons as key components of a spinal gate for pain and itch. Neuron *85*, 1289-1304.

Garraway, S.M., Petruska, J.C., and Mendell, L.M. (2003). BDNF sensitizes the response of lamina II neurons to high threshold primary afferent inputs. The European journal of neuroscience *18*, 2467-2476.

Gauriau, C., and Bernard, J.F. (2004). A comparative reappraisal of projections from the superficial laminae of the dorsal horn in the rat: the forebrain. J Comp Neurol *468*, 24-56.

Girard, F., Venail, J., Schwaller, B., and Celio, M.R. (2015). The EF-hand Ca(2+)-binding protein super-family: a genome-wide analysis of gene expression patterns in the adult mouse brain. Neuroscience *294*, 116-155.

Groth, R., and Aanonsen, L. (2002). Spinal brain-derived neurotrophic factor (BDNF) produces hyperalgesia in normal mice while antisense directed against either BDNF or trkB, prevent inflammation-induced hyperalgesia. Pain *100*, 171-181.

Han, L., Ma, C., Liu, Q., Weng, H.J., Cui, Y., Tang, Z., Kim, Y., Nie, H., Qu, L., Patel, K.N.*, et al.* (2013). A subpopulation of nociceptors specifically linked to itch. Nature neuroscience *16*, 174-182.

Harper, A.A., and Lawson, S.N. (1985). Electrical properties of rat dorsal root ganglion neurones with different peripheral nerve conduction velocities. The Journal of physiology *359*, 47-63.

Henry, A.M., and Hohmann, J.G. (2012). High-resolution gene expression atlases for adult and developing mouse brain and spinal cord. Mammalian genome : official journal of the International Mammalian Genome Society *23*, 539-549.

Herzog, E., Bellenchi, G.C., Gras, C., Bernard, V., Ravassard, P., Bedet, C., Gasnier, B., Giros, B., and El Mestikawy, S. (2001). The existence of a second vesicular glutamate transporter specifies subpopulations of glutamatergic neurons. J Neurosci *21*, RC181.

Herzog, E., Gilchrist, J., Gras, C., Muzerelle, A., Ravassard, P., Giros, B., Gaspar, P., and El Mestikawy, S. (2004). Localization of VGLUT3, the vesicular glutamate transporter type 3, in the rat brain. Neuroscience *123*, 983-1002.

Hokfelt, T., Johansson, O., Ljungdahl, A., Lundberg, J.M., and Schultzberg, M. (1980). Peptidergic neurones. Nature *284*, 515-521.

Hokfelt, T., Kellerth, J.O., Nilsson, G., and Pernow, B. (1975). Substance p: localization in the central nervous system and in some primary sensory neurons. Science *190*, 889-890.

Hökfelt, T., Zhang, X., Xu, X., and Wiesenfeld-Hallin, Z. (2013). Wall and Melzack's textbook of pain: Central consequences of peripheral nerve damage., 6th edn (Philadelphia, pp 902-914: Elsevier Saunders).

Horch, K.W., Tuckett, R.P., and Burgess, P.R. (1977). A key to the classification of cutaneous mechanoreceptors. The Journal of investigative dermatology *69*, 75-82.

Iketani, M., Imaizumi, C., Nakamura, F., Jeromin, A., Mikoshiba, K., Goshima, Y., and Takei, K. (2009). Regulation of neurite outgrowth mediated by neuronal calcium sensor-1 and inositol 1,4,5-trisphosphate receptor in nerve growth cones. Neuroscience *161*, 743-752.

Ikoma, A., Steinhoff, M., Stander, S., Yosipovitch, G., and Schmelz, M. (2006). The neurobiology of itch. Nature reviews Neuroscience *7*, 535-547.

Jensen, T.S., Baron, R., Haanpaa, M., Kalso, E., Loeser, J.D., Rice, A.S., and Treede, R.D. (2011). A new definition of neuropathic pain. Pain *152*, 2204-2205.

Ji, R.R., Chamessian, A., and Zhang, Y.Q. (2016). Pain regulation by non-neuronal cells and inflammation. Science *354*, 572-577.

Ji, R.R., Samad, T.A., Jin, S.X., Schmoll, R., and Woolf, C.J. (2002). p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. Neuron *36*, 57-68.

Johnson, K.O., and Hsiao, S.S. (1992). Neural mechanisms of tactual form and texture perception. Annual review of neuroscience *15*, 227-250.

Julius, D. (2013). TRP channels and pain. Annual review of cell and developmental biology *29*, 355-384.

Kerr, B.J., Bradbury, E.J., Bennett, D.L., Trivedi, P.M., Dassan, P., French, J., Shelton, D.B., McMahon, S.B., and Thompson, S.W. (1999). Brain-derived neurotrophic factor modulates nociceptive sensory inputs and NMDA-evoked responses in the rat spinal cord. The Journal of neuroscience : the official journal of the Society for Neuroscience *19*, 5138-5148.

Kilo, S., Harding-Rose, C., Hargreaves, K.M., and Flores, C.M. (1997). Peripheral CGRP release as a marker for neurogenic inflammation: a model system for the study of neuropeptide secretion in rat paw skin. Pain *73*, 201-207.

Kuraishi, Y., Nanayama, T., Ohno, H., Fujii, N., Otaka, A., Yajima, H., and Satoh, M. (1989). Calcitonin gene-related peptide increases in the dorsal root ganglia of adjuvant arthritic rat. Peptides *10*, 447-452.

Lagerstrom, M.C., Rogoz, K., Abrahamsen, B., Persson, E., Reinius, B., Nordenankar, K., Olund, C., Smith, C., Mendez, J.A., Chen, Z.F., *et al.* (2010). VGLUT2-dependent sensory neurons in the TRPV1 population regulate pain and itch. Neuron *68*, 529-542.

Laing, I., Todd, A.J., Heizmann, C.W., and Schmidt, H.H. (1994). Subpopulations of GABAergic neurons in laminae I-III of rat spinal dorsal horn defined by coexistence with classical transmitters, peptides, nitric oxide synthase or parvalbumin. Neuroscience *61*, 123-132.

Lallemend, F., and Ernfors, P. (2012). Molecular interactions underlying the specification of sensory neurons. Trends in neurosciences *35*, 373-381.

LaMotte, R.H., and Campbell, J.N. (1978). Comparison of responses of warm and nociceptive C-fiber afferents in monkey with human judgments of thermal pain. Journal of neurophysiology *41*, 509-528.

LaMotte, R.H., Thalhammer, J.G., Torebjork, H.E., and Robinson, C.J. (1982). Peripheral neural mechanisms of cutaneous hyperalgesia following mild injury by heat. The Journal of neuroscience : the official journal of the Society for Neuroscience *2*, 765-781.

Lawson, S.N. (1979). The postnatal development of large light and small dark neurons in mouse dorsal root ganglia: a statistical analysis of cell numbers and size. Journal of neurocytology *8*, 275-294.

Lawson, S.N., Harper, A.A., Harper, E.I., Garson, J.A., and Anderton, B.H. (1984). A monoclonal antibody against neurofilament protein specifically labels a subpopulation of rat sensory neurones. The Journal of comparative neurology *228*, 263-272.

Lawson, S.N., and Waddell, P.J. (1991). Soma neurofilament immunoreactivity is related to cell size and fibre conduction velocity in rat primary sensory neurons. The Journal of physiology *435*, 41-63.

Le Greves, P., Nyberg, F., Terenius, L., and Hokfelt, T. (1985). Calcitonin gene-related peptide is a potent inhibitor of substance P degradation. European journal of pharmacology *115*, 309-311.

Le Maitre, E., Barde, S.S., Palkovits, M., Diaz-Heijtz, R., and Hokfelt, T.G. (2013). Distinct features of neurotransmitter systems in the human brain with focus on the galanin system in locus coeruleus and dorsal raphe. Proceedings of the National Academy of Sciences of the United States of America *110*, E536-545.

Lee, K.H., Chung, K., Chung, J.M., and Coggeshall, R.E. (1986). Correlation of cell body size, axon size, and signal conduction velocity for individually labelled dorsal root ganglion cells in the cat. The Journal of comparative neurology *243*, 335-346.

Lein, E.S., Hawrylycz, M.J., Ao, N., Ayres, M., Bensinger, A., Bernard, A., Boe, A.F., Boguski, M.S., Brockway, K.S., Byrnes, E.J.*, et al.* (2007). Genome-wide atlas of gene expression in the adult mouse brain. Nature *445*, 168-176.

Leissring, M.A., Yamasaki, T.R., Wasco, W., Buxbaum, J.D., Parker, I., and LaFerla, F.M. (2000). Calsenilin reverses presenilin-mediated enhancement of calcium signaling. Proceedings of the National Academy of Sciences of the United States of America *97*, 8590-8593.

Li, C.L., Li, K.C., Wu, D., Chen, Y., Luo, H., Zhao, J.R., Wang, S.S., Sun, M.M., Lu, Y.J., Zhong, Y.Q.*, et al.* (2016). Somatosensory neuron types identified by high-coverage single-cell RNA-sequencing and functional heterogeneity. Cell research *26*, 83-102.

Li, L., Rutlin, M., Abraira, V.E., Cassidy, C., Kus, L., Gong, S., Jankowski, M.P., Luo, W., Heintz, N., Koerber, H.R., *et al.* (2011). The functional organization of cutaneous low-threshold mechanosensory neurons. Cell *147*, 1615-1627.

Littlewood, N.K., Todd, A.J., Spike, R.C., Watt, C., and Shehab, S.A. (1995). The types of neuron in spinal dorsal horn which possess neurokinin-1 receptors. Neuroscience *66*, 597-608.

Liu, Y., Abdel Samad, O., Zhang, L., Duan, B., Tong, Q., Lopes, C., Ji, R.R., Lowell, B.B., and Ma, Q. (2010). VGLUT2-dependent glutamate release from nociceptors is required to sense pain and suppress itch. Neuron *68*, 543-556.

Louis, S.M., Johnstone, D., Russell, N.J., Jamieson, A., and Dockray, G.J. (1989). Antibodies to calcitonin-gene related peptide reduce inflammation induced by topical mustard oil but not that due to carrageenin in the rat. Neuroscience letters *102*, 257-260.

Lu, Y., Dong, H., Gao, Y., Gong, Y., Ren, Y., Gu, N., Zhou, S., Xia, N., Sun, Y.Y., Ji, R.R., *et al.* (2013). A feed-forward spinal cord glycinergic neural circuit gates mechanical allodynia. The Journal of clinical investigation *123*, 4050-4062.

Ma, Q. (2012). Population coding of somatic sensations. Neuroscience bulletin *28*, 91-99.

Malmberg, A.B., Chen, C., Tonegawa, S., and Basbaum, A.I. (1997). Preserved acute pain and reduced neuropathic pain in mice lacking PKCgamma. Science *278*, 279-283.

Mannion, R.J., Costigan, M., Decosterd, I., Amaya, F., Ma, Q.P., Holstege, J.C., Ji, R.R., Acheson, A., Lindsay, R.M., Wilkinson, G.A., *et al.* (1999). Neurotrophins: peripherally and centrally acting modulators of tactile stimulus-induced inflammatory pain hypersensitivity. Proceedings of the National Academy of Sciences of the United States of America *96*, 9385-9390.

Mantyh, P.W. (2014). Bone cancer pain: from mechanism to therapy. Current opinion in supportive and palliative care *8*, 83-90.

Mantyh, P.W., DeMaster, E., Malhotra, A., Ghilardi, J.R., Rogers, S.D., Mantyh, C.R., Liu, H., Basbaum, A.I., Vigna, S.R., Maggio, J.E., *et al.* (1995). Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation. Science *268*, 1629-1632.

Marmigere, F., and Ernfors, P. (2007). Specification and connectivity of neuronal subtypes in the sensory lineage. Nature reviews Neuroscience *8*, 114-127.

McKemy, D.D., Neuhausser, W.M., and Julius, D. (2002). Identification of a cold receptor reveals a general role for TRP channels in thermosensation. Nature *416*, 52-58.

McMahon, S.B. (1996). NGF as a mediator of inflammatory pain. Philosophical transactions of the Royal Society of London Series B, Biological sciences *351*, 431-440.

McMahon, S.B., and Priestley, J.V. (2005). The Neurobiology of Pain (Oxford: Oxford University Press).

McMahon, S.B., Russa, F.L., and Bennett, D.L. (2015). Crosstalk between the nociceptive and immune systems in host defence and disease. Nature reviews Neuroscience *16*, 389-402.

Melzack, R., and Wall, P.D. (1965). Pain mechanisms: a new theory. Science *150*, 971-979.

Menetrey, D., Gannon, A., Levine, J.D., and Basbaum, A.I. (1989). Expression of c-fos protein in interneurons and projection neurons of the rat spinal cord in response to noxious somatic, articular, and visceral stimulation. The Journal of comparative neurology *285*, 177-195.

Michael, G.J., Averill, S., Nitkunan, A., Rattray, M., Bennett, D.L., Yan, Q., and Priestley, J.V. (1997). Nerve growth factor treatment increases brain-derived neurotrophic factor selectively in TrkA-expressing dorsal root ganglion cells and in their central terminations within the spinal cord. The Journal of neuroscience : the official journal of the Society for Neuroscience *17*, 8476-8490.

Mikhaylova, M., Hradsky, J., and Kreutz, M.R. (2011). Between promiscuity and specificity: novel roles of EF-hand calcium sensors in neuronal Ca2+ signalling. Journal of neurochemistry *118*, 695-713.

Mishra, S.K., Tisel, S.M., Orestes, P., Bhangoo, S.K., and Hoon, M.A. (2011). TRPV1-lineage neurons are required for thermal sensation. The EMBO journal *30*, 582-593.

Molander, C., Xu, Q., and Grant, G. (1984). The cytoarchitectonic organization of the spinal cord in the rat. I. The lower thoracic and lumbosacral cord. The Journal of comparative neurology *230*, 133-141.

Morris, C.J. (2003). Carrageenan-induced paw edema in the rat and mouse. Methods in molecular biology *225*, 115-121.

Mulder, J., Bjorling, E., Jonasson, K., Wernerus, H., Hober, S., Hokfelt, T., and Uhlen, M. (2009a). Tissue profiling of the mammalian central nervous system using human antibody-based proteomics. Molecular & cellular proteomics : MCP *8*, 1612-1622.

Mulder, J., Spence, L., Tortoriello, G., Dinieri, J.A., Uhlen, M., Shui, B., Kotlikoff, M.I., Yanagawa, Y., Aujard, F., Hokfelt, T.*, et al.* (2010). Secretagogin is a Ca2+-binding protein identifying prospective extended amygdala neurons in the developing mammalian telencephalon. The European journal of neuroscience *31*, 2166-2177.

Mulder, J., Zilberter, M., Spence, L., Tortoriello, G., Uhlen, M., Yanagawa, Y., Aujard, F., Hokfelt, T., and Harkany, T. (2009b). Secretagogin is a Ca2+-binding protein specifying subpopulations of telencephalic neurons. Proceedings of the National Academy of Sciences of the United States of America *106*, 22492-22497.

Neumann, S., Braz, J.M., Skinner, K., Llewellyn-Smith, I.J., and Basbaum, A.I. (2008). Innocuous, not noxious, input activates PKCgamma interneurons of the spinal dorsal horn via myelinated afferent fibers. The Journal of neuroscience : the official journal of the Society for Neuroscience *28*, 7936-7944.

Peier, A.M., Moqrich, A., Hergarden, A.C., Reeve, A.J., Andersson, D.A., Story, G.M., Earley, T.J., Dragoni, I., McIntyre, P., Bevan, S.*, et al.* (2002). A TRP channel that senses cold stimuli and menthol. Cell *108*, 705-715.

Peng, C., Li, N., Ng, Y.K., Zhang, J., Meier, F., Theis, F.J., Merkenschlager, M., Chen, W., Wurst, W., and Prakash, N. (2012). A unilateral negative feedback loop between miR-200 microRNAs and Sox2/E2F3 controls neural progenitor cell-cycle exit and differentiation.

The Journal of neuroscience : the official journal of the Society for Neuroscience *32*, 13292-13308.

Perl, E.R. (2007). Ideas about pain, a historical view. Nature reviews Neuroscience *8*, 71-80.

Pertin, M., Gosselin, R.D., and Decosterd, I. (2012). The spared nerve injury model of neuropathic pain. Methods in molecular biology *851*, 205-212.

Petitjean, H., Pawlowski, S.A., Fraine, S.L., Sharif, B., Hamad, D., Fatima, T., Berg, J., Brown, C.M., Jan, L.Y., Ribeiro-da-Silva, A.*, et al.* (2015). Dorsal Horn Parvalbumin Neurons Are Gate-Keepers of Touch-Evoked Pain after Nerve Injury. Cell Rep *13*, 1246-1257.

Polgar, E., Durrieux, C., Hughes, D.I., and Todd, A.J. (2013a). A quantitative study of inhibitory interneurons in laminae I-III of the mouse spinal dorsal horn. PloS one *8*, e78309.

Polgar, E., Fowler, J.H., McGill, M.M., and Todd, A.J. (1999). The types of neuron which contain protein kinase C gamma in rat spinal cord. Brain Res *833*, 71-80.

Polgar, E., Sardella, T.C., Tiong, S.Y., Locke, S., Watanabe, M., and Todd, A.J. (2013b). Functional differences between neurochemically defined populations of inhibitory interneurons in the rat spinal dorsal horn. Pain *154*, 2606-2615.

Ramón y Cajal, S., and Azoulay, L. (1952). Histologie du système nerveux de l'homme & des vertébrés, [Centenary] édition française / edn (Madrid: Consejo Superior de Investigaciones Científicas).

Ramón y Cajal, S., Azoulay, L., Swanson, N., and Swanson, L.W. (1995). Histology of the nervous system of man and vertebrates (New York ; Oxford: Oxford University Press).

Ren, K., and Dubner, R. (1999). Inflammatory Models of Pain and Hyperalgesia. ILAR J *40*, 111-118.

Ren, K., Ruda, M.A., and Jacobowitz, D.M. (1993). Immunohistochemical localization of calretinin in the dorsal root ganglion and spinal cord of the rat. Brain research bulletin *31*, 13-22.

Renier, N., Adams, E.L., Kirst, C., Wu, Z., Azevedo, R., Kohl, J., Autry, A.E., Kadiri, L., Umadevi Venkataraju, K., Zhou, Y.*, et al.* (2016). Mapping of Brain Activity by Automated Volume Analysis of Immediate Early Genes. Cell *165*, 1789-1802.

Renier, N., Wu, Z., Simon, D.J., Yang, J., Ariel, P., and Tessier-Lavigne, M. (2014). iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging. Cell *159*, 896-910.

Rexed, B. (1952). The cytoarchitectonic organization of the spinal cord in the cat. The Journal of comparative neurology *96*, 414-495.

Rogstam, A., Linse, S., Lindqvist, A., James, P., Wagner, L., and Berggard, T. (2007). Binding of calcium ions and SNAP-25 to the hexa EF-hand protein secretagogin. The Biochemical journal *401*, 353-363.

Romanov, R.A., Alpar, A., Zhang, M.D., Zeisel, A., Calas, A., Landry, M., Fuszard, M., Shirran, S.L., Schnell, R., Dobolyi, A.*, et al.* (2015). A secretagogin locus of the mammalian hypothalamus controls stress hormone release. The EMBO journal *34*, 36-54.

Safieh-Garabedian, B., Poole, S., Allchorne, A., Winter, J., and Woolf, C.J. (1995). Contribution of interleukin-1 beta to the inflammation-induced increase in nerve growth factor levels and inflammatory hyperalgesia. British journal of pharmacology *115*, 1265-1275.

Sakamoto, H., Spike, R.C., and Todd, A.J. (1999). Neurons in laminae III and IV of the rat spinal cord with the neurokinin-1 receptor receive few contacts from unmyelinated primary afferents which do not contain substance P. Neuroscience *94*, 903-908.

Salio, C., and Ferrini, F. (2016). BDNF and GDNF expression in discrete populations of nociceptors. Ann Anat *207*, 55-61.

Salio, C., Lossi, L., Ferrini, F., and Merighi, A. (2005). Ultrastructural evidence for a pre- and postsynaptic localization of full-length trkB receptors in substantia gelatinosa (lamina II) of rat and mouse spinal cord. The European journal of neuroscience *22*, 1951-1966.

Scherrer, G., Low, S.A., Wang, X., Zhang, J., Yamanaka, H., Urban, R., Solorzano, C., Harper, B., Hnasko, T.S., Edwards, R.H.*, et al.* (2010). VGLUT2 expression in primary afferent neurons is essential for normal acute pain and injury-induced heat hypersensitivity. Proceedings of the National Academy of Sciences of the United States of America *107*, 22296-22301.

Schmidt, R., Schmelz, M., Forster, C., Ringkamp, M., Torebjork, E., and Handwerker, H. (1995). Novel classes of responsive and unresponsive C nociceptors in human skin. The Journal of neuroscience : the official journal of the Society for Neuroscience *15*, 333-341.

Scholz, J., and Woolf, C.J. (2002). Can we conquer pain? Nature neuroscience *5 Suppl*, 1062-1067.

Schwaller, B. (2009). The continuing disappearance of "pure" Ca2+ buffers. Cellular and molecular life sciences : CMLS *66*, 275-300.

Sherrington, C.S. (1906). The integrative action of the nervous system (New York: Scribner).

Shi, T.J., Xiang, Q., Zhang, M.D., Tortoriello, G., Hammarberg, H., Mulder, J., Fried, K., Wagner, L., Josephson, A., Uhlen, M.*, et al.* (2012). Secretagogin is expressed in sensory CGRP neurons and in spinal cord of mouse and complements other calcium-binding proteins, with a note on rat and human. Molecular pain *8*, 80.

Steffens, H., and Schomburg, E.D. (1993). Convergence in segmental reflex pathways from nociceptive and non-nociceptive afferents to alpha-motoneurones in the cat. The Journal of physiology *466*, 191-211.

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.*, et al.* (2003). ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. Cell *112*, 819-829.

Sugita, S., Ho, A., and Sudhof, T.C. (2002). NECABs: a family of neuronal Ca(2+)-binding proteins with an unusual domain structure and a restricted expression pattern. Neuroscience *112*, 51-63.

Sugita, S., and Sudhof, T.C. (2000). Specificity of Ca2+-dependent protein interactions mediated by the C2A domains of synaptotagmins. Biochemistry *39*, 2940-2949.

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

Thompson, S.W., Bennett, D.L., Kerr, B.J., Bradbury, E.J., and McMahon, S.B. (1999). Brain-derived neurotrophic factor is an endogenous modulator of nociceptive responses in the spinal cord. Proceedings of the National Academy of Sciences of the United States of America *96*, 7714-7718.

Todd, A.J. (2006). Chapter 6 Anatomy and neurochemistry of the dorsal horn. Handbook of clinical neurology *81*, 61-76.

Todd, A.J. (2010). Neuronal circuitry for pain processing in the dorsal horn. Nature reviews Neuroscience *11*, 823-836.

Todd, A.J. (2015). Plasticity of inhibition in the spinal cord. Handbook of experimental pharmacology *227*, 171-190.

Todd, A.J., Hughes, D.I., Polgar, E., Nagy, G.G., Mackie, M., Ottersen, O.P., and Maxwell, D.J. (2003). The expression of vesicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn. Eur J Neurosci *17*, 13-27.

Todd, A.J., McGill, M.M., and Shehab, S.A. (2000). Neurokinin 1 receptor expression by neurons in laminae I, III and IV of the rat spinal dorsal horn that project to the brainstem. The European journal of neuroscience *12*, 689-700.

Todd, A.J., and Sullivan, A.C. (1990). Light microscope study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat. The Journal of comparative neurology *296*, 496-505.

Tominaga, M., and Caterina, M.J. (2004). Thermosensation and pain. Journal of neurobiology *61*, 3-12.

Uhlen, M., Bandrowski, A., Carr, S., Edwards, A., Ellenberg, J., Lundberg, E., Rimm, D.L., Rodriguez, H., Hiltke, T., Snyder, M.*, et al.* (2016). A proposal for validation of antibodies. Nature methods *13*, 823-827.

Usoskin, D., Furlan, A., Islam, S., Abdo, H., Lonnerberg, P., Lou, D., Hjerling-Leffler, J., Haeggstrom, J., Kharchenko, O., Kharchenko, P.V.*, et al.* (2015). Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. Nature neuroscience *18*, 145-153.

Vale, W., Rivier, C., Brown, M.R., Spiess, J., Koob, G., Swanson, L., Bilezikjian, L., Bloom, F., and Rivier, J. (1983). Chemical and biological characterization of corticotropin releasing factor. Recent Prog Horm Res *39*, 245-270.

van Hecke, O., Austin, S.K., Khan, R.A., Smith, B.H., and Torrance, N. (2014). Neuropathic pain in the general population: a systematic review of epidemiological studies. Pain *155*, 654-662.

Verge, V.M., Richardson, P.M., Wiesenfeld-Hallin, Z., and Hokfelt, T. (1995). Differential influence of nerve growth factor on neuropeptide expression in vivo: a novel role in peptide suppression in adult sensory neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience *15*, 2081-2096.

Wagner, L., Oliyarnyk, O., Gartner, W., Nowotny, P., Groeger, M., Kaserer, K., Waldhausl, W., and Pasternack, M.S. (2000). Cloning and expression of secretagogin, a novel neuroendocrine- and pancreatic islet of Langerhans-specific Ca2+-binding protein. The Journal of biological chemistry *275*, 24740-24751.

Wall, P.D., Devor, M., Inbal, R., Scadding, J.W., Schonfeld, D., Seltzer, Z., and Tomkiewicz, M.M. (1979). Autotomy following peripheral nerve lesions: experimental anaesthesia dolorosa. Pain *7*, 103-111.

Wiesenfeld-Hallin, Z., Hokfelt, T., Lundberg, J.M., Forssmann, W.G., Reinecke, M., Tschopp, F.A., and Fischer, J.A. (1984). Immunoreactive calcitonin gene-related peptide and substance P coexist in sensory neurons to the spinal cord and interact in spinal behavioral responses of the rat. Neuroscience letters *52*, 199-204.

Wiesenfeld-Hallin, Z., Villar, M.J., and Hokfelt, T. (1988). Intrathecal galanin at low doses increases spinal reflex excitability in rats more to thermal than mechanical stimuli. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale *71*, 663-666.

Willis, W.D., and Coggeshall, R.E. (2004). Sensory mechanisms of the spinal cord (New York, N.Y. ; London: Kluwer Academic/Plenum Publishers).

Willis, W.D., Jr. (2007). The somatosensory system, with emphasis on structures important for pain. Brain research reviews *55*, 297-313.

Woolf, C., and Wiesenfeld-Hallin, Z. (1986). Substance P and calcitonin gene-related peptide synergistically modulate the gain of the nociceptive flexor withdrawal reflex in the rat. Neuroscience letters *66*, 226-230.

Woolf, C.J. (1983). Evidence for a central component of post-injury pain hypersensitivity. Nature *306*, 686-688.

Woolf, C.J. (1989). Recent advances in the pathophysiology of acute pain. British journal of anaesthesia *63*, 139-146.

Woolf, C.J., and Ma, Q. (2007). Nociceptors--noxious stimulus detectors. Neuron *55*, 353-364.

Wu, H., Li, D., Shan, Y., Wan, B., Hexige, S., Guo, J., Wu, C., and Yu, L. (2007). EFCBP1/NECAB1, a brain-specifically expressed gene with highest abundance in temporal lobe, encodes a protein containing EF-hand and antibiotic biosynthesis monooxygenase domains. DNA sequence : the journal of DNA sequencing and mapping *18*, 73-79.

Zhang, L., Hoff, A.O., Wimalawansa, S.J., Cote, G.J., Gagel, R.F., and Westlund, K.N. (2001). Arthritic calcitonin/alpha calcitonin gene-related peptide knockout mice have reduced nociceptive hypersensitivity. Pain *89*, 265-273.

Zhang, M.D., Barde, S., Szodorai, E., Josephson, A., Mitsios, N., Watanabe, M., Attems, J., Lubec, G., Kovacs, G.G., Uhlen, M.*, et al.* (2016). Comparative anatomical distribution of neuronal calcium-binding protein (NECAB) 1 and -2 in rodent and human spinal cord. Brain structure & function *221*, 3803-3823.

Zhang, M.D., Tortoriello, G., Hsueh, B., Tomer, R., Ye, L., Mitsios, N., Borgius, L., Grant, G., Kiehn, O., Watanabe, M.*, et al.* (2014). Neuronal calcium-binding proteins 1/2 localize to dorsal root ganglia and excitatory spinal neurons and are regulated by nerve injury. Proceedings of the National Academy of Sciences of the United States of America *111*, E1149-1158.

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

Zimmermann, B., Girard, F., Meszar, Z., and Celio, M.R. (2013). Expression of the calcium binding proteins Necab-1,-2 and -3 in the adult mouse hippocampus and dentate gyrus. Brain research *1528*, 1-7.