CANCER-INDUCED BONE PAIN:
PERIPHERAL AND CENTRAL MECHANISMS
IN RODENT MODELS OF THE DISEASE

Mateusz Wojciech Kucharczyk

A thesis submitted to University College London for the degree of Doctor of Philosophy

Department of Neuroscience, Physiology and Pharmacology
Gower Street | London | WC1E 6BT

This work was supported by grant from the European Union’s Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No.642720
I, Mateusz Kucharczyk, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Abstract

Cancer pain remains a major area of unmet medical need. One common form of chronic cancer pain, affecting 400,000 people each year in the US alone, is associated with skeletal metastases. These pains are typically mechanoceptive in nature and poorly managed by available analgesics. Here, I employed in vivo imaging using GCaMP6s to assess the properties of the nerve fibres that transduce bone cancer pains. I showed that a subclass of nociceptors, those that are normally mechanically insensitive, are recruited and activated in a rodent model of bone cancer, and that this dramatically increases sensory input from the diseased tissue to the central nervous system. The recruitment of these so-called silent afferents was shown to be Piezo2-dependent.

Next, utilising in vivo spinal electrophysiology, I showed that the increased peripheral input described above leads to the reorganization of descending noradrenergic controls, potently increasing inhibitory tone. I hypothesise the existence of a spino-pontine-spinal loop that modulates spinal excitability in a noradrenergic fashion and is strongly altered in the advanced metastatic disease. This changes the spinal pharmacology of α₂-adrenoceptor antagonist atipamezole, which now has inhibitory actions at the level of the spinal cord.

Diffuse Noxious Inhibitory Controls (DNIC), a unique form of descending noradrenergic controls, was studied also. This phenomenon, whereby application of a noxious stimulus to one part of the body inhibits pain perceived in a remote body region, acts through inhibitory descending pathways. Interestingly, DNIC were found dynamic with the progression of bone tumours. I hypothesise that DNIC originate from brain regions separate to those that govern tonic NA-controls, and I conclude that the expression status of DNIC can be used clinically as a diagnostic tool to tailor pain pharmacotherapy in patients suffering from chronic bone cancer pain.

The unique properties of bone silent afferents, which show plasticity in chronicity, combined with ensuing central events offer several novel opportunities for targeting metastatic bone pain. Due to the dynamic nature of the disease and resulting pains, it is crucial to match pain therapy with the stage of the disease.
Significance statement

The mechanisms that underlie pain resulting from metastatic bone disease remain elusive. This translates to a clinical and socioeconomic burden; targeted therapy is not possible, and patients do not receive adequate analgesic relief for their chronic pain state. Complicating matters is the heterogeneous nature of metastatic bone disease. Not only does the individual sufferer's pain phenotype depend on genetic, emotional and sensory factors, but also on the progression of the disease. Early stage cancers are molecularly very different to their late stage counterparts and so too is the pain associated with infant and advanced tumours. In this thesis I have begun to dissect the mechanisms that underlie pain resulting from metastatic bone disease using a rodent model of cancer induced bone pain (CIBP). I studied both peripheral and central mechanisms of pain generation and in doing so was able to identify several new targets directly linked to this unique pain phenotype.

Regarding peripheral mechanisms I have shown that bone cancer engages previously silent nociceptors, ultimately increasing sensory input to the central nervous system. I identified novel molecular targets responsible for the activation of the previously silent afferents and thus was able to suggest a novel mechanism underlying cancer-induced bone pain. The results obtained provide a basis for future, more detailed, mechanistic studies as well as hinting at translational pharmacological interventions toward the revealed markers.

Regarding central mechanisms, I showed disease progression plasticity in the modulatory noradrenergic system in terms of the regulation of spinal and supraspinal activity. By demonstrating that the inhibitory noradrenergic tone in the dorsal horn of the spinal cord could be triggered by the selective activation of lamina I projection neurons, central targets for cancer pain pharmacotherapy were revealed. Crucially the hypothesised spino-pontine-spinal loop responsible for modulating spinal excitability in a noradrenergic fashion was strongly altered in the advanced metastatic disease. This is a novel mechanism of cancer-induced bone pain, suggestive of an as-yet unexplored mechanism by which pontine noradrenergic systems can orchestrate analgesic action. There is a strong potential to translate these findings to the clinic by repurposing already approved drugs (that modulate the noradrenergic system) to combat CIBP.

Finally, I explored a unique form of descending control that utilises noradrenergic systems. Diffuse Noxious Inhibitory Controls (DNIC) expression was shown dynamic with cancer progression since it was compromised in the early cancer stage yet restored in the late, advanced stage. I hypothesise a separate origin for descending noradrenaline operating via this particular descending pathway. This has a strong translational potential
since the human equivalent of DNIC, conditioned pain modulation (CPM), is a simple diagnostic test in terms of functional descending modulation that could be utilised to diagnose a patient and thereafter offer better suited pain pharmacotherapy depending on the presence or absence of DNIC/CPM expression.

In total I can conclude that, due to the dynamic nature of the bone cancer and resulting pains, it is crucial to match pain therapy with the stage of the disease. This has a significant translational implication in the clinic.
Acknowledgements

To my supervisors and colleagues: I owe you a huge debt of gratitude. Thank you for all your support during these 3+ years of work together. You are the cleverest people I met and without you this research would not be possible.

Kirsty, you were the best supervisor one can imagine and I was very lucky, simply could NOT be better. Thank you for supporting me and teaching me. You are a true educator and all these students are lucky to have you!

Mac, you are the best scientist I have ever met: a combination of smartness, vast knowledge and absolutely wonderful personality. Thank you for mentoring me and being my friend, that has been and is a privilege.

Tony, thank you for giving me this opportunity to work at both UCL and King’s and to pursue such a fantastic research.

Specially, I would like to thank: Kim, Fran, Doug, Martyn, Sara, Ryan and Leonor for your constant help, support and brilliant science we did and still do together.

To Alan: Doing PhD shares some similarities with chronic pain – it is chronic and painful, and affects relatives. Thank you Alan for sharing this pain with me.

To my family: Kochani jesteście wspaniali – bardzo Wam dziękuję za wsparcie i wszystko co dla mnie robicie.
# Table of Contents

Abstract.................................................................................................................................................. 3

Significance statement............................................................................................................................ 4

Acknowledgements................................................................................................................................. 6

Table of Contents .................................................................................................................................. 7

List of figures .......................................................................................................................................... 11

List of tables ......................................................................................................................................... 12

List of other materials ........................................................................................................................... 12

1. Chapter I. General introduction ......................................................................................................... 14
   1.1 Nociception and pain .................................................................................................................... 14
   1.2 Periphery: A somatosensory nervous system .............................................................................. 16
      1.2.1 Classification .......................................................................................................................... 16
   1.3 Central processing of somatosensory information ...................................................................... 19
      1.3.1 Spinal cord anatomy .............................................................................................................. 19
      1.3.2 Ascending pathways ............................................................................................................. 21
      1.3.3 Descending modulation of the sensory input ........................................................................ 22
      1.3.4 Sensory coding ...................................................................................................................... 25
   1.4 Cancer-induced bone pain ........................................................................................................... 26
      1.4.1 Bone innervation .................................................................................................................. 29
      1.4.2 Mechanisms of cancer-induced bone pain ......................................................................... 31
   1.5 General aims of thesis .................................................................................................................. 33

2. Chapter II. Materials and Methods ................................................................................................... 35
   2.1 Cell lines ...................................................................................................................................... 35
   2.2 Animals ....................................................................................................................................... 35
   2.3 Cancer-induced bone pain model ............................................................................................... 35
   2.4 Administration of tracers and calcium indicators ......................................................................... 36
   2.5 In vivo calcium imaging of sensory neurons .............................................................................. 37
      2.5.1 Activation of sensory neurons for GCaMP in vivo imaging .................................................. 37
      2.5.2 Calcium imaging data analysis ............................................................................................ 38
      2.5.3 Markov Cluster Analysis ...................................................................................................... 38
      2.5.4 Principal Component Analysis ............................................................................................ 39
   2.6 Behavioural testing ....................................................................................................................... 39
      2.6.1 Von Frey test ........................................................................................................................ 39
      2.6.2 Acetone test .......................................................................................................................... 40
      2.6.3 Static Weight Bearing .......................................................................................................... 40
   2.7 Micro-computed tomography of cancer-bearing legs ................................................................... 40
2.8 Immunohistochemistry ................................................................. 41
2.9 PACT ......................................................................................... 42
2.10 Spinal cord \textit{in vivo} electrophysiology ........................................ 43
  2.10.1 Diffuse Noxious Inhibitory Controls ........................................ 45
  2.10.2 \textit{In vivo} spinal pharmacology with electrophysiological monitoring .... 45
  2.10.3 Brain injections .................................................................. 46
  2.10.4 Optogenetics .................................................................... 47
2.11 Quantification and statistical analysis ........................................... 48
2.12 Methodological limitations ......................................................... 49

3. Chapter III. Encoding pressure by rat somatosensory neurons in health and bone cancer disease .................................................................................. 53
  3.1 Introduction ............................................................................. 53
  3.2 Materials and Methods ........................................................... 54
  3.3 Results .................................................................................... 55
    3.3.1 A high proportion of deep body CGRP afferents express little or no Advillin ................................................................. 55
    3.3.2 Cancer progression affects bone innervation ......................... 58
    3.3.3 The number of mechanically-responsive sensory neurons is tripled in animals with bone cancer ......................................................... 61
    3.3.4 Leg compression and position are differentially coded by DRG sensory neurons ................................................................. 64
    3.3.5 Intratibial afferent function in health and CIBP ....................... 66
    3.3.6 Muscle and periosteum afferents are recruited and sensitized by bone cancer ................................................................. 66
  3.4 Discussion ............................................................................... 67
    3.4.1 A high proportion of deep body CGRP afferents express little to no Advillin 67
    3.4.2 Cancer progression affects bone innervation ......................... 68
    3.4.3 Leg compression and position are differentially coded by DRG sensory neurons ................................................................. 68
    3.4.4 The number of mechanically-responsive sensory neurons is tripled in animals with bone cancer ......................................................... 69
    3.4.5 Silent nociceptors in CIBP originate from muscle or periosteum .... 71
    3.4.6 Conclusions ...................................................................... 71
  3.5 Author Contributions ............................................................... 72

4. Chapter IV. Spinal cord – secondary mechanical hyperalgesia ........... 74
  4.1 Introduction ............................................................................. 74
  4.2 Rationale, hypothesis and aims .................................................. 76
  4.3 Materials and Methods ........................................................... 76
4.4 Results:

4.4.1 Rats with bone cancer present secondary mechanical hyperalgesia

4.4.2 Deep dorsal horn wide-dynamic range neurons are not hyperexcitable in our rodent model of CIBP

4.4.3 Tonic descending noradrenergic controls are altered in our rodent model of CIBP

4.4.4 Inhibition of mechanosensation by spinal $\alpha_2$-ARs block depends on a functioning opioid system in the late stage CIBP rats

4.4.5 Pharmacological blockade of the locus coeruleus in the late stage CIBP rats abolishes spinal atipamezole-mediated inhibitions

4.4.6 Optoactivation of the locus coeruleus in naïve animals mimics the altered spinal noradrenergic-mediated actions observed in late stage CIBP rats

4.4.7 Optoactivation of lamina I projection neurons inhibits deep dorsal horn wide dynamic range neurons

4.5 Discussion

4.5.1 Deep dorsal horn wide-dynamic range neurons are not hyperexcitable in CIBP

4.5.2 Tonic descending noradrenergic controls are altered in CIBP

4.5.3 Inhibition of mechanosensation by spinal $\alpha_2$-ARs block depends on a functioning opioid system in the late stage CIBP rats

4.5.4 Pharmacological blockade of the locus coeruleus in the late stage CIBP rats abolished spinal atipamezole-mediated inhibition

4.5.5 Optoactivation of the locus coeruleus in naïve animals mimics the altered spinal noradrenergic-mediated actions observed in late stage CIBP rats

4.5.6 Optoactivation of lamina I projection neurons inhibits deep dorsal horn wide dynamic range neurons

4.5.7 Conclusions

4.6 Author Contributions

5. Chapter V. DNIC expression is dynamic in the progression of CIBP

5.1 Introduction

5.2 Rationale, hypothesis and aims

5.3 Materials and Methods

5.4 Results

5.4.1 DNIC expression in early and late stage of CIBP

5.4.2 Spinal $\alpha_2$-AR controls DNIC expression in early CIBP

5.4.3 Spinal $\alpha_2$-AR controls DNIC expression in late CIBP

5.4.4 Spinal opioid receptors do not control DNIC expression

5.4.5 Spinal $\alpha_2$-AR control of DNIC do not depend on spinal opioids

5.4.6 Locus coeruleus does not mediate DNIC expression
5.5 Discussion .............................................................................................................................. 111
  5.5.1 DNIC are dynamic throughout the course of the metastatic disease progression ................................................................. 111
  5.5.2 Spinal α2-ARs modulate DNIC expression ........................................................................ 112
  5.5.3 Spinal opioid receptors do not modulate DNIC expression ............................................... 112
  5.5.4 Locus coeruleus output does not modulate DNIC expression ........................................ 112
  5.5.5 Conclusions .............................................................................................................. 114
5.6 Author Contributions .......................................................................................................... 114
6. Chapter VI. Thermoception in CIBP .................................................................................... 116
  6.1 Introduction ...................................................................................................................... 116
  6.2 Rationale, hypothesis and aims ...................................................................................... 118
  6.3 Materials and Methods ................................................................................................. 118
  6.4 Results ............................................................................................................................ 119
    6.4.1 The recruitment of cold-responding neurons is enhanced in bone cancer 119
    6.4.2 Cold allodynia develops with the progression of bone metastatic disease 120
    6.4.3 Thermally-evoked activity of DDH WDR neurons is not altered by bone cancer .................................................................................................................................................. 121
    6.4.4 Tonic descending noradrenergic controls are altered in a rat model of CIBP .................................................................................................................................................................................. 123
    6.4.5 Spinal opioid system mediates tonic noradrenergic actions in the late stage CIBP rats .................................................................................................................................................................................. 125
  6.5 Discussion ........................................................................................................................ 126
    6.5.1 Bone cancer recruits more cold-responding neurons .................................................. 126
    6.5.2 Cold allodynia develops with the progression of bone metastatic disease 126
    6.5.3 Thermally-evoked activity of DDH WDR neurons is not altered by bone cancer .................................................................................................................................................. 127
    6.5.4 Tonic descending noradrenergic controls are altered in CIBP ................................. 127
    6.5.5 Spinal opioid system mediates tonic noradrenergic actions in the late stage CIBP rats .................................................................................................................................................................................. 128
  6.6 Author Contributions ........................................................................................................ 128
7. Chapter VII. General discussion............................................................................................ 131
References .................................................................................................................................... 139
Appendix 1 (related to Chapter 3) .......................................................................................... 151
Appendix 2 (related to Chapter 4) .......................................................................................... 159
Appendix 3 (related to Chapter 6) .......................................................................................... 161
Appendix 4 – on the CD (related to Chapter 3) ...................................................................... 162
List of figures

Figure 1.1. Sensory neuron classification proposed by Usoskin et al. 2014. based on the single cell whole RNA transcriptome analyses................................. 17
Figure 1.2. Peripheral mediators sensitise nociceptors after injury................................. 18
Figure 1.3. Spinal dorsal horn neurons classification based on the single cell whole RNA sequencing analyses by Häring et al. 2018................................. 21
Figure 1.4. Scheme representing the adrenergic receptor classes and their downstream signalling.......................................................... 23
Figure 1.5. Cancer-induced bone pain (CIBP) – main features associated with CIBP conditions .................................................................................. 27
Figure 1.6. Schematic representation of bone anatomy.................................................... 30
Figure 3.1. A high proportion of deep body CGRP afferents express little or no Advillin ............................................................................. 55
Figure 3.2. Cancer progression affects bone innervation.................................................. 57
Figure 3.3. The number of mechanically-responsive sensory neurons is tripled in animals with bone cancer............................................................. 60
Figure 3.4. Leg compression and position are differentially coded by DRG sensory neurons ................................................................................. 63
Figure 3.5. Intra- and peri-tibial afferent function in health and bone cancer.............. 65
Figure 4.1. Rats with bone cancer present secondary mechanical hyperalgesia. ... 77
Figure 4.2. Deep dorsal horn wide-dynamic range neurons are not hyperexcitable in our rodent model of CIBP................................................................. 78
Figure 4.3. Tonic descending noradrenergic controls are altered in our rodent model of CIBP............................................................... 80
Figure 4.4. Inhibition of mechanosensation by spinal α2-ARs block depends on a functioning opioid system in the late stage CIBP rats................................. 83
Figure 4.5. Pharmacological blockade of the locus coeruleus in the late stage CIBP rats abolishes spinal atipamezole-mediated inhibitions................................. 85
Figure 4.6. Spinal noradrenaline originates from the locus coeruleus. ....................... 86
Figure 4.7. Optoactivation of lamina I projection neurons inhibits deep dorsal horn wide dynamic range neurons.............................................. 89
Figure 4.8. Schematic representation of the suggested mechanism.................................. 94
Figure 4.9. Proposed mechanism of spino-pontine-spinal loop.................................. 99
Figure 5.1. DNIC expression in early and late stage of CIBP.......................................... 104
Figure 5.2. Spinal α2-AR controls DNIC expression in early CIBP................................. 105
Figure 5.3. Spinal α2-AR controls DNIC expression in late CIBP................................. 106
Figure 5.4. Spinal opioid receptors do not control DNIC expression......................... 107
Figure 5.5. Spinal α2-AR control of DNIC do not depend on spinal opioids.............. 108
Figure 5.6. Locus coeruleus activity does not influence DNIC expression................. 109
Figure 6.1. The recruitment of cold-responsive neurons is enhanced in bone cancer; Cold allodynia develops with the progression of bone metastatic disease........ 119
Figure 6.2. Thermally-evoked activity of DDH WDR neurons is not altered by bone cancer.......................................................... 121
Figure 6.3. Tonic descending noradrenergic controls are altered in CIBP..................... 122
Figure 6.4. A spinal opiodergic system modulates tonic noradrenergic actions in the late stage CIBP rats.............................................................. 124
In Appendices:

Appendix 1:
- Figure S.1.1……………………………………………………………….151
- Figure S.1.2……………………………………………………………….152
- Figure S.1.3……………………………………………………………….153
- Figure S.1.4……………………………………………………………….154
- Figure S.1.5……………………………………………………………….155
- Figure S.1.6……………………………………………………………….156
- Figure S.1.7……………………………………………………………….158

Appendix 2:
- Figure S.2.1……………………………………………………………….159
- Figure S.2.2……………………………………………………………….160

Appendix 3:
- Figure S.3.1……………………………………………………………….161
- Figure S.3.2……………………………………………………………….162

List of tables

Table 1.1. Peripheral nerve fibres characteristics .............................................. 16
Table 4.1. Pharmacological comparison of selected alpha-2 adrenergic receptors antagonists. .................................................................................. 79
Table 4.2. Comparison of noradrenaline (NA) affinities to selected adrenergic receptors. .................................................................................. 93
Table 7.1. Analysis of public dataset of single cell RNA sequencing of spinal inhibitory (GABA) and excitatory (GLUT) neurons.............................................. 133
Table 7.2. Suggested differences between selected factors underlaying pains with different aetiology................................................................. 137

List of other materials

In Appendix on the CD:

Appendix 4:
- Movie 1……………………………………………………………….162
- Movie 2……………………………………………………………….162
- Movie 3……………………………………………………………….162
- Movie 4……………………………………………………………….162
Chapter I
Chapter I. General introduction

1.1 Nociception and pain

Acute, chronic, burning, throbbing, stabbing, stinging, shooting, crushing...

There are many words to describe pain. But what is pain? Why can the experience of pain be described with so many different words? Does every organism experience pain? Can we truly understand another’s unique level of pain perception? Why do we need pain?

According to the International Association for Study of Pain (IASP), pain is: “An unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage”. This substantially differs from the definition of nociception (also known as nociperception), from the Latin nocere ‘to harm or to hurt’. Accordingly, IASP define nociception as: ‘The neural process of encoding noxious stimuli’, which does not necessarily imply the presence of a painful sensation. Nociception triggers a variety of physiological and behavioural responses to the noxious stimulus and pain usually follows in sentient beings. The multifaceted aspect of pain experience is difficult to judge in non-communicating subjects. In rodents, pain is often studied as the approximation of the measures of nociception (i.e. by measuring time required for the paw withdrawal following non-noxious and noxious heat). Whether the measures of nociception are associated with a full cognitive and emotional experience, which jointly defines the percept of pain, can be partly judged from other behavioural readouts (i.e. place avoidance in which the animal experience pain following similar noxious stimuli as in the measures of nociception). Some stimulus-independent tests (i.e. gait or weight bearing in unilateral pain models) are believed to analyse both nociception and non-evoked ‘pain’ (Deuis et al., 2017). Utilisation of human pain disease models and their behavioural verification with ‘gold-standard’ analgesics (i.e. morphine) could also be informative in a sense of the overall ‘painful’ experience and underlying mechanisms.

Broadly simplified, pain can be divided into 3 categories: nociceptive (caused by mechanical, thermal or chemical stimulation of sensory nerve fibres), neuropathic (caused by damage or disease affecting any part of the somatosensory system), and idiopathic (evoked by unidentified cause) pain.

*In the presented thesis, nociception was the main subject of studies. Pain as such, could have been judged based on the behavioural responses of investigated rodents, as I consider these animals quails (sentient) beings. Most of the presented studies of nociception, at different levels of the pain neuroaxis, are a good approximate of the pain perceived by patients.*
Based on the duration, pain can be **acute** or **chronic**. Adaptive reaction to acute painful stimulation usually results in fast withdrawal of the affected body part from the stimulus. Acute pain has an extremely important function since it preserves bodily integrity immediately following a peripheral injury. While the ability to react to a tissue-damaging stimulus is evolutionary conserved throughout all the life kingdoms, the ability to modulate (either inhibit or facilitate) an incoming impulse is restricted to more complex neuronal systems. Here, other factors including the emotional wellbeing of an individual, plays a significant role. Therefore, it is now widely accepted that pain experience is complex with sensory-discriminative, affective-motivational, and cognitive-evaluative components (Melzack, 1999).

In specific situations, pain becomes chronic thus outliving its biologically advantageous ‘acute’ effect. Chronic pain is frequent. Around 19% of European adults experience chronic pain, and 59% of them suffer for at least 2-15 years. Analgesic treatment of chronic pain is insufficient in over 40% of cases (Breivik et al., 2006) and, in the best case scenarios, only 1 in 3 patients achieve over 50% of pain reduction (Finnerup et al., 2010). Diseases that result in chronic pain are often dynamic in the sense of their progression and follow-up symptoms. Heterogenous disease progression (and associated pains) results in a great variation of number needed to treat (NNT) between disease states and therapeutics used for pain management. Pain chronicity frequently drags behind an avalanche of negative psycho-social events. Depression, anxiety and sleep disorders are amongst the most common co-morbidities impacting social and economic aspects. They also complicate the therapy, both due to the complex pharmacology needed for management of multisymptomatic illness as well as the decreasing willingness of patients to undergo treatment (Nicholson and Verma, 2004).

Chronic pain translates to public burden of $250 billion annually in the US alone, while in Europe it is estimated to cost €390-1300 billion a year (which renders 3-10% of gross domestic product cost) (Breivik et al., 2013). It is crucial to understand pain mechanisms such that, in turn, disease-specific pain management may be facilitated, and the socioeconomic burden eased. **Mechanistically, how do we get pain?**

Chronic pain manifests at peripheral sites, at the level of the spinal cord, and in the brain. This thesis will first introduce the basic anatomy of these different locations and how they have been implicated in the processing of chronic pain, before discussing the more specific case of cancer-induced bone pain.
1.2 Periphery: A somatosensory nervous system

1.2.1 Classification

A typical pain pathway involves activation of the peripheral fibres: Aδ- and C-fibres that carry noxious sensory information, as well as Aβ-fibres that carry non-noxious sensory information. Each of these fibres possesses different characteristics that allow segregated transmission of sensory information (Table 1.1). Dorsal root ganglia (DRG) and trigeminal ganglia (TG) house cell bodies of virtually all sensory neurons innervating the body and the head, respectively. The afferent branch of these pseudounipolar neurons terminates in the dorsal horn (DH) of the spinal cord. The dorsal horn can be divided histologically into layers called Rexed laminae (Rexed, 1954). Aδ- and C-fibres transmit information mainly to nociceptive-specific neurones in Rexed lamina I and II, whereas Aβ-fibres terminate in deeper laminae (Millan, 1999). Aδ-fibres can also send deep dorsal horn projections within lamina IV-V. Complex interactions occur in the dorsal horn between afferent neurones, interneurones and terminals of descending modulatory (either inhibitory or facilitatory) pathways. These interactions determine net activity of the secondary afferent neurones. Followed by activation of the second order neurons in the spinal cord, the nociceptive impulse propagates towards multiple brain regions where it is further integrated. Therein a percept of pain may be generated.

Table 1.1. Peripheral nerve fibres characteristics.

<table>
<thead>
<tr>
<th>Fibre type</th>
<th>Axone diameter [μm]</th>
<th>Myelination</th>
<th>Conduction velocity [m/s]</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ fibres</td>
<td>Large (&gt;10)</td>
<td>Thickly myelinated</td>
<td>Fast (30–100)</td>
<td>Low activation threshold and usually respond to light touch and transmit non-noxious stimuli</td>
</tr>
<tr>
<td>Aδ fibres</td>
<td>Medium (2–6)</td>
<td>Thinly myelinated</td>
<td>Intermediate (12–30)</td>
<td>Responding to mechanical and thermal stimuli. Carrying rapid, sharp pain and are responsible for the initial reflex response to acute pain</td>
</tr>
<tr>
<td>C fibres</td>
<td>Small (0.4–1.2)</td>
<td>Unmyelinated</td>
<td>Slow (0.5–2.0)</td>
<td>Polymodal; responding to chemical, mechanical and thermal stimuli. C fibre activation leads to slow, burning pain</td>
</tr>
</tbody>
</table>

There is also another system, separate to the neuroanatomical one, that classifies primary afferent neurons based on their molecular profiles. This complex and developing histochemical taxonomy provides us with the information that, for example, the heavy chain 200 kDa-neurofilament (NF200) positive cells are A-fibre neurons, whereas C-fibres can be split broadly into three groups based on their expression of calcitonin gene-related peptide (CGRP) – a peptidergic C-fibres; isolectin B4 (IB4) – a
non-peptidergic C-fibres (Snider and McMahon, 1998); tyrosine hydroxylase – a low-threshold mechanoreceptor C-fibres (Li et al., 2012).

Peripheral neurons were more recently clustered according to their genetic profile. Primary sensory neurons for example were divided into eleven types with significantly different molecular properties (see Fig. 1.1 for details) (Usoskin et al., 2014). Both single-cell and collective high-throughput RNA sequencing databases were generated providing unprecedented information relating to the whole-transcript characteristics of primary afferents in health and disease, as well as with regard to an animal’s sex. These data are open-access and ready for mining (Lopes et al., 2017a, 2017b; Usoskin et al., 2014; Zeisel et al., 2018) and often tightly fit with previous experimental findings highlighting roles of certain molecules involved in pain perception and transmission. These data can also facilitate our understanding of the primary afferent’s biology, providing more precise markers for discrete neuronal populations (like specific for bone afferents). This in turn will allow a more selective targeting for both research and for therapy.

Peripheral neurons express a plethora of pain-signalling targets. Pain can originate in the periphery, where pain sensors are activated when tissue is damaged. Chemicals released include the prostanoids, bradykinin, nerve growth factor (NGF), CGRP, and ATP, as well as many cytokines and chemokines. Multiple molecules are also to sense immediate physical damage that may be thermal (i.e. Trpm8 for cold and Trpv1 for hot) or mechanical (Piezos, some Trp channels) in nature. The release of numerous tissue-damage mediators leading to vasodilation and loosening the blood

Figure 1.1. Sensory neuron classification proposed by Usoskin et al. 2014. based on the single cell whole RNA transcriptome analyses. Gene products in red are new suggested markers and in black – markers previously known. The bottom panel points at markers used in the classical histological characterisation. LTMR-low-threshold mechanoreceptor, C-LTMRC-fibre LTMR. Myel.-myelinated, Unmyel.-unmyelinated. Taken from Usoskin et al., 2014.
vessels epithelium allowing for an infiltration of inflammatory cells often further enhances pain signalling. These local events sensitize entrapped primary afferents, lowering pain thresholds of the affected area and consecutively activating second order neurons within spinal dorsal horn (Fig. 1.2).

Figure 1.2. Peripheral mediators sensitise nociceptors after injury. After tissue damage a variety of mediators are released by local and blood/immune cells and these may sensitise primary afferents. Activated nociceptors have lowered excitability thresholds (peripheral sensitization) and release transmitters in the central terminals inducing central sensitization. AC-adenyl cyclase, PLC-phospholipase C, His-histamine, NGF-nerve growth factor, PG-prostaglandins, NO-nitric oxide, ATP-adenosine triphosphate, SP-substance P, CGRP-Calcitonin gene-related peptide, NA-noradrenaline, 5-HT-serotonin, Glu-glutamine, [Ca\textsuperscript{2+}]-intracellular calcium concentration. Modified after Donovan-Rodriguez T. PhD thesis, 2005.

The ensuing signal propagation relies on the presence of ion channels, some of which are more specific for pain transmission than others. Amongst a large family of sodium channels, NaV1.7 and NaV1.8 are proposed to be preferentially expressed by small diameter fibres. These large pore-forming protein complexes allow for sodium influx leading to neuronal membrane depolarization and facilitation of nociceptive
information propagation along C-fibres. Human and animal channelopathies resulting from these two channels mutations can lead to the loss of function (pain insensitivity) or gain of function (pain hypersensitivity) (Minett et al., 2014). There is considerable interest in finding specific blockers for these channels to selectively abolish nociceptive transmission without affecting other sensations not least since, following nerve injury, these channels tend to be upregulated. Also, following nerve injury, inhibitory potassium channels are downregulated on the afferents, suggesting an alternative therapeutic target. Unfortunately, similarly to sodium channels, selective potassium channel modulators remain elusive.

Another attractive targets for pain therapy are calcium channels. This large protein superfamily received particular attention in pain management following the discovery of the analgesic properties of intrathecally administered ω-conotoxins that target CaV2.2 and lead to a decrease in neurotransmitter release from central terminals of primary afferents (Snutch et al., 2001). Furthermore, an anticonvulsant, gabapentin, was found to selectively block nociceptive responses in neuropathy, inflammation and CIBP models by binding to the regulatory α2-δ subunit of voltage-gated calcium channels and altering its movement to the cell membrane (Chapman et al., 1998; Donovan-Rodriguez et al., 2005; Matthews and Dickenson, 2002; Stanfa et al., 1997). Gabapentin is widely used in clinics to treat neuropathic pain and preferentially inhibits evoked rather than on-going pain, forming a basis for differentiation of patients who might respond to them (Patel and Dickenson, 2016). Also, new analgesics were developed to selectively target modality-selective sensing channels. Namely Trpv1, Trpm8 and Trpa1 sensors are amongst the main targets.

1.3 Central processing of somatosensory information

1.3.1 Spinal cord anatomy

Incoming nociceptive information is first relayed within the dorsal horn of the spinal cord. Laminae I-VI composes the sensory dorsal horn and laminae VII-IX form the motoneuronal ventral horn. The central canal milieu is known as laminae X. A large region of the dorsal horn with the gelatinous appearance (known as substantia gelatinosa of Rolando) due to the low concentration of myelinated fibres encompass lamina II. Substantia gelatinosa (SG) can be further subdivided into functionally distinct outer (IIo) high cellular density part receiving nociceptive input, and inner (Iii) part receiving innocuous input (Brown, 1982).

Sensitized peripheral nerves release neurotransmitters on the spinal sites to activate second order neurons therein. Painful impulses are mainly relayed by nociceptive-specific (NS) neurons, the vast majority of which reside within superficial
dorsal horn (SDH). Another type of cell, **wide-dynamic range** (WDR) neurons of deep dorsal horn (DDH) lamina IV-V, are multi-receptive, with large cell bodies, extensive dendritic spreads in all directions and axons that ascend in the contralateral ventral white matter (Ritz and Greenspan, 1985). Some of these WDR cells can be also found within SDH. These are convergent cells responsive to all somatosensory modalities (thermal, chemical and mechanical), and a broad range of stimulus intensities fed in from peripheral nerves. Further, WDR neurons code stimuli in such a way that their firing rate increases as the stimulus intensity rises to the noxious range. The continuous stimulation of C-fibres leads to the activation of NMDA receptors in the corresponding spinal cord DH causing an event known as **pain wind-up**, which is an increase in pain intensity over time when a given stimulus is delivered repeatedly above a critical rate. This is a specific form of **temporal summation** which occurs at the spinal site.

Within laminae IV-VI, WDR neurons synapse mainly with Aβ-fibres and with some Aδ-collaterals and they have also been shown to possess dendritic projections to the SDH where they can receive direct input from nociceptors. Classically, DDH WDR neurons receive C-fibre input via **interneurons**. Interneurons play key role in the responsiveness of DDH WDR neurons. Residing mainly in lamina II and III, interneurons mediate impulses between superficial and deep laminae and have a potential to modulate the incoming messages. In total, the majority of SDH neuronal cells are interneurons (up to 98% of lamina I-III) (Graham et al., 2007). Interneurons can be broadly classified as excitatory (glutamatergic; around 75% of SDH lamina I and II cells) and inhibitory (both GABA- and Glycinergic; around 25% of SDH lamina I and II neurons) (Polgár et al., 2013).

Aside from an anatomical and electrophysiological classification, DH cells can be classified based on their morphology into islet (always inhibitory), radical, vertical (both mainly excitatory) and central types. DH neurons can be also divided based on their expression patterns of several unique markers for inhibitory and excitatory cells (Todd, 2010). As for the primary afferents, recent attempts utilising high-throughput single-cell RNA sequencing and various murine transgenic lines phenotyping revealed differential organisation of the spinal cord and allowed for in-depth classification of DH neurons (Abraira et al., 2017; Häring et al., 2018). In that according to Haring et al. there are 15 inhibitory (Gaba) and 15 excitatory (Glut) distinct interneuron classes within the DH (Häring et al., 2018). Some of these classes tightly reflect previously established classes of spinal interneurons based on morphological, cytochemical and electrophysiological properties (Fig. 1.3).
1.3.2 Ascending pathways

The pain signal is transduced by the DH secondary projection neurones to the higher pain centres via ascending tracts. Almost all the ascending tracts decussate either at the spinal or at the medullary level. Two main tracts, the spinothalamic and the spinoreticular, predominate in the transmission of ‘pain’ signals. In primates, the former involves lamina I (50%), lamina IV-V (25%) and lamina VII-VIII (25%) projection cells, ascending via the contralateral tract to nuclei within the thalamus (Trevino and Carstens, 1975). Interestingly there are some species differences, and in the rat thalamic input from lamina V dominates (Todd, 2002; Todd et al., 2002). Third order thalamic neurones terminate in the somatosensory cortex (mainly insula and anterior cingulate cortex). There are also projections to the periaqueductal grey (PAG). Ultimately the spinothalamic tract transmits signals that are important for pain localisation.
Comparatively the spinoreticular tract involves mainly laminae VII and VIII and also laminae V, I and X as well as lateral spinal nucleus projection cells. This tract also decussates in the cord and ascends, contralaterally, to the brainstem reticular formation, before projecting to the thalamus, hypothalamus and amygdala (Menétrey et al., 1983), with many further projections to the cortex. This pathway is involved in the emotional aspects of pain. Other parallel ascending pathways involving, for example the lateral spinal nucleus and dorsal column, exist also, although uncertainty exists regarding their exact role in pain perception. Spinomesencephalic and spinoparabrachial tracts will be described later in research chapters IV and VI.

1.3.3 Descending modulation of the sensory input

Descending pathways, which modulate the responses of both SDH and DDH neurons, can be broadly divided into facilitatory or inhibitory descending modulatory controls. The former are, broadly speaking, mainly orchestrated via activation of spinal 5-HT3 receptors when serotonin is released from the midbrain/brainstem efferents, while the latter are primarily driven by the actions of brain-stem derived noradrenaline acting on spinal α2-adrenergic receptors.

The periaqueductal grey (PAG) is considered a central hub that coordinates descending pain controls and thus maintains a balance between descending facilitatory and inhibitory drive to the spinal DH. A reciprocal connection exists between PAG and cortex, amygdala, pontine nuclei (parabrachial nuclei or the LC), as well as medullary cell groups (eg. RVM) (Mantyh, 1983b, 1983b, 1983a; Mantyh and Kemp, 1983; Mantyh and Peschanski, 1983; Millan, 2002). The majority of PAG actions are relayed via other nuclei localised caudally to the PAG (like the LC or RVM), and direct projections from this structure to the spinal cord are sparse (Bajic et al., 2001; Heinricher et al., 2009). Projections from the limbic structures to the PAG suggest a possible route via which affective components of the pain experience can influence nociception (Heinricher et al., 2009; Keay and Bandler, 2001).

1.3.3.1 Noradrenergic controls

Noradrenaline orchestrates its action via adrenergic receptors (AR). ARs belong to the G-coupled receptor superfamily (GPCRs) and can couple with either inhibitory or facilitatory guanine nucleotide-binding proteins (G-proteins). Two main classes of adrenergic receptors exist: α and β. In the spinal cord only the α type is expressed. The α-ARs can be further subdivided into α1 and α2, where the former couple with Gq/11 proteins and are facilitatory, while the latter couple with heterotrimeric Gi proteins and are thus inhibitory. Each of the ARs can be further subdivided based on their coding
sequences in the genome, resulting in up to 9 known ARs (Fig. 1.4, see more about the receptors expression and localisation in the spinal cord in chapter IV and VII).

Figure 1.4. Scheme representing the adrenergic receptor classes and their downstream signalling. cAMP-cyclic adenosine monophosphate, PKA-protein kinase A, IP3-inositol trisphosphate, PLC-phospholipase C, DAG-diacylglycerol, CaM-kinase-calmodulin kinase, PKC-phosphokinase C.

Noradrenergic projections originate largely from pontine noradrenergic A6 (locus coeruleus), A5 and A7 cell groups (Kwiat et al., 1992). The main source of spinal noradrenaline is the locus coeruleus (LC) (Aston-Jones et al., 1986; Jones and Gebhart, 1986; Jones SL, 1986; Miller and Proudfit, 1990; West et al., 1993). In rats, this bilateral pontine nucleus holds up to 3000 noradrenergic neurons. Projections from the LC to the DH of the spinal cord are well documented, terminating mainly within the SDH and around the central canal. Terminal sites are scarce in the DDH (Kwiat et al., 1992). Electrical stimulation of the LC was shown to be analgesic in naïve rats, and this action was reversible following spinal block of $\alpha_2$-ARs (Jones and Gebhart, 1986; Miller and
Proudfit, 1990). Recent opto- and chemogenetic studies exposed the LC as a heterogenous structure; dorsal LC neurons project to the medial prefrontal cortex and are pro-nociceptive while ventrally positioned neurons within the LC are antinociceptive and project to the spinal cord (Hickey et al., 2014; Hirschberg et al., 2017).

Since the LC is strongly interconnected with other brain and spinal regions there are numerous feedback loops, including those that form dynamic descending modulatory pathways. These projections can control spinal excitability during extended noxious stimulation (Pertovaara, 2006). Descending noradrenergic axons have marginal connections with the central terminals of sensory afferents (Hagihira et al., 1990). Interestingly, these descending fibres rarely form axon-somatic synapses within the spinal DH and the majority of them resemble ‘free endings’. This axonal organisation suggests a global release of noradrenaline in the DH, thus an unrestricted volumetric transmission to a synapse in question (Rajaofetra et al., 1992).

Descending noradrenergic inhibitions are particularly engaged during prolonged noxious stimulation (Azami et al., 2001). In murine α2A, α2B, and α2C receptor knockouts normal mechanical and heat pain thresholds are preserved (Malmberg et al., 2001). Another study, utilising either selective ablation of noradrenergic neurons with saporin-dopamine-β-hydroxylase conjugate or pharmacological block of spinal α-ARs, did not report an effect on acute nociception (Hylden et al., 1991; Jasmin et al., 2003). These results suggest that noradrenaline does not have a major role in the tonic control of spinal neuronal excitability.

1.3.3.2 Serotonergic controls

Serotonin (or 5-hydroxytryptamine, 5-HT) acts via serotonin receptors. There are seven known classes of these receptors (5-HT1-7) and each class can be further subdivided yielding a vast diversity of potential targets. All bar 5-HT3 receptors are metabotropic GPCRs. Many are expressed across the neuroaxis, as well as in the periphery. There are complex 5-HT receptor interactions at the spinal level since, similarly to the ARs, some are inhibitory and some facilitatory. Upon binding to the ligand-gated 5-HT3 receptor, serotonin opens the central pore allowing fast influx of sodium ions and cell excitation; 5-HT3 mediates facilitatory actions of descending serotonin in the DH of the spinal cord (Bannister et al., 2017; Suzuki et al., 2002).

Serotonergic projections originate chiefly from the rostroventromedial medulla (RVM), where nuclei including the nucleus raphe magnus (RMg), nucleus raphe pallidus (RPa) and gigantocellular reticular nucleus (Gi) are located. Since parallel spinal facilitatory and inhibitory pathways originate from the RVM, electrical stimulation of this structure can have a confounding effect on the excitability of spinal DH neurons (Zhuo...
et al., 1992). However following stimulation of the RVM an inhibitory drive predominates (Basbaum et al., 1976). In naïve animals, lidocaine block of the RVM inhibits excitability of the spinal DH, pointing at the net facilitatory output from this structure (Bee and Dickenson, 2007; Rahman et al., 2006). The RVM is strongly interconnected with other brain regions including the hypothalamus (lateral part and PVN), PAG, amygdala and parabrachial nuclei (Hermann et al., 1997).

Electrophysiologically speaking, neurons within the RVM are incredibly heterogenous. ‘ON-cells’ fire action potentials following noxious stimulation but before the withdrawal reflex occurs. ‘OFF-cells’ fire action potentials constantly but stop abruptly when the ON-cells become active. Meanwhile ‘neutral-cells’ fire continuously. The ON-cells are therefore believed to mediate descending facilitation, whereas OFF-cells are said to mediate inhibitory actions, important for opioid-driven analgesia (Fields et al., 1983a, 1983b; Heinricher et al., 1989).

Serotoninergic controls were previously shown exaggerated in a rat model of CIBP, potently facilitating the excitability of both SDH and DDH WDR neurons (Donovan-Rodriguez et al., 2006). It was also shown that, following electrostimulation of the RVM, there is a substantial release of 5-HT at spinal sites (Bourgoin et al., 1980). Again, analogically to noradrenaline, spinal serotonin acts volumetrically; there are no synaptic contacts with primary afferent terminals, but presynaptic receptors for 5-HT are abundant (Li et al., 1998). The existence of synapses between RVM efferents and spinal DH neurons however is well described. RMg is the main source of spinal serotonin, but 5-HT positive neurons often colocalise with other neurotransmitters including GABA, glycine and acetylcholine (Millan, 2002). Therefore, the actions of other transmitters could be restricted to the spinal synapses. Indeed, GABAergic neurons originating from the RVM were shown to act locally, within synapses in the SDH lamina I and IIo. For this study authors utilised selective trans-synaptic labelling with rabies virus to show that RVM GABAergic neurons can facilitate mechanical pain by inhibiting spinal enkephalinergic/GABAergic interneurons (François et al., 2017). This research, amongst others, highlights the existence of descending controls that mediate effect independently from the monoamines.

**1.3.4 Sensory coding**

Through the centuries coding of sensory information was a subject for many philosophical debates. How modality-reflecting information is encoded in the sensory nervous system is still not unequivocally resolved. Recent technological advancements are now available to facilitate our understanding of the sensory code of our bodies.

Two main concepts were introduced to explain somatosensory coding. First, the concept of **graded coding** can be seen as the number of cells being activated and their
strength of activation, namely firing frequency. In contrast, combinatorial coding describes a situation where information is encoded by a matrix of cells being activated. I believe that combinatorial coding can be extended to the firing patterns of a cell, namely if neuron fires intermittently, or constantly, or with longer or shorter gaps etc. Recent insights into the temperature coding by DRG cells revealed that heat is coded in a graded fashion as opposed to cold, which seems to be rather combinatorial (Wang et al., 2018). Coding of mechanical forces, especially by a deep body afferents, remains poorly studied on the populational level (Cordo et al., 2011).

1.4 Cancer-induced bone pain

A cancer diagnosis inevitably has a large emotional toll on patients, who not only have to confront their own mortality, but also must suffer a whole range of unpleasant side effects resulting from cancer growth and/or treatment. One very significant side effect of certain cancers is severe, chronic pain. Improvement in the early cancer diagnosis of some cancers (mainly breast, prostate and colon carcinomas), followed by aggressive therapy often leads to long (over 5 years) disease-free survival from cancer (Edwards et al., 2005; Jemal et al., 2004; Mantyh, 2006a). Diagnosis of lung, pancreatic or ovarian cancers is usually delayed translating to disease progression. For these tumours the first signal of the disease is pain (Jemal et al., 2004) and most patients will experience cancer-related pain in the progress of the disease. Cancer pain results from the neoplastic disease by itself, and also from its treatment (chemo-, radiotherapy etc.) and diagnostic procedures (biopsies, resections etc).

Bone cancers can be broadly classified as primary (mainly sarcomas, and osteosarcomas, but also leukaemia) or secondary (metastatic disease). Each type of cancer has unique characteristics, which dictate treatment. Some tumours disrupt bone integrity by digesting (osteolytic) or depositing (osteoblastic) bone. Others are responsive to a specific chemotherapeutic, or combination of different drugs and radiotherapy. It is believed that the chronic pain resulting from different cancer types has its own characteristics, implying that personalised treatment is the optimal way to sufficiently target the pain. Malignant tumours can escape their original place of birth and invade distant places of the body to form secondary tumours. A frequent direction for metastasis is the skeleton. Around 75% of patients with advanced bone cancer will suffer from bone metastatic pain (Foley, 2004).

Breast and lung cancers are commonly recognized as highly metastatic neoplasms, particularly targeting skeleton to develop secondary tumours. Cancer metastases to bone usually induce chronic bone pain that significantly alters the patients’

*Time after treatment during which no cancer is found.
The quality of life (Mantyh, 2006a; Wu et al., 2017). What is more, unlike other types of cancer that also extensively spread to the skeleton, breast cancer affects women who are in the middle of their careers. Men, however, more seldom, can also be affected by breast carcinomas. Nowadays, a survival rate following the breast cancer treatment is significantly increased, leaving patients with long-lasting pain even if the cancer itself was successfully challenged (Bloom et al., 2011; Foley, 2004). Recent findings have shown the complexity of the bone-cancer-associated pain, including a convoluted relationship between inflammatory and neuropathic pain mechanisms with some unique changes occurring at peripheral and central sites (see Fig. 1.6 for further cancer-induced bone pain characteristics) (Falk and Dickenson, 2014).

**Complex nature of CIBP:**
- **Additional CIBP hallmarks**
- **Neuropathic pain**
  - **Inflammatory pain**
  - Increased innervation of tissue
  - Compression and invasion of nerves by tumor cells
  - Breakdown of bone
  - Distention and denervation of sensory fibers or neurons
  - Altered ion channel function
  - Chemical activation of nociceptors by mediators
  - Mediators produced by damaged tissue and immune cells
  - Increased levels of cytokines and growth factors
  - Altered vascular events
  - Peripheral sensitization
  - Enhanced inputs into spinal cord
  - Gain of somatosensory function
  - Induction of central sensitization and windup
  - Increased transmission to sensory and affective brain areas
  - Gain of descending facilitation
  - Ongoing and evoked pain
  - Comorbidities

**Figure 1.5. Cancer-induced bone pain (CIBP) – main features associated with CIBP conditions.** Red-inflamatory pain features, blue highlighted-neuropathic pain features, green-CIBP hallmarks. Followed by Falk S. and Dickenson AH, 2014.

Cancer pain remains a major area of unmet medical need, with few studies existing in proportion to that need. One common form, affecting 400,000 people each year in the US alone, is associated with skeletal metastases. These pains are typically mechanoeceptive in nature and poorly managed by available analgesics. Cancer-induced bone pain (CIBP) is a mix of nociceptive and neuropathic/inflammatory pain, which can persist even in remission (Clohisy and Mantyh, 2003; Mantyh, 2006b; Mantyh et al., 2002). CIBP is unique in that the sufferers experience tonic, spontaneous and movement-evoked pain. The background (tonic) pain intensity typically increases with the progression of the disease. It is often described as a dull, continuous pain. This
component of CIBP is often manageable by common analgesics. Spontaneous and movement-evoked pains are also referred as “break-through” or suprathreshold pains. Unlike the background pain, these types of pain, being mechanoceptive in nature, are difficult to manage in mobile subjects, as by definition, they ‘break-through’ the barriers of analgesia (Mantyh et al., 2002). Since they are also unpredictable, it is extremely challenging to suit sufficient therapies without adverse effects of high doses of painkillers being continuously administered.

The chronic nature of CIBP (including its persistence in remission) requires particular attention, since as in any chronic pain type, prolonged treatment with analgesics results in multiplicity of side-effects. Due to the severity, CIBP are typically managed with opioids, the prolonged usage of which inevitably leads to the development of drug-tolerance and associated side-effects (i.e. dizziness, drowsiness, addiction, heavy constipation, and in severe cases opioid-induced hyperalgesia). Therefore, understanding the underlying mechanisms of CIBP is crucial to suit successful, mechanism-based treatment (see section 1.4.2.).

CIBP is also dynamic. It often mirrors the disease progression and associated side-effects of the state-dependent treatment. With a high degree of bone tissue damage, locally-entrapped afferents in ischemic and toxic conditions, CIBP echoes distinctive and rapid modifications to the tissue. Complicating the clinical picture, CIBP does not always correlate with the number or size of metastatic sites: on one occasion certain malignancies are entirely painless, whereas others constitute the roots of severe pain. This likely reflects the dialogue between peripheral and central aspects of CIBP in that multiple metastases are not necessarily more painful than a single cancer-evoked lesion to the bone. This could be explained by the adaptation of the CNS to increasing painful signals from the periphery (Falk and Dickenson, 2014). For example, unlike nerve trauma models of neuropathy, spinal dorsal horn and DRG levels of substance P and CGRP are not altered in CIBP (Honoré et al., 2000). Other central mechanisms have been described in the course of CIBP development, including the altered ratio of WDR to NS cells in the dorsal horn or increased superficial dorsal horn neurons’ excitability, likely mediated by a facilitatory action of descending serotonin (see more in Chapter IV, V and VI) (Donovan-Rodriguez et al., 2004).

The past decade brought new ways of studying CIBP. New models closely reflecting the clinical picture of the disease were developed allowing the precise studies of the disease-underlying mechanisms (see section 1.4.2.) (Medhurst et al., 2002; Schwei et al., 1999). These models recapitulate anatomical (bone damage: both bone lesion and deposition (Halvorson et al., 2006; Honoré et al., 2000), neuronal sprouting (Jimenez-Andrade et al., 2011; Mantyh et al., 2010a), physiological (acidification,
necrosis, allodynia, hyperalgesia (Donovan-Rodriguez et al., 2005; Halvorson et al., 2006; Honore et al., 2000; Honoré et al., 2000; Sabino et al., 2002) and pharmacological (morphine (Jemal et al., 2004; Urch et al., 2005), anti-NGF (Jimenez-Andrade et al., 2011; Sopata et al., 2015) profiles of the human disease (Falk and Dickenson, 2014; Mantyh, 2006a).

One of the obstacles current and past physiological studies of CIBP face is the non-invasive way of stimulating bone afferents. Achieving direct bone afferents’ stimulation with natural stimuli (i.e. pressure), while maintaining a physiological and anatomically-intact system is extremely challenging. The Randall-Sellito test was proposed as a good stimulus-induced behavioural measure of bone afferents’ activation in health and in the rat model of CIBP (Falk et al., 2015d). Other behavioural measures of CIBP included: weight bearing (likely reflecting both primary afferents activity as well as overall CNS adaptation) (Falk et al., 2015d), rota-rod (movement-evoked pain) (Urch et al., 2003), von Frey filaments or acetone applied to the paw of the cancer-bearing leg (measure of central sensitisation) (Urch et al., 2003). In in vivo electrophysiological recordings of the tibial nerve, intratibial afferents were directly stimulated by the implantation of elastic tubing into the bone cavity and intraosseous pressure was increased by a surge of saline pumped into the sealed bone (Nencini and Ivanusic, 2017; Nencini et al., 2017). While this approach offers direct modulation and monitoring of the intraosseous pressure, it also has obvious limitations: extensive bone and surrounding tissue damage due to the tubing implantation, inability to activate periosteum afferents and those located close to the bone, inability to be used in models resulting in bone lesions (like CIBP). Therefore, in order to study bone afferents responses in physiological and pathological conditions, a new means of stimulation must be developed.

1.4.1 Bone innervation

Anatomically, every bone can be divided into cortical (compact outer layer) and trabecular (spongiform inner layer) segments. The former comprises almost 80% of the skeletal mass and is built from tightly packed osteons. A central part of each osteon is called a haversian canal which houses nerves and blood vessels. The canal is surrounded by lamellae within which the bone matrix (called lacunae) holds bone cells – osteocytes. There are also smaller canals between lacunae and haversian canal known as canaliculi, which allow blood vessels and nerves to be connected between osteons. Osteocytes can be typically divided into bone forming cells (osteoblasts) and bone resorbing cells (osteoclasts). Trabecular (or cancellous) bone has a high turnover rate, is elastic and far less dense than the cortical part. Blood vessels and nerves run between the cavities, where apart from their normal physiological functions, they can
regulate the osteoclastic activity (Hara-Irie et al., 1996). The densest network of nerves is found within the periosteum (Fig. 1.5) (Mach et al., 2002).

Figure 1.6. Schematic representation of bone anatomy. Taken from Pearson Education and Antranik.org and modified.

Bone is innervated mainly by small (C-fibres) and medium size (Aδ-) peptidergic fibres, and almost never by large, thickly myelinated Aβ-fibres. Bone innervation is particularly rich in peptidergic afferents and a triad of peptides, substance P (SP), CGRP and NGF predominate. Thinly and unmyelinated neurons can be broadly divided into two major classes: CGRP and isolectin B4 (IB4) positive. The former commonly co-express tropomyosin receptor kinase A (TrkA), a receptor for NGF, as well as substance P. All three peptides strongly contribute to nociception, and have been extensively reported to modulate CIBP (Nencini and Ivanusic, 2016a). As expected, bone afferents often stain positively for CGRP and the other two peptidergic markers, but are rarely positive for IB4, a marker for mainly non-peptidergic neurons (Bloom et al., 2011; Nencini and Ivanusic, 2016a; Nencini et al., 2017).

Over 80% of fibres innervating bone are TrkA positive (Castañeda-Corral et al., 2011). It has been also shown that, following bone cancer, sensory and sympathetic nerve fibres innervating bone undergo a pathological ectopic sprouting and
reorganization. These pathologically branched neurons, forming neuroma-like structures around the cancer-affected bone, are predominantly classified as CGRP positive sensory nerve fibres co-expressing TrkA and growth associated protein-43 (GAP43) (Bloom et al., 2011; Jimenez-Andrade et al., 2010a). A growing body of evidence supports the view that sprouting is mediated by NGF. NGF acts via TrkA, as well as via p75 neurotrophin receptor (p75NTR) that belongs to the TNF – receptors superfamily. Some studies illustrated the importance of NGF-mediated signalling by the NGF-sequestration strategies in CIBP pain (Bloom et al., 2011; Jimenez-Andrade et al., 2010a; Mantyh et al., 2010b). NGF is likely involved both in sensitizing TrkA+ sensory nerve fibres and ectopic sprouting in the diseased tissue. However, there are still controversies as to whether or not the mechanism of ectopic sprouting is mediated by binding of NGF to the TrkA or p75NTR receptor, or both.

1.4.2 Mechanisms of cancer-induced bone pain

Upon bone cancer conditions, tumour development stimulates an infiltration of inflammatory cells, as well as the release of numerous mediators including growth factors (particularly NGF), cytokines, interleukins, chemokines, prostanoids, and endothelins. Simultaneous ongoing bone remodelling in close proximity to cancer cell colonies leads to osteoblastic bone lesions, an acidification of the surrounding area (a reduction of pH to below 5) and direct deformation of primary afferents (of which extensive sprouting of sensory and sympathetic afferents is the most abundant) (Jimenez-Andrade et al., 2010a; Safieh-Garabedian et al., 1995; Sorkin et al., 1997; Suzuki and Yamada, 1994; Watkins et al., 1994). All these processes contribute to the development of cancer-induced bone pain.

NGF is a crucial component of sensitization of primary afferent nociceptors associated with tissue inflammation. In carrageenan-inflamed skin, NGF levels increase in inflamed tissue, and neutralization of endogenous NGF prevents the hyperalgesia that normally develops during inflammation of the skin (Koltzenburg, 1999).

Neurons and cancer cells are engaged in bi-directional crosstalk. For instance, cancer causes a reorganization of normal anatomy, driving neurons to sprout and more densely innervate the tumour-bearing bone (Bloom et al., 2011; Jimenez-Andrade et al., 2010c; Mantyh, 2006a). Conversely, neurons release factors which support tumour growth and vascularization (Boilley et al., 2017; Hayakawa et al., 2017; Toda et al., 2008). This complex dialogue involves numerous mediators and different local cells, including fibroblasts, osteoclasts and newly recruited immune cells (Hayakawa et al., 2017; Mantyh, 2002). Injection of canine prostate cancer cells into mouse bone induces a notable sprouting of CGRP+ and NF200+ sensory fibres (Bloom et al., 2011). What is more, almost all sensory nerve fibres that undergo sprouting additionally co-expressed
Thus, a new, dense neuronal network, developed around the cancer-affected bone, may lead to extensive firing at the first synapse in the DH due to an increased number of fibre branches receiving stimuli from the periphery. Consequently, it may lead to observed changes in the excitability of spinal neurones in CIBP.

The phenomenon of afferent sprouting is not restricted to bone cancer conditions. It has been reported in many soft-tissue tumours too (Hayakawa et al., 2017), as well as osteoarthritis (Jimenez-Andrade and Mantyh, 2012), bone breaks (Chartier et al., 2014; Koewler et al., 2007; Mantyh, 2018), etc. The exact role of neuronal sprouting in these conditions remains elusive. It is known that sprouted fibres have the potential to not only transmit sensory information to the CNS, but to also regulate events in situ by releasing various agents like CGRP (Toda et al., 2008). CGRP acting on local cells mediates vasodilation, neovascularization, inflammation etc. As mentioned earlier, nerves could also modulate the activity of osteocytes.

Afferents express receptors for inflammatory molecules including the cytokines and most importantly NGF, which has been demonstrated to mediate inflammatory pain (McMahon, 1996). Treatment targeting this molecule or its receptor – TrkA, is an elegant example of mechanism-targeted therapy. Currently there are several monoclonal antibodies targeting the NGF-TrkA system, for example tanezumab, fulranumab, and fasinumab, in the clinical trials for pain management. The analgesic effects reported are typically long-lasting (up to several weeks post single injection). Tanezumab, now in the 3rd phase of clinical trials, is shown to be effective for the management of osteoarthritic, chronic low back, diabetic peripheral neuropathic, and cancer-induced bone pains (Katz et al., 2011; Lane et al., 2010; Sopata et al., 2015). Initially, there were considerable side effects discovered from the anti-NGF antibodies leading to osteonecrotic activity and therefore a need for premature joint replacement. This problem was resolved by dose adjustment and by the prevention of unwanted drug interactions (Bramson et al., 2015; Ekman et al., 2014; Schnitzer and Marks, 2015). One such interaction was identified between the non-steroidal anti-inflammatory drugs (NSAIDs), which are often used to alleviate inflammatory pains. Tanezumab monotherapy, in its lower therapeutic doses, does not elevate the risk of total joint replacements, but when co-administered with NSAIDs, the risk is notably higher (Schnitzer and Marks, 2015).
1.5 General aims of thesis

Neurobiology of cancer emerges as a new discipline to dissect the complex interaction of the nervous system with the tumour environment (Boilly et al., 2017). From an oncological perspective, it tries to unravel how cancer progression and metastasis depend on nerves. From a neuroscience site, the focus is on the mechanisms underlaying the cancer-evoked neuronal reorganization and changes in perception, including pain.

In the context of CIBP, peripheral nerves were never studied functionally. Therefore, here I will investigate their role using in vivo GCaMP6s imaging expressed by DRG neurons. Utilising specific neuronal tracing strategies, I will analyse neuronal responses of the discrete neuronal populations innervating bone.

Little is known about central processing of CIBP. Therefore, I aim to study evoked spinal excitability using combined in vivo spinal electrophysiology with pharmacological/optogenetic manipulations of descending systems. My focus will be on the descending noradrenergic controls, since they were not studied in the context of metastatic bone disease before.

I will also study a specific form of descending modulation that is largely mediated by spinal α2-adrenergic receptor mechanisms. The expression status of Diffuse Noxious Inhibitory Controls (DNIC) has not knowingly previously been studied in the context of metastatic bone disease.
Chapter II
Chapter II. Materials and Methods

2.1 Cell lines

Syngeneic rat mammary gland adenocarcinoma cells (MRMT-1, Riken cell bank, Tsukuba, Japan) isolated from female Sprague-Dawley rat, were cultured in RPMI-1640 medium (Invitrogen, Paisley, UK) supplemented with 10% FBS, 1% L-glutamine and 2% penicillin/streptomycin (Invitrogen, Paisley, UK). All cells were incubated at 5% CO₂ in a humidity-controlled environment (37 °C, 5% CO₂; Forma Scientific).

2.2 Animals

Male Sprague-Dawley rats (UCL Biological Services, London, UK or Charles-River, UK), C57/BL6 mice (Charles-River, UK), and Avil-GFP mice [see gensat.org: STOCK Tg (Avil-EGFP) QD84Gsat/Mmucd for BAC expression levels] were used for experiments. Avil-GFP mice were bred in-house for several generations onto a C57/BL6J background. In all experiments, adult, age-matched (3–6 month) littermate controls from both genders were used. Animals were group housed on a 12:12-hour light–dark cycle. Food and water were available ad libitum. Animal house conditions were strictly controlled, maintaining stable levels of humidity (40-50%) and temperature (22±2°C). All procedures described were approved by the Home Office and adhered to the Animals (Scientific Procedures) Act 1986. Every effort was made to reduce animal suffering and the number of animals used in accordance with IASP ethical guidelines (Zimmermann, 1983).

2.3 Cancer-induced bone pain model

On the day of surgery, MRMT-1 cells were released by brief exposure to 0.1% w/v trypsin-ethylenediaminetetraacetic acid (EDTA) and collected by centrifugation in medium for 5 min at 1000 rpm. The pellet was washed with Hanks’ balanced salt solution (HBSS) without calcium, magnesium or phenol red (Invitrogen, Paisley, UK) and centrifuged for 5 min at 1000 rpm. MRMT1 cells were suspended in HBSS to a final concentration of 300,000 cells/ml and kept on ice until use. Only live cells were counted with the aid of Trypan Blue (Sigma) staining. Cell viability after incubation on ice was checked after surgery, and no more that 5-10% of cells were found dead after 4 h of ice-storage.

Sprague-Dawley rats weighting 120-140 g (for late-stage CIBP, 14 days post-surgery) or 180-200 g (for early-stage CIBP, 7 days post-surgery), following complete induction of anaesthesia with isoflurane (induction 5%, maintenance 1.5-2%) in 2 l/min O₂ and subcutaneous perioperative meloxicam injection (50 μl 2 mg/kg, Metacam®, Boehringer Ingelheim, Berkshire, UK), were subjected to the surgical procedure of cancer cell implantation into the right tibiae (Medhurst et al., 2002). Briefly, in aseptic
conditions, a small incision was made on a shaved and disinfected area of the tibia’s anterior-medial surface. The tibia was carefully exposed with minimal damage to the surrounding tissue. Using a 0.7 mm dental drill, a hole was made in the bone through which a thin polyethylene tube (I.D. 0.28 mm, O.D. 0.61 mm; Intramedic, Becton Dickinson and Co., Sparks, MD, USA) was inserted 1-1.5 cm into the intramedullary cavity. Using a Hamilton syringe, either $3 \times 10^3$ MRMT-1 carcinoma cells in 10 $\mu$l HBSS or 10 $\mu$l HBSS alone (Sham) was injected into the cavity. The tubing was removed, and the hole plugged with bone restorative material (IRM, Dentsply, Surrey, UK). The wound was irrigated with saline and closed with Vicryl 4-0 absorbable sutures and wound glue (VetaBond 3M, UK). The animals were placed in a thermoregulated recovery box until fully awake.

2.4 Administration of tracers and calcium indicators

Male Sprague-Dawley rats weighing 60-70 g, following complete induction of anaesthesia with isoflurane (induction 5%, maintenance 1.5-2%) in $O_2$ (2 l/min) were maintained at around 37°C using a homeothermic heating mat and 50 $\mu$l of Meloxicam (2 mg/kg, Metacam®, Boehringer Ingelheim, Berkshire, UK) was subcutaneously administered for post-operative pain management. Animals were fixed in a stereotaxic apparatus (Kopf, Germany), their lumbar region was clamped and spinal T12-L1 intervertebral space was exposed by bending the lumbar region rostrally providing easy access to the underlaying dura without the need for laminectomy. A small puncture in the dura was made and a thin catheter of 0.2 mm diameter (Braintree Scientific) was inserted in the caudal direction. 10 $\mu$l of AAV9.CAG.GCaMP6s.WPRE.SV40 (a gift from Douglas Kim & GENIE Project via Addgene viral prep #100844-AAV9, US) was infused into the intrathecal space at 1.2 $\mu$l/min (titer $\geq 1 \times 10^{13}$ vg/ml). Due to the length of the inserted cannula, the infusion was close to L4 DRG. The catheter was left in place for 2 minutes before slow withdrawal. The incision was closed with wound clamps and postsurgical glue (Vetabond, 3M, UK). After 7 days of recovery, left tibia was injected with 5 $\mu$l of 4% Fast Blue neuronal tracer (Polysciences Inc., Germany) as described above, allowing tracing of bone afferents. The muscle layer adjacent to Fast Blue injected bone was injected at the same time with 5 $\mu$l of pAAV-CAG-tdTomato (titer $7 \times 10^{12}$ vg/ml, a gift from Edward Boyden via Addgene viral prep #59462-AAVrg, US) to allow tracing of muscle and periosteum afferents. After a 7-day recovery period, animals were randomly divided into two groups receiving either cancer cells or sham HBSS buffer treatment into the left tibia (injection was through the same hole in the bone to prevent further damage). Two weeks after cancer implantation animals were subjected to terminal in vivo calcium imaging. Throughout the whole period, body mass was carefully
monitored, and animals steadily gained weight resulting in 250-280 g at the day of imaging.

2.5 In vivo calcium imaging of sensory neurons

Rats were anaesthetised using urethane (12.5% w/v in saline, Sigma, UK). Starting with an initial dose of 0.5 ml given i.p., subsequent (0.5ml) given at approximately 10-15-minute intervals, depending on hind limb reflex activity, until surgical depth was achieved. The core body temperature was maintained close to 37°C using a homeothermic heating mat with a rectal probe (Harvard Apparatus). Tracheotomy was performed to secure steady breathing. An incision was made to the skin on the back and the muscle overlying the L3, L4 and L5 vertebral segment was removed. The bone around either the L3 or L4 DRG was carefully removed and the underlying epineurium and dura mater over the DRG were washed and moistened with normal saline. The position of the animal’s body was varied between prone and lateral recumbent to orient the DRG in a more horizontal plane. The exposure was then stabilised at the neighbouring vertebrae using spinal clamps (Precision Systems and Instrumentation) attached to a custom-made imaging stage. The exposed cord and DRG were covered with silicone elastomer (World Precision Instruments, Ltd) to avoid drying and to maintain a physiological environment. The rat was then placed under the Eclipse Ni-E FN upright confocal/multiphoton microscope (Nikon) and the microscope stage was variably diagonally orientated to optimise focus on the DRG. The ambient temperature during imaging was kept at 32°C throughout. All images were acquired using a 10X dry objective. To obtain confocal images a 488 nm Argon ion laser line was used. GCaMP signal was collected at 500-550 nm. Time series recordings were taken with an in-plane resolution of 512 x 512 pixels and a fully open pinhole for video-rate acquisition. Image acquisition varied between 2-4 Hz depending on the experimental requirements and signal strength. At the end of the experiment, rats were sacrificed by clamping the trachea tube and left for 1h for the DRG to fill up with calcium for maximum signal control.

2.5.1 Activation of sensory neurons for GCaMP in vivo imaging

Throughout the experiment, care was taken to provide sufficient breaks between stimuli (usually 3-5 min) in order for the tissue to equilibrate back to its baseline state. Mechanical stimulation consisted of brushing the ipsilateral calf, stretching the leg and pressure application to the leg. Brushing was performed for 10 s to the shaven surface of the cancer-bearing calf. The leg was stretched by a cycle of 10 x gentle pulling and pushing of the ipsilateral leg rostro-caudally along the body axis. Finally, incremental pressure (50 mmHg increments every 10 s, in the range of 0-400 mmHg) was applied to the leg using a neonatal cuff connected to a manometer and air pump (air-filled 20 ml
The cuff was consecutively positioned in 3 places: knee-tibial head, calf, and calf-ankle. Cold stimulation was achieved by application of 5 drops of ethyl chloride topically on same places as cuff, and 5 min period between stimuli was allowed for the system to retrieve to baseline.

2.5.2 Calcium imaging data analysis

Drift in time-lapse recordings was corrected using NIS Elements AR 4.30.01 (Nikon, align application). Further image processing was done using Fiji/ImageJ Version 1.52h, and graphing and statistical analysis was undertaken with a combination of Microsoft Office Excel 2013, IBM SPSS Statistics 25 package and RStudio 0.99.893. In order to generate traces of calcium signals from time lapse images, regions of interest (ROIs) surrounding cell bodies were chosen using a free hand selection tool in Fiji. ROIs were chosen with minimal overlap to ensure less interference from surrounding somata. A region of background was selected, and its signal subtracted from each ROI. To generate normalised data, a baseline period of fluorescence was recorded for each ROI and changes from this baseline fluorescence were calculated as $\Delta F/F$ and expressed in percentages (Chisholm et al., 2018). Implemented here are stringent criteria, where an average signal reaching 70% above baseline fluorescence plus 4 standard deviations were qualified as a response. Percentage of responders was quantified in a binary fashion within all selected ROIs. The fluorescence intensity and size analysis was performed only for responders. Non-responding cells were not analysed for their fluorescence intensity levels, as it would artificially introduce biased zero values for these cells which were either non-responding for the particular modality, or were outside of the stimulated receptive field. Thus, only those cells were analysed for intensity and size, which responded at least once to the given stimulus modality (i.e. knee compression 0-400 mmHg).

2.5.3 Markov Cluster Analysis

Markov Cluster Analysis was utilised to cluster hundreds of neurons responding to the predefined stimuli (Enright et al., 2002). BioLayout Express (under GNU Public License, Kajeka Ltd, UK) was used to run the analysis (Theocharidis et al., 2009). The data derived from the range of neuronal responses ($\Delta F/F$ values averaged for each stimulus length i.e. all frames from the entire duration of the 50 mmHg compression) to the defined stimuli, originating from all responders across the time. Constructed csv files with all responders’ values were used to construct network graphs (see step 1 in Fig. S1.5A). Initially, the similarity between individual cell responses was determined by the Pearson correlation. Pairwise Pearson correlation coefficients were calculated for every cell-set after defined sensory stimuli and correlation coefficients above a predefined
threshold (R>0.9) were used to draw edges between cells (nodes) in the construction of network graphs. The nodes over the preselected value were removed from the graph (see step 2 in Fig. S1.5A). Several trials with different R-values were tested and the value of 0.9 was selected as the best ‘trade-off’ between overpopulated graphs with overwhelming number of clusters and no biological meanings (R<0.85) and exclusion or too many cells from the analysis (R>0.9). Next, the generated non-weighted graphs were clustered with the MCA with the following parameters: pre-inflation = 1.8, inflation = 1.8, scheme = 3, minimal number of clusters = 5. The most restrictive parameter – inflation (defines granularity of the clustering) was chosen experimentally to most tightly represent clean clusters without losing too many cells from the analysis (see step 3 in Fig. S1.5A). Post-hoc analysis of all visualised clusters allowed for user-defined merging of clusters with biologically-relevant similarity. This last part was supervised (see step 4 in Fig. S1.5A). Final core data was exported with cluster’s tag for each cell.

2.5.4 Principal Component Analysis

A csv file containing final core data after MCA was used in Principal Component Analysis (PCA) to demonstrate that the classical analysis of variances is unable to detect different patterns in the longitudinal datasets (i.e. fluorescence changes to the pressure ramp across hundreds of cells) (Fig. S1.5B). PCA was run in R (RStudio, Version 1.1.419) and analysis and visualisation were performed utilising the following packages: factoextra, corrplot, FactoMineR, ggfortify, cluster.

2.6 Behavioural testing

Behaviour was assessed 2-4 hours before surgery (day 0) and at 2, 7 and 14 days following cancer cells injection. Testing was preceded by a 30 min acclimatisation period. Rooms conditions used for behavioural testing were strictly controlled, maintaining stable levels of humidity (40-50%) and temperature (22±2°C).

2.6.1 Von Frey test

Mechanical hypersensitivity was assessed by application of increment von Frey filaments starting from 0.16 g up to 26 g – cut off (Touch-test, North Coast Medical Inc., San Jose CA, USA). Each hair was applied 5 times to the plantar surface proximal to the digits of the ipsilateral and contralateral hind paws. Withdrawal responses and whole paw lifts elicited by von Frey hairs were scored as positive remark. Five subsequent positive responses to the same filament were considered as the overall positive reaction, the force of the filament noted, and further testing with higher force filaments abandoned. Results are presented as a mean ± SEM.
2.6.2 Acetone test

Cold hypersensitivity was assessed using a few drops of acetone applied onto the plantar surface of the hind paws, both ipsilateral and contralateral to the injected leg. A marked flinching or shaking behaviour was considered as a positive reaction. The test was repeated a total of three times on each side with a minimum of 3 min between each application. The resulting score was averaged from all rats from the same group and presented as mean ± SEM.

2.6.3 Static Weight Bearing

Behaviour was assessed 2-4 hours before surgery (day 0) and at 2, 7 and 14 days following cancer cells injection. Testing was preceded by a 30 min acclimatisation period. Weight bearing was assessed using a tester (Linton Instrumentation, Norfolk, UK) in which rats were placed in a plexiglass enclosure that each hindpaw laid on a separate weighing plate. After a few minutes of habituation, the force exerted by each hind paw was measured 5 times with a 10-20 s gap between measurements. Measurements from each paw separately were averaged and then results were transformed to give the percentage of weight borne on each side to the total rear legs bearing (taken as 100%).

2.7 Micro-computed tomography of cancer-bearing legs

Rats were sacrificed by overdose of isoflurane (5% vol/vol) and transcardially perfused with 250 ml of cold phosphate buffer saline solution (PBS, pH=7.5, Invitrogen, Paisley, UK) followed by 4% paraformaldehyde solution in 0.1 M phosphate buffer (250 mL, pH=7.5, Sigma, UK). Bones were stored frozen in -20°C until analysis. Rat tibiae, cleared of excess muscle and soft tissue, were placed into a micro-computed tomography scanner (μCT, Skyscan1172) with Hamamatsu 10 Mp camera. Recording parameters were set as follows: source voltage at 40 kV, source current at 250 μA, rotation step at 0.600 deg, with 2 frames averaging and 0.5 mm aluminium filter. For reconstruction NRecon software (version: 1.6.10.4) was used. In total, over 500, 34 μm thick virtual slices were collected per bone. Because the reference point for rat’s tibia for micro-CT analysis was not previously described, we established an anatomically relevant reference point which was not affected by cancer, from which a region of interest was chosen for further analysis. Reference point for bone mineral density analysis (BMD) was defined as the internal tip of the intercondylar area, which was consistently located at 5 mm from the centre of the cancer growth zone. For BMD, the cancer growth zone encompassing space between 3 to 7 mm caudally from the reference point was quantified. A total of 119 scanned planes, each with a thickness of 34 μm, was analysed (see Fig. S1.3 for more details). Comparison to two known density standards allowed us to quantify BMD values in mg/cm³ of both trabecular and cortical bone (utilising
Representative visualisations were prepared with Fiji with 3D viewer plugin.

2.8 Immunohistochemistry

For experiments which did not involve in vivo imaging, animals were sacrificed by overdose of pentobarbital (Euthanal, Merial, UK) and transcardially perfused with 250 ml of cold phosphate buffer saline solution (PBS, pH=7.5, Invitrogen, Paisley, UK) followed by 4% paraformaldehyde solution in 0.1 M phosphate buffer (250 mL, pH=7.5, Sigma, UK). The lumbar 1-6 dorsal root ganglia (DRG) from both injured (ipsilateral) and intact (contralateral) sides were collected and post-fixed overnight in 4% paraformaldehyde (PFA) at 4°C followed by cryoprotection in 30% sucrose (with 0.02% sodium azide) for 24 hours. Alternatively, at the end of each GCaMP imaging experiment L1-L5 ipsi/contra DRG were collected and post-fixed overnight in 4% paraformaldehyde (PFA) at 4°C followed by cryoprotection in 30% sucrose (with 0.02% sodium azide) for 24 hours. After cryoprotection all DRG were embedded in Optimal Cutting Temperature (Tissue-Tek) and stored at -80°C for further analysis. The DRG embedded in OTC mould were cryosectioned (Bright Instruments, UK) to 10 μm thick slices collected on Menzel-Gräser Superfrost Plus Slides (25x75x1.0 mm) and stored in -20°C freezer until staining. Once dried (45°C for 2 hours) and briefly washed with 50% ethanol, sections were outlined with a hydrophobic marker (PAP pen, Japan), rehydrated and blocked with 10% donkey serum in washing solution (0.01% NaN₃, 0.3% Triton X-100 in PBS, pH=7.5) for two hours prior to overnight incubation at room temperature with primary antibodies against Atf3 (rabbit, 1:200, Santa Cruz, (C-19): sc-188, US), TubβIII (mouse, 1:1000, G712A, Promega, UK), GFP (to visualise GCaMP6s; chicken, 1:1000, ab13970, Abcam, UK), TrkA (NGF receptor; 1:400, Abcam, ab8871, UK), Piezo2 (Rabbit; 1:200, NBP1-78624SS, NovusBio, UK), CGRP (marker of small peptidergic neurons; 1:500, CA1134, Enzo Life Sciences, UK), CGRP (marker of small peptidergic neurons; 1:1000, ab81887, Abcam, UK), IB4 (conjugated to Alexa Fluor 647; marker of small, non-peptidergic fibres; 1:250, I32450, Molecular Probes, UK), Advillin (Rabbit, 1:500, Abcam, ab72210, UK), dopamine-β-hydroxylase (DBH, Mouse, 1:400, Millipore, MAB308, CA, USA), Tyrosine hydroxylase (TH, Rabbit, 1:1000, Millipore, AB152, CA, USA). Slides were then incubated with the appropriate fluorophore-conjugated secondary antibodies (Goat anti-Chicken, Alexa Fluor 488, A11039, Invitrogen, Eugene, OR, US; Goat anti-Rabbit, Alexa Fluor 594, A11037, Invitrogen, Eugene, OR, US; Goat anti-Rabbit, Alexa Fluor 568, A10042, Invitrogen, Eugene, OR, US; Goat anti-Mouse, AlexaFluor 647, A31571, Invitrogen, Eugene, OR, US; Goat anti-Rabbit, AlexaFluor 647, A21244, Invitrogen, Eugene, OR, US; all used at 1:1000 dilution) for 2 hours at room temperature. Slides
were coverslipped using media (Fluoromount-G without DAPI, eBioscience, UK) and stored in darkness at 4°C until imaging.

Samples were typically imaged with an LSM 710 laser-scanning confocal microscope (Zeiss) using 10x (0.3 NA) and 20 x (0.8 NA) dry objectives and analysed with Fiji Win 64. For quantification, samples were imaged with 20x dry objective on Zeiss Imager Z1 microscope coupled with AxioCam MRm CCD camera. The acquisition of images was made in multidimensional mode and the MosaiX function was used to construct the full view. 3-6 DRG were imaged per lumbar region. Cell counting was carried out on the Fiji Win 64 utilising cell counter plugin. For Atf3 analysis, cells were counted as positive only when the cell's nucleus was stained red. The percentage of Atf3 positive cells relative to the total number of neurons (TubβIII) and FB positivity was calculated. On average, 20-30 DRG sections were imaged for quantification.

2.9 PACT

A passive CLARITY tissue clearing technique (PACT) (described in detailed in: (Treweek et al., 2015)) has been implemented to allow whole-mount DRG or spinal cord (1 mm cross-sections) imaging from Avil-GFP mice or rats with minor modifications. Briefly, Avil-GFP mice or rats were deeply anesthetized with pentobarbital and transcardially perfused with cold PBS followed by a cold 4% PFA solution in phosphate buffer, pH=7.5. Lumbar DRG or lumbar spinal cords were extracted and post-fixed in 4% PFA overnight in 4°C. After fixation, samples were transferred directly to ice-cold A4P0 solution consisting of: 4% acrylamide monomer (40% acrylamide solution, cat. 161-0140, Bio-Rad, UK), 0.25% VA-044 (thermoinitiator, Wako, US) in 0.01 M PBS, pH=7.4, and incubated at 4°C overnight in prewashed distilled water (to remove anticoagulant) in dried vacutainer tubes (Vacutainer, cat 454087, Greiner GmbH, Austria). The next day, samples were degassed by piercing the septum with a 20G needle connected to a custom-build vacuum line. The residual oxygen was replaced with nitrogen by 5 min bubbling of the solution with pure nitrogen (BOC, UK) via a long, bottom-reaching 20G needle, and a second short needle pierced to allow gases to exhaust. Throughout, samples were kept on ice to prevent heating and consequent premature A4P0 polymerisation. After achieving oxygen-free conditions, samples were polymerised by 3 h incubation in a 37°C water bath. Following polymerisation, the excess honey-like polyacrylamide gel was removed with tissue paper, and samples were transferred to 15-50 ml falcon tubes filled with clearing solution. 10% SDS (cat. L3771, Sigma-Aldrich, UK) in PBS, pH=8.0, was used for passive clearing. Samples were incubated on a rotary shaker at 37°C and 70 rpm (Phoenix Instruments, UK) until reaching the appropriate transparency (usually overnight).
Next, all samples were washed with PBS pH=7.5 on rotary shaker at room temperature, by replacing the solution 4-5 times throughout the course of 1 day in order to remove the SDS. Following washing, samples were treated with primary antibodies in 2% normal donkey serum in 0.1% Triton X-100 in PBS, pH=7.5 with 0.01% sodium azide. The following primary antibodies were used: anti-eGFP (chicken, 1:400, ab13970, Abcam, UK), anti-CGRP (marker of small peptidergic neurons; 1:200, ab81887, Abcam, UK), dopamine-β-hydroxylase (DBH, Mouse, 1:200, Millipore, MAB308, CA, USA). 250 µl antibody solution was used per DRG and 500 µl antibody solution was used for spinal cord in a 2 ml Eppendorf tube. Samples were incubated with primary antibodies at room temperature, with gentle shaking for 3-4 days. This was followed by 4-5 washing steps with PBS over the course of 1-2 days. Next, we used secondary antibodies conjugated with fluorophores (1:200): Goat anti-Chicken, Alexa Fluor 488, A11039, Invitrogen, Eugene, OR, US; Goat anti-Mouse, AlexaFluor 647, A31571, Invitrogen, Eugene, OR, US; all were used in the same buffer solution as described for primary antibodies above and incubated in darkness at room temperature with gentle agitation for 3-4 days. After that time, samples were washed extensively with PBS at least 5 times over 1-2 days at room temperature. Finally, samples were incubated in the refractive index-matching solution (RIMS, refractive index = 1.47) consisting of 40 g of Histodenz (cat. D2158, Sigma-Aldrich, UK) dissolved in 30 ml of PBS, pH=7.5 with 0.01% sodium azide. 200 µl of RIMS was used per DRG. Samples were allowed to equilibrate in RIMS overnight in darkness. Before imaging samples were placed in fresh RIMS in custom-made glass slide chambers and covered with coverslips. DRG were ready to image 4 hrs after final equilibration in RIMS.

Samples were imaged with a Zeiss LSM 780 single-photon confocal upright microscope, equipped with EC Plan-Neofluar 10x 0.3 NA, Ph1 dry objective (w.d=5.3 mm, cat. 420341-9911, Zeiss, Germany) and laser lines: 488, 633 nm. Scans were taken with 2048x2048 pixel resolution, with 4-5 µm optical section typically spanning 400-700 µm of scanned depth (resulting in 100-150 planes) with auto Z brightness correction to ensure uniform signal intensity throughout the sample. Images were exported from Zen 2012 Blue Edition software (Carl Zeiss Microscopy GmbH, Germany). Next graphical representations, 3D-rendering, animations, maximal intensity projections within selected z-stacks and further analysis were obtained with open-source Fiji (ImageJ) equipped with appropriate plugins.

2.10 Spinal cord in vivo electrophysiology

In vivo electrophysiology was performed on animals weighing 250-300 g (post-operative days 14 to 16 for sham and CIBP animals), and on weight-/age-matched naive
rats as previously described (Urch and Dickenson, 2003). Briefly, after induction of anaesthesia, a tracheotomy was performed, and the rat was maintained with 1.5% of isoflurane in a gaseous mix of N\textsubscript{2}O (66%) and O\textsubscript{2} (33%). A laminectomy was performed to expose the L3–L5 segments of the spinal cord. Core body temperature was monitored and maintained at 37°C by a heating blanket unit with rectal probe. Using a parylene-coated, tungsten electrode (125 µm diameter, 2 MΩ impedance, A-M Systems, Sequim, WA, USA), wide dynamic range neurons in deep laminae IV/V (~650–900 µm from the dorsal surface of the cord) receiving afferent A-fibre and C-fibre input from the hind paw were sought by periodic light tapping of the glabrous surface of the hind paw. Extracellular recordings made from single neurones were visualized on an oscilloscope and discriminated on a spike amplitude and waveform basis. Sampling parameters were set as follows: 30-40k amplification (preamp+amp), band-pass filtering between 1k and 3k Hz and the signal was digitalised at 20 kHz sampling rate. HumBag (Quest Scientific, Canada) was used to remove low frequency noise (50-60 Hz). Electrical stimulation (NeuroLog system, Digitimer, UK) was given via two tuberculin needles inserted into the receptive field and a train of 16 stimuli was given (2 ms pulse duration, 0.5 Hz at three times C-fibre threshold). Responses evoked by A\textsubscript{β}, A\textsubscript{δ}, and C-fibres were superimposed and separated according to latency (0–20 ms, 20–90 ms and 90–350 ms, respectively), on the basis that different fibre types propagate action potentials at different conduction velocities. Neuronal responses occurring after the C-fibre latency band of the neuron were classed as post-discharge, a result of repeated stimulation leading to wind-up neuronal hyperexcitability. The “input” (non-potentiated response) and the “wind-up” (potentiated response, evident by increased neuronal excitability to repeated stimulation) were calculated. Input = (action potentials evoked by first pulse at three times C-fibre threshold) × total number of pulses. Wind-up = (total action potentials after 16 train stimulus at three times C-fibre threshold) – input. A wide range of natural stimuli, including brush, von Frey filaments (2 g, 8 g, 26 g and 60 g), heat (42°C, 45°C and 48°C – constant water jet), and cold (evaporation of a few drops of acetone or ethyl chloride), were applied to the receptive field for 10 s per stimulus. For each stimulus, the evoked responses were recorded and quantified as the number of neuronal events counted during the 10 s duration of a given stimulation. Data were captured and analysed by a CED 1401 interface coupled to a Pentium computer with Spike 2 software (Cambridge Electronic Design, Cambridge, UK; peristimulus time histogram and rate functions). Stabilization of neuronal responses to the range of electrical and natural stimuli was confirmed with at least three consistent recordings (<10% variation in the action potential) to all measures. Means of these baseline responses were calculated and used as the ‘pre-drug’ controls.
2.10.1 Diffuse Noxious Inhibitory Controls

Diffuse Noxious Inhibitory Controls (DNIC) were induced analogically to previously published methodology (Bannister et al., 2015). Briefly, extracellular recordings were made from 1 WDR neuron per animal by stimulating the hind paw peripheral receptive field and then repeating in the presence of the ear pinch (conditioning stimulus - DNIC). The number of action potentials fired in 10 seconds was recorded for each test. Baseline responses were calculated from the mean of 3 trials. Each trial consisted of 3 consecutive stable responses to 8, 26, and 60 g von Frey filaments applied to the hind paw (where all neurons met the inclusion criteria of 10% variation in action potential firing for all mechanically evoked neuronal responses). This was then followed by consecutive responses to the same mechanical stimuli (8, 26, and 60g von Frey filaments) in the presence of DNIC. Precisely, DNIC was induced using a noxious ear pinch (15.75 x 2.3 mm Bulldog Serrefine; InterFocus, Linton, United Kingdom) on the ear ipsilateral to the neuronal recording, whilst concurrent to this, the peripheral receptive field was stimulated using the von Frey filaments listed. Diffuse noxious inhibitory control was quantified as an inhibitory effect on neuronal firing during ear pinch. A minimum 1-minute non-stimulation recovery period was allowed between each test in the trial. After this, for predrug neuronal recordings, a 10-minute non-stimulation recovery period was allowed before the entire process was repeated for control trial number 2 and 3.

2.10.2 In vivo spinal pharmacology with electrophysiological monitoring

After collection of predrug baseline control data as outlined above, atipamezole (a α2-AR antagonist: 10 and 100 μg; Sigma-Aldrich, Gillingham, United Kingdom, dissolved in 97% normal saline, 2% Cremophor [Sigma, UK], 1% dimethyl sulfoxide [DMSO; Sigma, UK] vehicle), naloxone HCl (a non-selective opioid receptors antagonist: 5 and 20 μg, Sigma-Aldrich, Gillingham, United Kingdom, dissolved normal saline) was administered topically to the spinal cord in 50 μl volumes. A co-administration of atipamezole (100 μg) and naloxone (20 μg) was performed in the same total volume of 50 μl, topically on the lumbar spinal cord surface. Each individual drug dose effect (one neuron per rat) was followed for up to 90 minutes with tests performed at 3 time points (starting at 15, 35 and 60 minutes). For each time point, a trial consisted of consecutive stable responses to all type of stimuli starting with electrical, mechanical, cold, and heat. The post-drug effects in subsequent individual modalities (as compared to mean pre-drug baseline) were judged by the maximal change in recorded action potential rate for noxious stimuli (60 g von Frey, 48°C, ethyl chloride evaporation). Drug effects on electrical stimulation were considered for the time point, in which the highest change was recorded for the C-fibre response. All data plotted represents the time point of peak change based on these criteria.
2.10.3 Brain injections

Stereotaxic injections were made utilizing a precise frame (Kopf Instruments, UK). The procedures for viral vector injections have been described in detail previously (Hickey et al., 2014). In brief, rats were anesthetized with i.p. ketamine (5 mg/100 g, Vetalar; Pharmacia) and medetomidine (30 µg/100 g, Dormitor; Pfizer) until loss of paw withdrawal reflex and perioperative analgesia was achieved by the s.c. injections of meloxicam (2 mg/kg, Metacam®, Boehringer Ingelheim, Berkshire, UK). The animal was placed in a stereotaxic frame and core temperature was maintained at 37°C using a homeothermic blanket (Harvard Apparatus, US). Aseptic surgical techniques were used throughout. Using a 0.7 mm dental drill a hole was made in the skull right above the targeted structure.

For the LC transduction the construct and the virus (CAV-sPRS-hChR2(H134R)-mCherry, a gift from Professor Anthony Pickering, University of Bristol, UK) production were described in details before (Hickey et al., 2014; Li et al., 2016). Briefly, injections of canine adenovirus (CAV) carrying channelrhodopsin 2 under the control of NA-specific synthetic promoter (PRS) were made in male Sprague-Dawley rats (220 g, Charles River) at the following coordinates: 10° rostral angulation (to avoid puncturing the sinus) from lambda: RC: -2.1 mm, ML: 1.3 mm, and -5.8-6.2 mm deep from the cerebellar surface. Three injections were made with a glass pulled micropipette coupled to electronically controlled nanoinjector (Nanoliter 2010, WPI, FL, US) facilitating precise delivery with minimal damage. Each injection was of 300 nl, every 200 µm starting from -6.2 mm (DV) with 2 nl/s delivery rate and minimal 3-5 minutes between slow pipette retraction. The wound was irrigated with saline and closed with Vicryl 4-0 absorbable sutures and wound glue (VetaBond 3M, UK). Anaesthesia was reversed with s.c. injection of atipamezole (Antisedan, 0.1 mg/100 g, i.p.; Pfizer). The animals were placed in a thermodregulated recovery box until fully awake. Two to three weeks were allowed for the transgene expression, after which animals were taken for terminal in vivo electrophysiology.

For transduction of the lamina I projection neurons, injections of viral particles were made to the contralateral to the aimed spinal cord recording site IPB area utilising analogical to the LC injections setup. The following coordinates were used: (from lambda with no angulation) RC: -0.36 mm, ML: 2.2 mm, and at -4.6 mm (150 nl injected) and -4.3 mm (another 150 nl injected) deep from the cerebellar surface. Adult, male Sprague-Dawley rats (220 g, Charles River) were used for injections. Retrograde serotype of adeno-associated virus (AAVrg) was used to express ChR2 under neuronal-specific promoter – synapsin (pAAVrg-Syn-ChR2(H134R)-GFP, titer >7x10^{12} vg/ml, Cat. 58880-
AAVrg, Addgene, US,(Boyden et al., 2005)) in the lamina I projection neurons back labelled from the IPB area.

For the lidocaine block of the LC activity, a hole in the skull was drilled right after performing lumbar laminectomy for DDH WDR neuronal recordings as described above. Utilising the same parameters to reach the ipsilateral to the spinal recording side ventral LC (~6.0 mm from the cerebellum surface), injections of lidocaine (2% in Saline, Cat. L5647 Sigma, UK) were made after collecting stable baseline recordings of DDH WDR neurons. A glass pulled pipette was used coupled to the electronically controlled nanoinjector (Nanoliter 2010, WPI, FL, US) to precisely deliver the drug (400 nl, 2 nl/s). After the drug delivery, pipette was left in place throughout the spinal recordings.

2.10.4 Optogenetics

Every experiment was fully controlled by the neurolog system (NeuroLog system, Digitimer, UK) and the data were acquired with a CED1401 connected to the PC with (Spike2 Cambridge Electronic Design, Cambridge, UK) as described in details above. Extracellular recordings (after filtration and amplification) from DDH WDR neurons, as well as from the LC implanted optrode were saved in Spike2 in parallel with TTL signals from the 470 nm diode controller (also controlled by the neurology, Cat. LEDD1B, Thorlabs, Germany) used for optoactivation. This allowed for the full temporal control of the complex experimental setup.

A simultaneous recording from the transduced LC and ipsilateral to the LC lumbar DDH WDR neurons were made as described above in the in vivo electrophysiology section. LC neurons were identified as described before (Hickey et al., 2014) by their large amplitude with duration of action potentials over 1 ms, spontaneous firing (0.5-7 Hz) and biphasic response following hindpaw pinch (activation followed by transient silent period, particularly strong for the contralateral hindpaw). For the LC recordings and optoactivation, an in-house manufactured optrode consisting of the recording electrode (parylene-coated, tungsten electrode (A-M Systems, Sequim, WA, USA) coupled to the bare optic fibre (multimodal, 200 µm core diameter, Cat. CFM12U-20, Thorlabs, Germany) with its recording tip 300 µm ahead of the fibre tip was used. The LC neurons were optoactivated by light pulses (470 nm diode, 15 mW, 20 ms pulse width, 5 Hz, Cat. M470F3, Thorlabs, Germany (Hickey et al., 2014)) 30 s before and throughout the tests (brush, 8, 15, 26, 60 g von Frey filaments, followed by von Frey filaments with ear pinch to evoke DNIC).

For lamina I optoactivation, a bare optic fibre (multimodal, 200 µm core diameter, Cat. Thorlabs, Germany) was placed in contact with the spinal cord surface 0.5-1 cm rostrally from the DDH recording site. Lamina I neurons were optoactivated (470 nm
diode, 10 mW, 25 ms pulse width, 5 Hz, Cat. M470F3, Thorlabs, Germany) 30 s before and throughout the recording of mechanically-evoked (brush, 8, 15, 26, 60 g von Frey filaments) action potentials from the ipsilateral lumbar DDH WDR neurons. These were contralateral to the IPB area to which virus was delivered. The optimal optoactivation regimen was found experimentally by recording light-evoked action potentials from SDH neurons (the recording electrode in this case was placed 50-150 µm below the cord surface and proximal to the optic fibre).

For combined optogenetics and spinal pharmacology, after collecting three stable baseline and three stable optoactivation responses (each averaged if stable), a drug (either 100 µg atipamezole or 20 µg naloxone) was applied topically on the exposed spinal cord surface, right above the recording site. Evoked responses were tested again immediately after the drug application and without the optoactivation to verify baseline stability after trains of optoactivation. To test simultaneous action of the drug and optoactivation, light pulses were delivered 30 s before and throughout each series of tests (approximately 5 minutes per series) and minimally 5 minutes of the recovery time was allowed between the tests. Pharmacology was monitored every 10 minutes for 50 minutes (each test with optoactivation) and the 60 minutes time point was to test neuron returning to the baseline (no optoactivation).

At the end of every experiment, animals were sacrificed by the overdose of isoflurane and transcardially perfused with cold saline followed by 4% paraformaldehyde. Extracted brains and spinal cords were postfixed in 4% paraformaldehyde for 48 h at 4°C followed by 48 h sucrose (30% in PBS) cryoprotection for anatomical verification.

2.11 Quantification and statistical analysis

Statistical analyses were performed using SPSS v25 (IBM, Armonk, NY). All data plotted in represent mean ± SEM. Detailed description of the number of samples analysed and their meanings, together with values obtained from statistical tests can be found in each figure legend. Symbols denoting statistically significant differences were also explained in each figure legend. Main effects from ANOVAs are expressed as an F-statistic and P value within brackets. Throughout, P-value below 0.05 was considered significant.

Behaviour: Kruskal–Wallis one-way analysis of variance (K-W, one-way ANOVA) test was used to analyse behavioural data for von Frey, acetone and weight bearing. Data for body mass between Sham and CIBP groups was analysed by 2-way repeated-measures analysis of variance (RM-ANOVA) with Bonferroni post hoc test. Data for body mass between Sham and CIBP groups was analysed by 2-way repeated-measures analysis of variance (RM-ANOVA) with Bonferroni post hoc test. Data from in-cage
monitoring was analysed by One-way RM-ANOVA with Greenhouse-Geisser correction [time] followed by Bonferroni post hoc test.

**GCaMP imaging:** Statistical differences in the neuronal responses from the GCaMP experiments, were determined using a 2-way repeated-measures analysis of variance (RM-ANOVA), where applicable with Bonferroni post hoc test. Unpaired t-test was used to compare EtCl responses.

**Electrophysiology:** Statistical differences in the neuronal responses observed after ear pinch and/or after drug administration were determined using a 2-way repeated-measures analysis of variance (RM-ANOVA) with Bonferroni post hoc test. The paired t-test was used for analysis of brush, electrical and cold responses after drug application. Populational studies of brush, electrical and cold responses were analysed with univariate ANOVA, where applicable with Bonferroni post hoc test. Optogenetic experiments were analysed by One-way RM-ANOVA with Greenhouse-Geisser correction and Bonferroni post hoc test.

**Other:** One-way ANOVA with Tukey post-hoc performed in the GraphPad Prism was used to analyse data for BMD and for Atf3 quantifications.

### 2.12 Methodological limitations

I utilised several types of stimuli to measure changes in nociception (and pain) related to bone cancer progression. Utilising weight bearing, I aimed to evaluate the animal’s ability to bear body mass between legs in this unilateral pain model as a stimulus-independent measure of nociception and an approximation of pain.

In *in vivo* preparation, I aimed to activate bone afferents by applying the miniature pressure cuff with a controlled compression pressure ramp. Utilised here, a neonatal pressure cuff offers a non-invasive (no tissue damage), continuous and homogenous (same pressure from every side of the limb) increase in pressure applied to the selected limb area (by the 1-1.5 cm long pressure cuff, allowing it to overlay 3 different areas along the tibia separately). Utilising this stimulus, I was able to activate both intratibial (FB-traced) as well as muscle/skin leg afferents.

This approach allows for a non-invasive activation of the majority of limb afferents from the selected region in the pressure ramp fashion from non-noxious to noxious ranges. There are at least three limitations of this approach: 1) the pressure ramp needs to be selected experimentally so it does not evoke ischemia (here I have experimentally chosen 50 mmHg increases every 10 s, which did not result in visible ischemic decolourisation of the paw below the cuff), 2) the pressure cuff can preferentially inhibit Ab fibres (Prescott et al., 2014a), 3) owing to tissue resistance, the limb’s compression
will likely result in the different pressure values acting on the deeper leg layers (pressure values in the bone are likely lower than those acting in the superficial muscle/skin layers).

Indirect measures of cancer-evoked hypersensitivity, likely reflecting spinal nociceptive processing, were behaviourally tested by stimulus application (von Frey filaments (mechanical hypersensitivity) or acetone drops (cold allodynia)) to the ipsilateral paw. As with behaviour, in in vivo electrophysiological preparations of anaesthetized rodents, I applied different electrical and natural stimuli to the spinal DDH-WDR neuronal receptive field located on the ipsilateral paw. Measurement of WDR activity reflects global changes in spinal nociceptive processing. Therefore, stimulation of the remote receptive field (paw) to the disease-affected region (bone), informs about the central mechanisms of nociception (i.e. central sensitisation). Additionally, DDH-WDR neurons often receive direct or indirect inputs from the whole leg area extending the interpretation of collected results.

In the electrophysiological experiments performed in this thesis, a search technique (a repetitive tapping to the localised on the hind paw receptive field) was used to find DDH WDR neurons. This restriction can be both positive and negative, depending on the addressed questions. DDH WDR neurons represent a unique population of spinal cells that code multimodal responses to the peripheral inputs. The search restriction can be positive in that we know which, physiologically-defined population we study. However, studies of these electrophysiologically-defined neurons can be in some cases misleading. For example, recording from SDH in the CIBP model was shown to yield in the increase in the ratio of WDR to NS neurons, suggesting a physiological shift in the electrical properties of SDH neurons in disease conditions (Urch et al., 2003). Therefore, if one searches for only WDR neurons to study their properties, discarding non-WDR neurons from the studies may introduce a bias for the whole populational representation. These could be especially altered when studying pharmacology of these electrophysiologically-defined neurons in different disease conditions. Also, neurons that were chosen based on their responsiveness to multiple peripheral stimuli (mechanical, heat, cold, presence of wind-up) with a wide range of stimulus strength can restrict their selection, omitting those cells that upon peripheral stimuli depolarise or burst (cells as such need to be excluded from the analysis as their fail to stabilise). Such experiments were therefore performed with the restriction to LV cells and utilising a large animal cohort studied.

It is also known that the anaesthetic can significantly influence neuronal excitability. To minimise the bias from the anaesthetic used, several stable (< 10% of variability in evoked action potentials) baseline recordings were made before pursuing with further pharmacological or physiological analysis. Isoflurane in oxygen/nitrous oxide mix was
used for all central nervous recordings, since it allows a precise adjustment of the depth of anaesthesia during the long recordings. Nitrous oxide additionally guarantees more stable anaesthesia and limits the amount of isoflurane needed.

Every effort was made to overcome these obstacles, especially the bias was minimised in few ways: 1) maintenance of the same experimental conditions, 2) introduction of proper controls for the experimental groups used (i.e. early sham vs. early cancer etc.), 3) analysing data also within internal control conditions (i.e. drug action as compared to the baseline obtained from the same animal), and aiming for a larger representative group of animals with respect to the predicted G-Power.
Chapter III
Chapter III. Encoding pressure by rat somatosensory neurons in health and bone cancer disease

3.1 Introduction

A significant side effect of certain cancer types is severe pain, which can persist even in remission (Clohisy and Mantyh, 2003; Mantyh, 2006b; Mantyh et al., 2002). Cancer pain is particularly prominent in cases where malignant tumours have invaded the skeleton (Mantyh, 2006b; Nyquist and Nelson, 2017). This type of pain, being mechanoreceptive in nature, is difficult to manage in mobile subjects, with the ability to 'break-through' the barriers of analgesia (Mantyh et al., 2002). Mechanistically, CIBP is transmitted via peripheral sensory neurons, the cell bodies of which are housed in DRG buried deep underneath the spinal cord vertebrae. These pseudounipolar somatosensory neurons are genetically and physiologically heterogenous (Chisholm et al., 2018; Lopes et al., 2017b; Prescott et al., 2014a; Schmelz et al., 2000; Usoskin et al., 2014; Wang et al., 2018; Zeisel et al., 2018) (see Chapter I for more).

How exactly do the somatosensory neurons encode different sensory information on the population level remains only partly explained (Prescott et al., 2014a; Wang et al., 2018). Largely, it is hypothesised that the somatosensory neurons encode stimulus intensity in two ways: by frequency coding (a number of action potentials fired in response to a given stimulus strength) and by population coding (the number of afferents engaged in response to a given stimulus). Recent tools like in vivo imaging of genetically-encoded calcium indicators offer sampling of large neuronal populations, the scale of which was until recently unachievable with other approaches (i.e. electrophysiology). Utilising this technique others reported that, for example, heating and cooling sensations are encoded differently by primary afferents (Wang et al., 2018).

Several studies utilised Cre recombinase-dependent expression of genetically-encoded calcium indicators (i.e. GCaMP6s) to study the physiology of DRG neurons (Chisholm et al., 2018; Emery et al., 2016). Advillin, an actin-binding protein, has been suggested as a specific marker for sensory neurons offering selective expression of a transgene in these cells (Hasegawa et al., 2007). However, the specificity of Avil-driven transgene expression for all DRG neurons was recently questioned (Cowie et al., 2018; Murthy et al., 2018; Woo et al., 2014). Having available in-house mice expressing transgenes (eGFP or GCaMP6s) under Avil promoter, I went to evaluate whether the bone afferents are covered by Avil, which could potentially preclude the model used.
Many questions remain regarding afferent function, both in health and disease. For instance, how do they encode mechanical stimuli i.e. body compression and position. Are those modalities encoded also by musculoskeletal afferents? And most importantly, are their functional responses altered in disease conditions? This study was designed to address these outstanding questions using in vivo functional imaging of rodent somatosensory neurons in health and in the context of bone cancer. Further, I used an unsupervised clustering of neuronal responses based on the graph theory to reveal major responder’s classes to defined stimuli. The data gathered reveal important new information about bone afferent expression patterns, encoding of mechanical stimuli and the potential functional mechanism explaining hyperexcitability in the presence of a tumour. Taken together, these findings could help identify new therapeutic avenues for treatment of patients with CIBM.

3.2 Materials and Methods
A detailed description of employed in this chapter procedures can be found in Chapter II. See pages referral below.

- Cell cultures and animals (detailed description, page 35)
- CIBM rat model (detailed description, page 35)
- Behaviour: Weight Bearing (detailed description, page 40)
- Micro-computed tomography of cancer-bearing legs (detailed description, page 40)
- In vivo calcium imaging in DRG neurons (detailed description, page 37-39)
- Markov Cluster Analysis and Principal Component Analysis (detailed description, page 48)
- Immunohistochemistry (detailed description, page 41)
- PACT clearing of DRG (detailed description, page 42)
- Quantification and statistical analysis (detailed description, page 48)
3.3 Results

Figure 3.1. A high proportion of deep body CGRP afferents express little or no Advillin. Representative images of lumbar 3 DRG immunostained for CGRP and Advillin (Avil) in the Fast Blue (FB) traced tibial afferents. Arrows indicate FB/CGRP double positive neurons. Scale bars, 100 µm (A). Quantification of (A). Mean ± SEM percentage of neurons positive for selected markers in analysed L3 DRG from n=3 rats (B). Example PACT-cleared L3 DRG from Avil/eGFP mice (green), immunolabelled for CGRP (red). Scale bars, 200 µm (C). See also Figure S1.1, S1.2 and Movie 1, Movie 2.

3.3.1 A high proportion of deep body CGRP afferents express little or no Advillin

To study bone afferents in the absence of a selective marker, I injected the retrograde tracer fast blue (FB) into the tibial cavity. All lumbar DRG, ipsi- and contralaterally to the injection were dissected, cryosectioned and analysed. I found 18%, 51%, 24% and 7% of all FB+ cells in L2, L3, L4 and L5 DRG respectively (Fig S1.1A-C
and Fig. 3.2F), and no FB+ cells on the contralateral side (Not shown), indicating that my tracing was specific. Next, I showed that the traced tibial afferents stained positively for markers of peptidergic nociceptors (e.g. calcitonin gene-related peptide (CGRP) and TrkA, a receptor for NGF), but were rarely positive for isolectin B4 (IB4), a marker for non-peptidergic neurons (Fig S1.1A, B).

I examined co-expression of a virally delivered genetically-encoded calcium indicator (AAV9-GCaMP6s) and a commonly used marker for sensory neurons, Advillin (Avil). I performed this analysis using a knock out-validated antibody (Lopes et al., 2017c). Surprisingly, around 40% of FB/GCaMP6s++ cells were Avil negative (Fig. S1.1C). To ensure validity, I investigated FB-traced tibial afferents from rats that were not subjected to GCaMP6s delivery. The tissue was co-stained for CGRP and TubulinβIII (TubbIII, a known pan-neuronal marker) to further ensure the presence of well-defined neuronal populations (Fig. 3.1A and S1.1D). Almost 41% of tibial afferents did not express Avil (Fig. 3.1B). These Avil negative fibres often (55%) co-localise with CGRP (Fig. 3.1B). Similarly, in the mouse, I employed a PACT-clearing technique (Treweek et al., 2015) to visualise all neurons within the DRG of Avil-eGFP mice. Counterstaining with CGRP revealed a discrete population of CGRP+, Avil- neurons (around 10% of the total) (Fig. 3.1C, S1.1E, See also Movie 1, 2). A search through two separate, publicly available single-cell RNA sequencing databases further supported our finding of differential expression between CGRP (and Tac1, a gene for substance P, another peptide found in peptidergic nociceptors) and Avil in sensory neurons (Fig. S1.2A, B) (Usoskin et al., 2014; Zeisel et al., 2018).
Figure 3.2. Cancer progression affects bone innervation. Example micro-computer tomography reconstructions of rat tibiae. Panels depict a sham-operated control, early (day 7/8) and late (day 14/15) cancer stage. Top panels represent a whole 3D-rendered tibia with corresponding orthogonal projection. Bottom panel shows a plantar representation of selected micro-scans from the top panel. An early cancer stage is characterised by the trabecular bone lesions and little to no cortical bone lesions, whereas the late stage is characterised by both trabecular and cortical bone lesions (A). Trabecular bone mineral density quantification. Volumetric bone mineral density quantification from 114 reconstructed micro-scans (every 34 μm) per bone. Selected planes for analysis were chosen to cover the tumour growth area (see methods for details). Each dot represents a single bone from a separate animal (n = 5-7 per group). Data represent the mean ± SEM (in mg/cm³). One-Way ANOVA: F₃, 18 = 6.272, p = 0.0042 (group), with Tukey post-hoc test: # vs. Early Sham, * vs. respective Sham. * or # p <
0.05, ## p < 0.01 (B). Cortical bone mineral density quantification. Volumetric bone mineral density quantification from 114 reconstructed micro-scans (every 34 µm) per bone. Selected planes for analysis were chosen to cover the tumour growth area (see methods for details). Each dot represents a single bone from a separate animal (n = 5-7 per group). Data represent the mean ± SEM (in mg/cm³). One-Way ANOVA: F₃, ₁₉ = 35.57, p < 0.0001 (group), with Tukey post-hoc test: # vs. Early Sham, * vs. respective Sham, $ vs. Early Cancer, ****p < 0.0001 (C). Static weight bearing measurement of rear legs. Within a timepoint, each dot represents a single animal (n = 13-20 per group). Each measurement was taken as an average of 5 consecutive readouts per animal per timepoint. Data represent the mean ± SEM. Kruskal-Wallis H for independent samples (Cancer vs. Sham): day 0: χ²(1) = 0.083, p = 0.773, day 2: χ²(1) = 1.602, p = 0.206, day 7: χ²(1) = 5.286, p = 0.022, day 14: χ²(1) = 15.384, p < 0.0001. *p < 0.05, ****p < 0.0001 vs. respective Sham (D).

Representative images selected from lumbar 3 DRG of IHC analysis of Atf3 and TubulinβIII protein expression in the Fast Blue (FB) traced tibial afferents. FB was injected a week before the cancer cells or vehicle (sham) implantation. Scale bars, 100 µm (E). Distribution of all FB+ afferents within analysed ipsilateral lumbar DRG (L2-L5). No FB positivity was noticed in the contralateral lumbar DRG (not shown) (F). Quantification of all FB+ afferents within ipsilateral L2-5 DRG, analysed as a percentage of all neurons (TubulinβIII) therein. Early (E, day 7/8 post cancer cells implantation) and late (L, day 14/15 post cancer cells implantation) stage groups. Each dot represents an average count from 4-6 10 µm sections per DRG from separate animals (n = 3-6). Data represent the mean ± SEM. One Way ANOVA [group]: F₃, ₁₇ = 0.0335, p = 0.992 (G). Quantification of all Atf3+ afferents within ipsilateral L2-5 DRG analysed as a percentage of all neurons therein (TubulinβIII). Analysed as in (G), data represent the mean ± SEM. One Way ANOVA [group] with Tukey post-hoc test. # vs. Sham early, * vs. Sham late, $ vs. CIBP late. *p < 0.05, **p < 0.01, ****p < 0.0001 (H). Quantification of all Atf3+ afferents within ipsilateral L2-5 DRG analysed as a percentage of all tibial cavity neurons (FB traced). Analysed as in (G), data represent the mean ± SEM. One Way ANOVA [group]: F₃, ₁₇ = 8.844, p < 0.001 with Tukey post-hoc test. # vs. Sham early, * vs. Sham late, $ vs. CIBP late. **p < 0.01 (I). See also Figure S1.3 and Movie 3.

3.3.2 Cancer progression affects bone innervation

I investigated how the presence of bone cancer affects these patterns of innervation by generating a validated rat CIBP model using syngeneic mammary gland carcinoma cells (MRMT1) (Medhurst et al., 2002). I evaluated bone damage caused by cancer growth using a high-resolution micro-computer tomography technique (µCT) at two different time points: days 7/8 and days 14/15. Significant damage to the trabecular bone occurred in both stages, while cortical bone was mostly impaired at days 14/15 only, suggestive of early versus late stage modelling of CIBP (Fig. 3.2A, see also 3D-visualisation in the Movie 3). Volumetric reconstruction enabled bone mineral density (BMD) quantification for the whole tumour growth area. Reduction of trabecular BMD from around 101-120 mg/cm³ in sham groups to 45-62 mg/cm³ in respective CIBP groups, and cortical BMD from 485 mg/cm³ in the sham late group to 146 mg/cm³ in the CIBP late group, tightly reflects visually evident bone lesions (One Way ANOVA [group]; trabecular BMD: F₃, ₁₈ = 6.272, P < 0.0042, Tukey post-hoc: CIBP early vs. Sham early P < 0.05, CIBP late vs. Sham early P < 0.01, CIBP late vs. Sham late P < 0.05; cortical
BMD: $F_{3,19} = 35.57, P < 0.0001$, Tukey post-hoc: CIBP late vs. all the others $P < 0.0001$) (Fig. 3.2 B, C, S1.3A).

Tumour progression is expected to relate to animal behaviour, and I monitored rats up to 14 days after surgery. While body weight gain remained stable in all groups (Fig. S1.3D), the behavioural data demonstrate that CIBP rats manifest mechanical hypersensitivity; significant changes in static weight bearing between rear legs is evident from day 7 post-surgery (Kruskal-Wallis H for independent samples (Cancer vs. Sham): day 0: $\chi^2(1) = 0.083, p = 0.773$, day 2: $\chi^2(1) = 1.602, p = 0.206$, day 7: $\chi^2(1) = 5.286, p = 0.022$, day 14: $\chi^2(1) = 15.384, p < 0.0001$) (Fig. 3.2D). These results correspond to other studies using similar rodent models of CIBP (Medhurst et al., 2002; Urch et al., 2003).

Damage to afferents innervating cancerous tissue was quantifiable using activating transcription factor 3 (Atf3), a protein induced by cellular stress (Peters et al., 2005). Representative micrographs of the L3 DRG ipsilateral to the injury site clearly demonstrated the characteristic nuclear expression pattern of Atf3 in bone and other afferents (Fig. 3.2E). This is especially evident in early stages of our CIBP model (Fig. 3.2H, S1.3B, C). Interestingly, by the late stage, Atf3 positivity normalises in both groups, with almost no occurrence in late-stage sham animals, suggesting full postsurgical recovery (One Way ANOVA [group]: $F_{3,17} = 14.37, P < 0.0001$, Tukey post-hoc: CIBP early vs. Sham early $P < 0.0001$, CIBP early vs. CIBP late $P < 0.01$, Sham late vs. Sham early $P < 0.01$) (Fig. 3.2H, S1.3B, C). Bone afferents are more likely than other afferents to express Atf3 at early disease stages, suggesting higher levels of stress in this population (One Way ANOVA [group]: $F_{3,17} = 8.844, P < 0.001$, Tukey post-hoc: CIBP early vs. all the others $P < 0.01$) (Fig. 3.2I). Moreover, there is a visible shift in the expression pattern of Atf3+/FB+ from L3 to L4 DRG between early and late CIBP (Fig. S1.3C).
Figure 3.3. The number of mechanically-responsive sensory neurons is tripled in animals with bone cancer. An experimental timeline. The injection of neuronal tracers and cancer cells implantation was performed via the same hole in the tibia to limit damage. FB – fast blue, GCaMP6s – genetically encoded calcium indicator, AAV9 – adeno-associated virus serotype 9, AAVrg – adeno-associated virus serotype 2 retrograde (A). A summary table representing the number of cells analysed in the in vivo calcium imaging experiment. In brackets are the numbers of animals studied (B). Representative frames from the end of an in vivo imaging session with circled DRG neuronal cell bodies for analysis. The confocal z-scan was taken over 30 minutes post mortem allowing for the calcium to build up in the neuronal cell bodies permitting indication of most of the GCaMP6s molecules (C). Selected video frames of imaged...
DRG cell bodies responding to the knee compression and the leg movements (Push/Pull along the body axis). Sham-operated (left) and Cancer rat model (right). Note the increase in number of responding neurons between the groups. Scale bars, 100 µm (D).

Schematic representation of the in vivo GCaMP6s imaging. DRG, dorsal root ganglia; GCaMP6s, genetically encoded calcium indicator, L3, L4 are lumbar vertebrae 3 and 4, respectively. Pressure was delivered utilising neonatal pressure cuff overlaying animal’s knee. Rats were stably anaesthetised with urethane (E). Percentage of responders from 1337 neuronal cell bodies analysed from L3 and L4 DRG during dynamic brushing of the leg surface in Sham and Cancer animals. The denominator for the total number of cells was established at the end of the experiment as explained in (C). Data represent the mean ± SEM (shaded areas) of n = 359 cells (sham) from 6 animals, and n = 978 cells from 6 animals (Cancer). Student t-test: p = 0.376 (F). Percentage of responders from 2505 neuronal cell bodies analysed from L3 and L4 DRG during knee compression in Sham and Cancer animals. Pressure in the neonatal cuff overlaying knee joint was increased in 50 mmHg steps every 10 s (see methods for more). The denominator for the total number of cells was established at the end of the experiment as shown in (C). Data represent the mean ± SEM (shaded areas) of n = 757 cells (sham) from 9 animals, and n = 1748 cells from 10 animals (Cancer). The increase in number of responding cells follows the logarithmic function of the pressure applied. RM-ANOVA: \( F_{1,2503} = 43.276, p < 0.0001 \) (vs. sham) (G). Percentage of responders from 1592 neuronal cell bodies analysed from L3 and L4 DRG during the gentle leg movement along the body axis in Sham and Cancer animals. Responses of 5 consecutive pull (PU) and push (PS) pairs are presented. The denominator for the total number of cells was established at the end of the experiment as shown in (C). Data represent the mean ± SEM (shaded areas) of n = 359 cells (sham) from 6 animals, and n = 1233 cells from 7 animals (Cancer). RM-ANOVA: \( F_{1,1590} = 17.396, p < 0.0001 \) (vs. sham) (H). Cell size analysis of all responders during: dynamic brushing of the leg surface, leg movement along the body axis (first pull and push shown), and knee compression in Sham and Cancer animals. Pressure was increased in 50 mmHg steps every 10 s. Data represent the mean ± SEM (shaded areas). Brush: unpaired t-test: p = 0.984, Movement (first Pull and Push only): Kruskal-Wallis for independent samples: \( F_{1,674} = 0.110, p = 0.740 \) (Sham vs. Cancer), Knee compression: Kruskal-Wallis for independent samples: \( F_{1,2429} = 28.469, p < 0.0001 \) (Sham vs. Cancer). Number of cells analysed are in brackets above each bar (I). See also Figure S1.4 and Movie 4.

### 3.3.3 The number of mechanically-responsive sensory neurons is tripled in animals with bone cancer

I implemented in vivo GCaMP6s imaging to thoroughly analyse bone afferent physiology in the late cancer stage since mechanical hypersensitivity was evident most clearly here. Considering my anatomical findings of low Avil expression in bone afferents, I opted for intrathecal delivery of an AAV9 viral vector containing GCaMP6s (Fig. 3.3A). This method of delivery was previously shown by our lab to ensure uniformly distributed expression between all subtypes of the DRG neurons (Chisholm et al., 2018) and Fig. S1.1. I analysed 757 DRG neuronal cell bodies from 9 sham-operated rats, and 1748 neurons from 10 CIBP animals transduced with AAV9/Syn.GCaMP6s (Fig. 3.3B, C). Lumbar DRG L3 and L4 were imaged (based on my FB tracing studies (Fig. S1.1, Fig. 3.2F, and (Kaan et al., 2010; Peters et al., 2005)), and 6-7 L3 DRG and 3 L4 DRG from
each group were imaged. No response difference was detected between L3 and L4 lumbar levels (not shown), hence results from all DRG were pooled.

To apply pressure stimuli, I implemented a novel method using a neonatal cuff connected to a manometer and air pump. The cuff was applied sequentially in all animals to the following rear limb regions: knee and tibial head, calf, and calf-ankle. Incremental pressure (50 mmHg every 10 s, in the range of 0-400 mmHg) was applied (Fig. 3.3E, G, S1.4A, B, C, E). In addition to cuff stimulation, we also examined proprioceptive responses, by gently moving the limb along the body axis in 5 consecutive push-pull stretching cycles. My results were analysed using in-house R scripts. Very stringent criteria were applied in order to select responses: fluorescence intensity was counted as a positive response when an average signal reached 70% above baseline fluorescence plus 4 standard deviations (Chisholm et al., 2018).

A striking difference in the number of neurons recruited between sham and CIBP groups, especially after the knee compression (which mostly covered the tumour-growth area) was clear. The effect was detected with the naked eye on video-rate recording (Movie 4). Selected frames from before and after stimulation (Fig. 3.3D, Fig. S1.4E) further illustrate the large magnitude of our effect. No difference in the number of primary afferent responders to dynamic brushing between sham-operated and CIBP animals was observed (Fig. 3.3F). An almost 3-fold increase in the total number of responding cells, from around 6% in sham to 18% in CIBP after knee compression (RM-ANOVA (vs. sham): $F_{1, 2503} = 43.276, p < 0.0001$) (Fig. 3.3G), and an around 2-fold increase for gentle leg movement without mass bearing (RM-ANOVA (vs. sham): $F_{1, 1590} = 17.396, p < 0.0001$) (Fig. 3.3H) was observed. Interestingly, increased compression forces (range 100-400 mmHg) were reflected in the logarithmic recruitment of responders in the CIBP group (Fig. 3.3G). In contrast, the number of responders in sham group did not appear to increase logarithmically. There appeared to be a threshold between 50-100 mmHg, after which all potential mechanoreceptors within the imaged field of view (FOV) were responding to the chosen receptive field stimulation (Fig. 3.3G).

I analysed the cell size distribution of responders in healthy and cancer states. I chose 700 $\mu$m$^2$ and 1200 $\mu$m$^2$ to crudely separate sensory neuron type (small and medium-size cells). My results suggest that pressure is encoded mainly by small to medium-size neurons. As expected, the average cell size decreased with the increase of the force applied (Fig. 3.3I, S1.4D). Intriguingly, there were significant differences in the average cell sizes of responders to knee compression (Kruskal-Wallis for independent samples (Sham vs. Cancer): $F_{1, 2429} = 28.469, p < 0.0001$) (Fig. 3.4I), but not brush (unpaired t-test: $p = 0.984$) or leg movement (Kruskal-Wallis for independent samples (Sham vs. Cancer): $F_{1, 874} = 0.110, p = 0.740$) (Fig. 3.4I), between cancer and
sham animals. Specifically, in sham animals, the number of medium-size responders increased with increasing stimulus pressure (Fig. S1.4D), while dynamic brushing of the calf only recruited a few large-sized neurons. Meanwhile, in my cancer group, an additional population of small diameter neurons (likely C nociceptors) was activated proportionally with increasing stimulus strength, reaching almost 3 times the number of cells that responded to the 400 mmHg than the initial 50 mmHg (Fig. S1.4D).

Figure 3.4. Leg compression and position are differentially coded by DRG sensory neurons. Normalised fluorescence intensities of all responding neuronal cell bodies from
L3 and L4 DRG to dynamic brushing of the leg. Data represent the mean ± SEM. N = 5 cells (sham) from 6 animals, and n = 21 cells from 6 animals (Cancer). Unpaired t-test: p = 0.737 (A). Normalised fluorescence intensities of all responding neuronal cell bodies from L3 and L4 DRG to knee compression. Pressure was increased in 50 mmHg increments every 10 s. Data represent the mean ± SEM (shaded areas). N = 70 cells (sham) from 9 animals, and n = 415 cells from 10 animals (Cancer). RM-ANOVA: F1, 483 = 0.177, p = 0.674 (vs. sham) (B). Normalised fluorescence intensities of all responding neuronal cell bodies from L3 and L4 DRG to leg movement along the body axis (PL – Pull, PS – Push). Data represent the mean ± SEM (shaded areas). N = 83 cells (sham) from 6 animals, and n = 493 cells from 7 animals (Cancer). RM-ANOVA: F1, 574 = 0.306, p = 0.580 (vs. sham) (C). Unsupervised clustering of neuronal responses to the knee compression. Markov Clustering Analysis (MCA) revealed 4 major clusters of responders to knee compression; those that responded with the gradual increase of fluorescence in function of the pressure increase (Ramp), or those that responded only to the lower pressures (Low), middle pressures (Mid) and high pressures (High). Due to the lack of differences in the clusters between the Sham and Cancer animals, groups were pooled (see Figure S4) (D). Clustering of neuronal responses to the limb’s movement. MCA revealed 3 major clusters of cells; those that responded stably throughout the movements (Const), or those that responded with the increase in fluorescence only to the leg pulling (Pull) or pushing (Push). Due to the lack of differences in the clusters between the Sham and Cancer animals, groups were pooled (see Figure S4) (E). Percentage of cells in each knee compression cluster with regards to the group classifiers. Numbers over the bars represent total numbers of cells identified in each cluster. Total numbers of cells after MCA in Sham and Cancer groups are given in brackets. Percentage of cells in each movement cluster with regards to the group classifiers. Numbers over the bars represent total numbers of cells identified in each cluster. Total numbers of cells after MCA in Sham and Cancer groups are given in brackets. Unpaired t-test: ‘Low’ P = 0.007, ‘Mid’ P = 0.007, ‘High’ P = 0.274, ‘Ramp’ P = 0.216. **p < 0.01, NS – non significant (F). Percentage of cells in each movement cluster with regards to the group classifiers. Numbers over the bars represent total numbers of cells identified in each cluster. Total numbers of cells after MCA in Sham and Cancer groups are given in brackets. Unpaired t-test: ‘Pull’ P = 0.342, ‘Push’ P = 0.720, ‘Const’ P = 0.384. NS – non significant (G). Example normalised GCaMP6s fluorescence traces from DRG neurons of the identified knee compression clusters. Pink line indicates the surge in cuff pressure (H). Example normalised GCaMP6s fluorescence traces from DRG neurons of the identified movement-evoked clusters. Pink line indicates events: higher points are reflecting pulling (PL) and lower pushing (PS) the leg along the body axis (I). See also Figure S1.5, S1.6, and Movie 4.

3.3.4 Leg compression and position are differentially coded by DRG sensory neurons

Contrary to cell number, the fluorescent intensity of responders was not altered in the CIBP rat model between sham and cancer groups, neither after brushing (unpaired t-test vs. sham: p = 0.737), nor knee compression (RM-ANOVA (vs. sham): F1, 483 = 0.177, p = 0.674), nor leg movement (RM-ANOVA (vs. sham): F1, 574 = 0.306, p = 0.580) (Fig. 3.4A, B, C, S1.6A, B). Fluorescent intensity increased proportionally with pressure intensity in both sham-operated and CIBP rats (Fig. 3.4B). The major responder pattern of firing was analysed using Markov Cluster Analysis (MCA). This largely unsupervised approach revealed 4 main clusters of neuronal responses to limb compression where afferent responses were classified as (1) triggered by ‘low’ pressure (<100 mmHg), (2) triggered by ‘middle’ pressure (peak at around 200 mmHg), (3) triggered by ‘high’
pressure (>300 mmHg), or (3) triggered by the pressure surge (0-400 mmHg, ‘ramp’) (Fig. 3.4D). Neuronal responses to limb movement revealed 3 clusters, classified as a fluorescence increase to limb (1) ‘pull’, (2) ‘push’ or (3) movement (‘const’) (Fig. 3.4E).

There were significantly more cells in the mid, and less in the low, clusters in CIBP rats compared with sham-operated animals (unpaired t-test: ‘Low’ P = 0.007, ‘Mid’ P = 0.007, ‘High’ P = 0.274, ‘Ramp’ P = 0.216) (Fig. 3.4F). Conversely, there was no difference in the number of responders in the movement clusters (unpaired t-test: ‘Pull’ P = 0.342, ‘Push’ P = 0.720, ‘Const’ P = 0.384) (Fig. 3.4G).

Figure 3.5. Intra- and peri-tibial afferent function in health and bone cancer. Representative z-stack collected at the end of the in vivo imaging experiment in order to identify Fast Blue (FB) traced neurons from the tibial cavity (blue) and all labelled GCaMP6s (green). Scale bars: 100 µm. Arrows indicate FB-traced neurons (A). Percentage of responders from all FB-traced tibial afferents within L3 and L4 DRG during knee compression in Sham and Cancer animals. Pressure was increased in 50 mmHg steps every 10 s (See methods for more). Data represent the mean ± SEM of N = 81 cells from 6 animals (sham) and 220 cells from 9 animals (Cancer). RM-ANOVA: F_{1, 299} = 0.045, p = 0.832 (B). Fluorescence intensities of all responding FB+ neuronal cell bodies to knee compression. Pressure was increased in 50 mmHg increments every 10 s. Data represent the mean ± SEM of N = 15 cells (Sham) from 6 animals, and n = 34 cells from 9 animals (CIBP). RM-ANOVA: F_{1, 47} = 0.552, p = 0.461 (C). Representative z-stack collected at the end of the in vivo imaging experiment in order to identify traced AAVrg/tdTomato (TD) traced neurons innervating the muscle and periosteum (MP)
surrounding tibia (red) and all labelled GCaMP6s (green). Scale bars: 100 µm. Arrows indicate TD-traced neurons (D). Percentage of responders of all TD-traced MP afferents within L3 and L4 DRG during knee compression in Sham and Cancer animals. Pressure was increased in 50 mmHg steps every 10 s (See methods for more). Data represent the mean ± SEM of N = 14 cells from 3 animals (Sham) and 43 cells from 5 animals (Cancer). RM-ANOVA: F1, 55 = 2.868, p = 0.096 (E). Fluorescence intensities of all responding TD+ neuronal cell bodies to knee compression. Pressure was increased in 50 mmHg increments every 10 s. Data represent the mean ± SEM of N = 9 cells (Cancer) from 3 animal. No between-groups comparison was done since in Sham no cells responded (F). Pie chart showing the number of cells in each pressure cluster (identified in Fig. 3D) after the knee compression in FB-traced neurons in sham animals. Numbers represent cells identified in each cluster. Total numbers of cells after MCA are given in brackets (G). Pie chart showing the number of cells in each pressure cluster (identified in Fig. 3D) after the knee compression in TD-traced neurons in cancer animals. Numbers represent cells identified in each cluster. Total numbers of cells after MCA are given in brackets. Since no TD-traced cells responded in the sham group MCA was not performed (I). See also Figure S1.6, S1.7.

3.3.5 Intratibial afferent function in health and CIBP

I next focused on bone afferents using FB tracing. Firstly, I investigated whether the neurons innervating the tibial cavity express Piezo-type mechanosensitive ion channel component 2 (Piezo2) and TrkA. As observed previously (Nencini and Ivanusic, 2017; Nencini et al., 2017; Prato et al., 2017) I confirmed the presence of both proteins on bone afferents, however no quantification was performed (Fig. S1.7A, B). Secondly, I investigated the functional responses of FB-traced intratibial afferents (Fig. 3.5A). Previously these fibres were shown to respond to intraosseous pressure in healthy animals (Nencini and Ivanusic, 2017; Nencini et al., 2017), while NGF sensitizes mechanically activated bone nociceptors (Nencini et al., 2017). I extended these findings by revealing that the sensory cells innervating the tibial cavity respond to whole limb mechanical stimulation; around 13% of traced tibial cavity afferents responded to knee compression. Interestingly, there was no difference between sham and cancer-bearing groups regarding the number of responders to knee compression (RM-ANOVA (vs. sham): F1, 299 = 0.045, p = 0.832) (Fig. 3.5B). Fluorescence intensity of bone afferents after knee compression was equally unaffected by the presence of cancer (RM-ANOVA (vs. sham): F1, 47 = 0.552, p = 0.461) (Fig. 3.5C).

3.3.6 Muscle and periosteum afferents are recruited and sensitized by bone cancer

I investigated whether periosteum and muscle afferents (rather than bone afferents) are sensitised in CIBP rats by tracing muscle and periosteum (MP) afferents using injection of AAV-retrograde virus expressing tdTomato outside the tibia (Fig. 3.5D). Piezo2 and TrkA were both present in the traced cells, however no quantification was performed (Fig. S1.7C, D). I showed that virtually no MP traced cells from the sham-
operated rats responded to mechanical stimulation (Fig. 3.5E). In CIBP rats however, 20% of traced MP afferents responded to knee compression (RM-ANOVA (vs. sham): \( F_{1, 55} = 2.868, p = 0.096 \)). As with total DRG cell analysis, the number of responders increased with compression intensity (Fig. 3.5E). Fluorescence intensity of MP afferents after knee compression increased with the pressure surge in cancer animals (Fig. 3.5F).

3.4 Discussion

Malignant tumours can escape their origin and invade distant regions of the body to form secondary tumours. A frequent direction for metastasis is the skeleton. In this study, I identified a discrete population of peripheral neurons that innervate bone and lack Avil expression. Using in vivo calcium imaging, I also revealed potential mechanisms that drive cancer-induced bone pain.

3.4.1 A high proportion of deep body CGRP afferents express little to no Advillin

I demonstrate that many bone afferents express markers of peptidergic (e.g. CGRP and TrkA), but rarely non-peptidergic (IB4) nociceptors, in agreement with previous reports (Ivanusic, 2009; Jimenez-Andrade et al., 2010b, 2011; Nencini and Ivanusic, 2016b). Further, I found that a large population of bone afferents rich in CGRP lack Avil expression. Over the last decade there have been repeated efforts to more accurately define sub-populations of sensory neurones (Usoskin et al., 2014). Avil was anticipated to be selectively expressed by up to 97% of all sensory DRG cells (Hasegawa et al., 2007). Following this announcement, several murine models were created to drive transgene expression utilising Avil. Recent research suggests that Avil is expressed not only in sensory neurons, but in most adult neural crest-derived neurons including sympathetic fibres (Lopes et al., 2017c). Others reported that Avil-driven transgene expression does not cover around 10-15% of sensory neurons. A restriction to any particular population of the DRG cells, however, was not described (Woo et al., 2015; Zappia et al., 2017). Publicly available single-cell RNA sequencing databases supported my finding of differential expression between CGRP and Avil on the whole population level (Usoskin et al., 2014; Zeisel et al., 2018). Overall the presence of a discrete (around 10% of all DRG neurons) but significant population of peptidergic afferents that are low or almost not expressing Avil, although enriched in CGRP, is reported. This type of afferent appears to be particularly abundant in bone innervation. The physiological meaning of the lack of Avil in bone afferents remains an open question, not least since the exact function of Avil is unknown.
3.4.2 Cancer progression affects bone innervation

I employed a recognised rat CIBP model (Medhurst et al., 2002) and analysed bone afferent response to the tumour. Firstly, based on the high-resolution micro-computer tomography results and individual animal's ability to weight bear, I divided the model into early and late stages of bone cancer: days 7/8, defined by a significant damage of trabecular but little to no cortical bone damage (as measured by BMD), and imbalance in weight bearing <10%; versus days 14/15, defined by damage of both trabecular and cortical bone (as measured by BMD), and imbalance in weight bearing >10%. Secondly, I showed that afferents innervating cancer-bearing bones are sensitized in the early stage with high expression of cellular stress marker Atf3. In the late cancer stage, the activation of Atf3 appears resolved. Multiple mechanisms may contribute to the reduction of Atf3 in the late stages of cancer. It is possible that CIBP afferents are undergoing cell death as a result of tumour invasion and toxic local conditions. Supporting this hypothesis is an observed shift in the expression pattern of Atf3+/FB+ from L3 to L4 DRG between early and late CIBP, further indicating the presence of a degenerative mechanism in L3 tibial afferents, and consecutive sprouting and/or activation of L4 afferents.

The reluctance of CIBP rats to bear weight on the cancer-loaded leg corresponds to the clinic, where CIBP often manifests as a result of musculoskeletal compression, both due to weight bearing in moving subjects and increased intraosseous pressure secondary to tumour expansion (Medhurst et al., 2002; Nencini and Ivanusic, 2017; Nencini et al., 2017; Urch et al., 2003). Indeed, mechanical pain is the most commonly reported type of pain in bone cancer patients (Mantyh et al., 2002). Given the above evidence, I studied mechanosensation in the CIBP rats using in vivo imaging with GCaMP6s.

3.4.3 Leg compression and position are differentially coded by DRG sensory neurons

Pressure and mechanosensation coding is investigated on both a molecular (Ranade et al., 2014; Szczot et al., 2017) and neuronal system levels (Prescott et al., 2014b; Wang et al., 2018). How is modality-reflecting information encoded by sensory neurons? I took the opportunity to analyse DRG cells that responded to mechanical simulation, observing closely whether these cells encoded the analysed stimuli.

The analysed DRG neurons responded globally to pressure with proportional increase in fluorescence to the compression force increase. Graded, frequency coding of compression, which is maintained in both health and disease, is thus likely. In contrast, gentle leg movement likely activating proprioceptors appears encoded by the same cells
responding to different leg positions. This most likely reflects combinatorial coding, where different firing patterns of a cell encode changes in leg position.

The averaged neuronal responses from all analysed cells ignore the individual afferent’s firing characteristics. To stratify whether averaged responses reflected uniform or rather more complex patterns I utilised MCA, an algorithm that is widely used in genetics to cluster sizeable expression datasets as a function of biological relevance. I found that MCA is particularly useful for large functional imaging datasets to reveal major responder’s types to defined longitudinal stimuli. This largely unsupervised approach revealed 4 main clusters of neuronal responses to leg compression and 3 clusters for leg position. In that pressure responders were classified as: (1) ‘low’ if they responded to lower pressures only (<100 mmHg), (2) ‘middle’ (peak at around 200 mmHg), (3) ‘high’ (>300 mmHg), or (4) ‘ramp’ (increased in line with 0-400 mmHg). High but not low responders likely represent nociceptive-specific afferents. The most abundant ramp cluster likely reflects graded frequency coding of compressive forces (similarly to heat (Wang et al., 2018)), which is maintained in both health and disease (Prescott et al., 2014a). Neuronal responses to limb movement were classified by a fluorescence increase to limb (1) ‘pull’, (2) ‘push’ or (3) movement (‘const’). The presumed proprioceptive response most likely reflects combinatorial coding, where different cells encode the different position of the limb (similarly to cold (Wang et al., 2018)) (Prescott et al., 2014a).

The subsequent analysis of the number of cells in each cluster between sham and CIBP groups revealed that there were significantly more cells in the mid, and less in the low, clusters in CIBP rats compared with sham-operated animals. This further suggests the recruitment of nociceptive, mechanically-responding cells in the disease. The decrease in low cluster cells could suggest an inhibition of Aβ-fibres, corresponding to the numbness experienced by patients, or the loss of this fibre type in CIBP rats. The lack of difference in the number of responders in the movement clusters suggests that no one limb position is particularly painful, simply that movement alone engages more cells in CIBP rather than sham-operated rats.

3.4.4 The number of mechanically-responsive sensory neurons is tripled in animals with bone cancer

I showed that pressure is encoded mainly by small to medium-size neurons. As expected, the average cell size decreased as the force applied increased (increasingly painful levels of pressure recruit small nociceptive afferents preferentially over large myelinated Aβ mechanosensors). Previously, dynamic brushing on the calf surface confirmed that light touch is encoded by Aβ fibres (Chisholm et al., 2018; Ma and Woolf, 1996).
By looking at the overall population of analysed DRG neurons, I found a three-fold increase in the number of cells responding to knee compression in CIBP, as compared to sham animals. Interestingly, these cells were small to medium-size, in contrast to results obtained from sham animals where pressure activated mainly medium-size neurons. The latter is in keeping with previous literature which suggests that in healthy animals, noxious compression is encoded preferentially by myelinated Aδ nociceptors (Nencini and Ivanusic, 2017).

The observed increase in sensory afferent response (by means of the robust recruitment of new cells, likely belonging to the ‘Mid’ cluster) was not due to increased individual afferent activity. I hypothesise that these were previously ‘silent’ nociceptive afferents, the recruitment of which has been reported previously in inflammatory conditions (Gold and Gebhart, 2010).

Silent nociceptors describe a type of sensory afferents that fire action potentials after electrical stimulation of the receptive field but are otherwise insensitive to noxious mechanical stimuli. This type of afferents is particularly abundant in deep body structures and extremely rare in rodent skin (Wetzel et al., 2007). Thus, for example colon, knee joint, urinary bladder or muscles were shown to be richly innervated by silent nociceptors, with current estimations suggesting that in mice up to 50% of all deep afferents are ‘silent’ (Feng et al., 2012; Prato et al., 2017; Schaible and Schmidt, 1985). These afferents can be sensitised by variety of inflammatory mediators and as result gain the ability to respond to mechanical stimuli (Feng et al., 2012; Gold and Gebhart, 2010; Prato et al., 2017; Schaible and Schmidt, 1985). Recently, both the histochemical marker (nicotinic acetylcholine receptor subunit alpha 3, CHRNA3) as well as a potential mechanism for ‘unsilencing’ of silent nociceptors by NGF-TrkA-Piezo2 has been proposed (Prato et al., 2017). Considering the observed increase in the number of responders in cancer conditions as well as the fact that the CIBP is NGF-dependent (Bloom et al., 2011; Tomotsuka et al., 2014), it is reasonable to predict that silent nociceptors would significantly contribute to the development of pain in the CIBP rats.

Since I have not tested the electrical activation of the receptive field, and since silent nociceptors are classically defined on the basis of their responsiveness to electrical but not to mechanical stimuli, unless ‘unsilenced’, I rely on the direct comparison of the number of responding afferents to the chosen receptive field stimulation with the pressure ramp. The major methodological limitation of the imaging sensitivity is a low sampling rate (typically 2-4 Hz) together with the lack of electrical activation of silent nociceptors in the control group (sham-operated animals). Therefore, whether the information is encoded in different temporal forms of impulses needs to be verified utilising methodologies offering higher temporal resolution (i.e. electrophysiology).
However, considering relatively slow kinetics of GCaMP6s (as compared to the length of typical action potential), the building up intracellular calcium concentration with each action potential fired, will likely reflect summation of impulses fired in response to the pressure ramp. Therefore, the increase in fluorescence with the continuous increasing stimulus would likely approximate the increase in firing frequency.

3.4.5 Silent nociceptors in CIBP originate from muscle or periosteum

Next, I went on to investigate where these silent nociceptors might originate from. I traced afferents from within the bone with FB and found that they respond to mechanical forces, as demonstrated previously by electrophysiology (Nencini and Ivanusic, 2017; Nencini et al., 2017). However, no additional recruitment was detected in this neuronal population suggesting that silent nociceptors in late cancer stage originate outside of the bone. The reduced fluorescence of bone afferents to the proprioceptive stimulation in CIBP animals suggests impaired bone proprioceptor functioning and/or their loss in tumour conditions.

These functional results support my anatomical findings (decreased Atf3 staining in the late CIBP group) (Fig. 3.2E, H, I) and suggesting a degree of bone afferent loss in advanced cancer states. This is also supported by the shift in FB positivity that occurs from L3 to L4 DRG in the late stage CIBP (Fig. S1.3C). The anaerobic and toxic conditions of the tumour are likely to evoke degeneration of locally entrapped afferents.

Since bone afferents themselves do not appear to be the neurons responsible for sensitization, I next used a viral approach to label MP afferents. I found them to be silent in sham operated animals, but responsive in cancer conditions. This suggests that in the late stage of the disease, cancer induces employment of silent nociceptors from bone surroundings, rather than the bone cavity itself. My anatomical results must be taken with some care. Virally-delivered tdTomato expression levels around the bone remained low and I had to limit the amount of virus used to ensure specificity and to avoid off-target labelling in contralateral DRG. This meant that our cell numbers were low compared to the rest of my analyses, though still considerably higher than those that can be achieved in electrophysiological studies from the same number of animals.

3.4.6 Conclusions

I conclude that the increase in the number of mechanically responding cells, rather than the increase in individual cell sensitivity, likely translates to the mechanical hypersensitivity observed in both CIBP rats and patients with CIBP. A probable mechanism is the activation of silent nociceptors surrounding the tumour. I show that primary afferents respond differently according to the mechanical stimulus, suggestive of specific differential coding of pressure and proprioception (graded vs. combinatorial)
by the somatosensory neurons. Finally, in sham animals, pressure is likely encoded by means of the frequency change, whereas in advanced bone cancer conditions frequency coding is largely dominated by the population coding resulting from the robust recruitment of silent nociceptors.

3.5 Author Contributions
I performed all presented experiments. The following individuals contributed to the collection of the presented data: Dr. Kim Chisholm jointly performed all the in vivo calcium imaging and wrote the R scripts used for GCaMP data analysis. I performed data analysis in R (utilising KC scripts) and other software. Dr. Douglas Lopes provided the primers for genotyping Avil/eGFP mice. Ms. Diane Derrien performed Atf3 quantifications and staining under my supervision.
Chapter IV
4.1 Introduction

In the previous chapter I demonstrated that silent nociceptors are recruited in late stage bone cancer, resulting in a three-fold increase in the number of responding cells to mechanical stimuli applied for example to the knee. Recently, silent nociceptors were shown to terminate strictly in the SDH (Prato et al., 2017), this in line with other nociceptors (Aδ, C-fibres) that also synapse with second order neurons within superficial laminae of the spinal dorsal horn (although some collaterals of Aδ-fibres also project to the deeper laminae) (Basbaum et al., 2009). It stands to reason that peripheral sensitization to mechanical stimulation should be reflected in central (spinal) events. Indeed, Urch et al., utilising the same CIBP rat model, reported the presence of hyperexcitable superficial dorsal horn (SDH) neurons (Urch et al., 2003).

SDH neurons can be broadly divided according to their electrophysiological characteristics into nociceptive specific (NS) and wide dynamic range (WDR) neuronal groups. The former dominates the SDH neuronal composition. In the late stage of the rat CIBP model however, a shift in favour of WDR neuronal-like responses was reported in the SDH (Urch et al., 2003). In the same study, a substantial increase in the peripheral receptive field size of the SDH neurons was described, likely reflecting the employment of previously dormant nociceptors, thus contributing to central sensitization. I showed in the previous chapter that silent nociceptors respond to a broad spectrum of pressures applied, including those from non-noxious range. Since more innocuous input will be delivered to the SDH because of medium diameter primary afferent termination sites, they will likely contribute to increase the ratio of WDR:NS described by Urch and colleagues (Urch et al., 2003). Interestingly, it was possible to reverse the increased WDR:NS ratio with chronic gabapentin treatment, implying that gabapentin normalises this peripheral and/or dorsal horn CIBP-induced plasticity (Donovan-Rodriguez et al., 2005). Such anatomical organisation of spinal inputs may explain the observed hyperexcitability of the superficial laminae in the CIBP animals, but there is also another population of cells responding to the nociceptive inputs.

Deep dorsal horn (DDH) (laminae IV-VI) WDR neurons are known to be a good approximate for peripheral innocuous and noxious inputs, with some projecting supraspinally, mainly to the thalamic nuclei, comprising the spino-thalamic tract (STT). In this chapter the responsiveness of DDH WDR neurons to a range of peripherally
applied stimuli in the rat model of CIBP is presented. The unique characteristics of the DDH WDR neurons are detailed in Chapter I, general introduction. There exists a dearth of publications regarding the activity of DDH WDR neurons in CIBP rodent models. Two reports have demonstrated no difference in the firing rates of DDH WDR cells after mechanical stimulation of the peripheral receptive field (Falk et al., 2015a; Urch et al., 2003). However increased input, C-fibre and post-discharge responses in the CIBP group as compared with sham were reported only in one of the studies, and this study also documented no expansion of the peripheral receptive field (Urch et al., 2003). It differs substantially from the spinal nerve ligation (SNL) model of neuropathy, where the opposite pattern was observed (Suzuki et al., 2000).

Utilising in vivo electrophysiology to record spinal neuronal responses to mechanical and electrical stimulation, the inhibitory effect of acute and chronic morphine in CIBP was demonstrated in terms of SDH and DDH WDR neuronal output (Urch et al., 2005), confirming proper functioning of the spinal opioidergic pain controls in the CIBP model. This back-translates from the clinic, where opioids are still the first and the most effective line for CIBP management. Acute spinal tapentadol, a dual acting μ-opioid receptor (MOR) agonist and norepinephrine reuptake inhibitor (NRI), strongly inhibited DDH WDR neuronal responses in the CIBP (Falk et al., 2015a). This effect was abolished by naloxone (a non-selective opioid receptor antagonist) application however, not by the application of atipamezole (a selective $\alpha_2$ adrenergic receptors antagonist). This is suggestive that in the CIBP model, effects of systemic tapentadol are mediated mainly by spinal MORs. In contrast, in the SNL model of neuropathy, the spinal NRI-related action predominates over the agonism of spinal MORs (Bee et al., 2011).

The direction of effect of spinal atipamezole likely exposes plasticity in top-down modulatory controls. Upon activation of locus coeruleus (LC) neurons (for example by a strong painful stimulus), NA is released in the spinal cord where it orchestrates its action via alpha adrenergic receptors ($\alpha$-ARs), which are either facilitatory ($\alpha_1$-ARs) or inhibitory ($\alpha_2$-ARs).

Importantly, the regulation and modulation of descending noradrenergic controls has not been studied in the CIBP rodent model previously. Therefore, the research described in this chapter was designed to dissect the role of descending noradrenergic modulatory controls in both early and late stage of rodent bone cancer progression.
4.2 Rationale, hypothesis and aims

Spinal processing in a rodent model of CIBP is largely unexplored. To date, 8 physiological reports on spinal CIBP mechanisms exist and have been gathered from the Dickenson lab at UCL and the McMahon lab at KCL. By utilising in vivo electrophysiology, six of these studies focused on superficial laminae neuronal responses, and 4 on the DDH WDR neuronal physiology. In this chapter a detailed populational study of DDH WDR neurons in different stages of bone cancer progression (precisely defined in Chapter III: early stage (day 7-8) and late stage (day 14-16) stage) is presented. Here, the pharmacological manipulation of spinal $\alpha_2$ adrenergic receptors is described following my analysis of the effect of tonic noradrenergic descending control systems on spinal WDR neuronal processing in the rodent model of CIBP. Further, I analysed the interaction of spinal opioid and noradrenergic systems and examined the supraspinal circuity underlaying descending noradrenergic inhibitions in this model.

4.3 Materials and Methods

A detailed description of employed in this chapter procedures can be found in Chapter II. See pages referral below.

- Cell cultures and animals (detailed description, page 35)
- CIBP rat model (detailed description, page 35)
- Behaviour: Von Frey (detailed description, page 39)
- In vivo spinal electrophysiology (detailed description, page 43-45)
  - Baseline responses (detailed description, page 43)
  - Spinal pharmacology (detailed description, page 45)
  - Brain pharmacology (detailed description, page 46)
- Optogenetic experiments:
  - Viral delivery of opsins (detailed description, page 46)
  - Optoactivation with in vivo spinal electrophysiological recordings (detailed description, page 47)
- Immunohistochemistry (detailed description, page 41)
- PACT clearing of DRG (detailed description, page 42)
- Quantification and statistical analysis (detailed description, page 48)
4.4 Results:

**Figure 4.1.** Rats with bone cancer present secondary mechanical hyperalgesia. Behavioural analysis of ipsilateral (A) and contralateral (B) leg with von Frey filaments in sham and CIBP rats. Data represent the mean ± SEM. N = 20 animals per group. Kruskal–Wallis for independent samples: Ipsi: overall sham vs. CIBP P<0.01, F[1, 132] = 10.321, pairwise comparison: day 0 – day 7: non-significant (N.S.), day 14: ****P < 0.0001; Contra: overall sham vs. CIBP P = 0.958, F[1, 132] = 0.003.

4.4.1 Rats with bone cancer present secondary mechanical hyperalgesia

I began by behaviourally assessing the presence of secondary mechanical sensitisation in both sham—operated (n = 20) and cancer-bearing (n = 20) animals using the von Frey test. CIBP rats exhibited mechanical allodynia on the side ipsilateral to cancer cell injection from day 14 post-surgery (Kruskal–Wallis for independent samples: Ipsi: overall sham vs. CIBP P<0.01, F[1, 132] = 10.321, pairwise comparison: day 0 – day 7: non-significant (N.S.), day 14: ****P < 0.0001) (Fig. 4.1A). Both animal groups experienced postsurgical pain in the week following surgery as revealed by a lowered mechanical threshold for von Frey filaments that lasted up until day 7 (Kruskal–Wallis one-way ANOVA [group]: P = 0.0001; within group Bonferroni: day 2 and day 7, as compared to day 0 within group: P = 0.0001, P = 0.029, respectively. Not indicated on the graphs) (Fig. 4.1A). No differences were detected on the contralateral sites in either analysed group (Kruskal–Wallis for independent samples: Contra: overall sham vs. CIBP P = 0.958, F[1, 132] = 0.003) (Fig. 4.1B).
4.4.2 Deep dorsal horn wide-dynamic range neurons are not hyperexcitable in our rodent model of CIBP

The activity of DDH WDR neurons was studied in rats under light isoflurane/N₂O/O₂ anaesthesia (slight toe pinch reflex maintained). Electrophysiological (in vivo) recordings of DDH WDR neurons were used to study von Frey and brush-evoked firing rates (Fig. 4.2A). A train of 16 electrical impulses to the receptive field (localised on the hind paw...
tees ipsilateral to injury) was also applied to verify changes in the basal spinal coding and temporal summation. Stable baseline neuronal recordings from sham early (SE, n = 6), CIBP early (CE, n = 11), sham late (SL, n = 31) and CIBP late (CL, n = 31) were made. One neuron was studied per animal. Animals with CIBP (early or late stage) showed no significant change in the basal firing rate of WDR neurons when compared to sham-operated WDR neuronal firing rates. Analysis of variance revealed significant changes in the basal von Frey-evoked activity only between CE and SL groups (univariate between-subject test [group]: $F_{3, 315} = 4.127$, $P = 0.007$; multiple comparison [group]: Bonferroni post hoc: all $P > 0.05$, but CE vs. SL: $P < 0.05$) (Fig. 4.2B). A comparison between each group for individual bending forces revealed a significant difference only for WDR response to 8 g filament application at the peripheral receptive field between CE and SL groups (one way ANOVA [group]: [2 g]: $F_{3, 72} = 1.391$, $P = 0.253$, [8 g]: $F_{3, 81} = 3.268$, $P < 0.05$, Bonferroni post hoc: all $P > 0.05$, but CE vs. SL: $P < 0.05$. [26 g]: $F_{3, 81} = 1.662$, $P = 0.182$, [60 g]: $F_{3, 81} = 0.882$, $P = 0.454$) (Fig. 4.2C). Dynamic brushing of the receptive field (localised typically on the paw) revealed no significant difference between all analysed groups (univariate ANOVA [group]: $F_{3, 81} = 0.476$, $P = 0.700$) (Fig. 4.2D). Electrically evoked parameters, $\alpha\beta$- ($F_{3, 75} = 0.655$, $P = 0.582$), $\alpha\delta$- ($F_{3, 75} = 0.655$, $P = 0.582$) and $C$- fibre ($F_{3, 75} = 2.667$, $P = 0.054$) evoked activity and post-discharge ($F_{3, 75} = 1.899$, $P = 0.137$), input ($F_{3, 75} = 1.213$, $P = 0.311$) and wind-up ($F_{3, 75} = 1.483$, $P = 0.226$) were all unchanged between groups (all: univariate ANOVA [group]) (Fig. 4.2E).

Table 4.1. Pharmacological comparison of selected alpha-2 adrenergic receptors antagonists. Modified from Pertovaara et al., 2005. $K_i$ values are for the displacement of the relevant receptor’s ligand: $[^{3}\text{H}]$-Prazosin for $\alpha_1$, $[^{3}\text{H}]$-clonidine for $\alpha_2$. Yohimbine is an alkaloid isolated from yohimbe tree (Pausinystalia johimbe), atipamezole and idazoxan are fully synthetic.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\alpha_2$</th>
<th>$\alpha_1$</th>
<th>$\alpha_2 / \alpha_1$</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atipamezole</td>
<td>1.6</td>
<td>13300</td>
<td>8300</td>
<td></td>
</tr>
<tr>
<td>Idazoxan</td>
<td>148</td>
<td>3960</td>
<td>27</td>
<td>40</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>130</td>
<td>5130</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$\alpha_1$, $[^{3}\text{H}]$-prazosin; $\alpha_2$, $[^{3}\text{H}]$-clonidine displacement

A. Pertovaara et al. CNS Drug Reviews 2005
Tonic descending noradrenergic controls are altered in our rodent model of CIBP. Following spinally applied atipamezole (100 µg) recordings were made of deep dorsal horn lamina V/VI WDR neuronal responses to punctate mechanical (A, D, G, J), dynamic brushing (B, E, H, K) and electrical (C, F, I, L) stimulation of the receptive field in early (day 7-8, top panels) and late (day 14-16, bottom panels) CIBP stage and corresponding sham-operated rats. PD-post discharge. All the data represent the mean ± SEM from sham early (n = 4), CIBP early (n = 6), sham late (n = 11) and CIBP late (n = 9). Each dot represents one animal. RM-ANOVA with Bonferroni post-hoc: *P < 0.05, **P < 0.01 vs. baseline (for punctate mechanical stimulation) and paired t-test: *P < 0.05 vs. baseline (for brush and electrical stimulation). See also Figure S2.1 for effects of 10 µg of atipamezole.

4.4.3 Tonic descending noradrenergic controls are altered in our rodent model of CIBP

The spinal WDR modulation (as described above) was unexpected, not least because SDH neurons were previously reported to be hyperactive in the CIBP-model (Urch et al., 2003). Explanations could include the plasticity of internal spinal circuitry and/or activation of potent inhibitory mechanisms. Knowing that peripheral input in bone cancer conditions...
is increased (see Chapter III), and knowing that descending facilitatory controls, orchestrated via spinal 5-HT₃ receptor activation, are enhanced in the CIBP rat model impinging on both SDH and DDH neurons (Donovan-Rodriguez et al., 2006), it was imperative for me to investigate whether plasticity occurs within the descending inhibitory controls. To challenge that question, a selective α₂-AR blocker atipamezole (see Table 4.1) was applied spinaly to sham operated and CIBP rats in two doses: 10 and 100 µg. Atipamezole is a small and largely hydrophobic molecule and a brain/spinal cord penetrant. In rats, atipamezole concentrations (at the peak of action) in the CNS are 2-3-fold higher than in plasma levels and its elimination via first-pass metabolism is fast with the half-life of 1.3 h (Haapalinna et al., 1997, 1999). After achieving 3 stable baseline readouts for a set of tested stimuli each, the drug was applied topically on the spinal cord (in 50 µl of vehicle) immediately above the recording site (lumbar L3-4 enlargement). Vehicle alone (97% saline, 2% cremophore, 1% DMSO) had no effect on WDR neuronal activity (not shown). For clarity, only results utilising the higher dose of atipamezole are shown throughout (10 µg is reported in the Appendix 2, Figure S2.1).

A brand-new set of in vivo electrophysiological experiments was completed. In the SE group (n = 4), spinal application of atipamezole had no effect on the DDH WDR neuronal responses, as measured using in vivo electrophysiology, to peripherally applied von Frey filaments (repeated-measures ANOVA [group]: F₁, ₃ = 0.737, P = 0.454) (Fig. 4.3A), nor to dynamic brushing (paired t-test: P = 0.825) (Fig. 4.3B). Electrically evoked parameters measured, including Aβ- (P = 0.064), Aδ- (P = 0.262) and C- fibre (P = 0.763) responses and post-discharge (P = 0.988), input (P = 0.632) and wind-up (P = 0.380), were not affected either (all: paired t-test) (Fig. 4.3E).

Similarly, in the CE group (n = 6), atipamezole had no effect on the DDH WDR neuronal activity, neither to bending force (repeated-measures ANOVA [group]: F₁, ₅ = 0.171, P = 0.248) (Fig. 4.3D), nor to dynamic brushing (paired t-test: P = 0.909) (Fig. 4.3E) applied to the receptive field. Electrically evoked parameters, Aβ- (P = 0.926), Aδ- (P = 0.144) and C- fibre (P = 0.473) evoked activity and post-discharge (P = 0.227), and input (P = 0.308) were not affected either (all: paired t-test) (Fig. 4.3F). Interestingly, wind-up was significantly decreased after spinal atipamezole application (P = 0.040) (Fig. 4.3F).

As in SE, in the late sham-operated (SL) group (n = 11), atipamezole had no effect on the DDH WDR neuronal activity, neither to bending force (repeated-measures ANOVA [group]: F₁, ₁₀ = 0.085, P = 0.776) (Fig. 4.3G), nor to dynamic brushing (paired t-test: P = 0.356) (Fig. 4.3H) applied to the receptive field. Electrically evoked parameters, Aβ- (P = 0.840), Aδ- (P = 0.521) and C- fibre (P = 0.556) evoked activity and post-
discharge (P = 0.503), input (P = 0.135) and wind-up (P = 0.562) were also unaffected (all: paired t-test) (Fig. 4.3I).

However, in the CL group (n = 9), spinal application of atipamezole induced an unexpected effect; interestingly, it dose-dependently inhibited DDH WDR neuronal activity. Precisely, responses to both innocuous and noxious mechanical stimuli were inhibited (repeated-measures ANOVA [group]: F_{1,8} = 23.965, P = 0.001, Bonferroni post hoc: [2 g]: P < 0.05, [8, 26, 60 g]: P < 0.01) (Fig. 4.3J). Responses to dynamic brushing of the receptive field, however, were not affected (paired t-test: P = 0.066) (Fig. 4.3K). As well as dynamic brushing, atipamezole did not modulate electrically evoked Aβ-related activity (paired t-test: P = 0.085), nor input (paired t-test: P = 0.266) or wind-up (paired t-test: P = 0.069) (Fig. 4.3L). Nociceptor-related actions following electrical stimulation of the receptive field (the ipsilateral hind paw toes) were, however, significantly inhibited by the spinal application of atipamezole: Aδ- (paired t-test: P = 0.034) and C- fibre (paired t-test: P = 0.023) evoked activity and post-discharge (paired t-test: P = 0.046) outputs (Fig. 4.3L).
Figure 4.4. Inhibition of mechanosensation by spinal α2-ARs block depends on a functioning opioid system in the late stage CIBP rats. Following spinally applied naloxone (20 µg) recordings were made of deep dorsal horn lamina V/VI WDR neuronal responses to punctate mechanical (A, D), dynamic brushing (B, E) and electrical (C, F) stimulation of the receptive field in late (day 14-16) CIBP stage and corresponding sham-operated rats. Similarly, following spinal co-administration of atipamezole (100 µg) and naloxone (20 µg) recordings of WDR neuronal action potentials were made to punctate mechanical (G, J), dynamic brushing (H, K) and electrical (I, L). PD-post discharge. All the data represent the mean ± SEM from sham (naloxone: n = 5, drug mix: n = 6 except electrical stimuli, where n = 3), CIBP (naloxone: n = 6, drug mix: n = 6). Each dot represents one animal. RM-ANOVA with Bonferroni post-hoc: *P < 0.05 vs. baseline (for punctate mechanical stimulation) and paired t-test: *P < 0.05, **P < 0.01 vs. baseline (for brush and electrical stimulation). See also Figure S2.2 for effects of 5 µg of naloxone.

4.4.4 Inhibition of mechanosensation by spinal α2-ARs block depends on a functioning opioid system in the late stage CIBP rats

A decrease in DDH WDR neuronal activity in CL rats following spinal α2-AR antagonism suggests a diminished descending NAergic control mediated by these receptors. Based on the results of previous research it is likely that an interplay of
opioidergic- and noradrenergic-inhibitory systems exists in the CIBP rat, with possible selective strengthening of spinal opioidergic controls over NA ones in cancer conditions (Falk et al., 2015a). To verify potential plasticity within the spinal opioid system in the late stage of CIBP, naloxone, a pan-opioid receptor antagonist, was applied directly onto the spinal cord following measurement of stable baseline WDR responses (concentrations of 5 µg (Figure S2.2), and 20 µg were applied in 50 µl saline).

In the SL group, spinal naloxone facilitated DDH WDR neuronal activity (n = 5) to both noxious von Frey filament stimulation (repeated-measures ANOVA [group]: F_{1, 4} = 12.438, P = 0.024, Bonferroni post hoc: [2, 8 g]: P > 0.05, [26, 60 g]: P < 0.05) (Fig. 4.4A), and dynamic brushing (paired t-test: P = 0.004) (Fig. 4.4B) of the receptive field. Interestingly, electrically-evoked parameters including Aβ- (P = 0.211), Aδ- (P = 0.304) and C-fibre (P = 0.238) evoked activity and post-discharge (P = 0.230), input (P = 0.313) and wind-up (P = 0.482) were not significantly changed (all: paired t-test) (Fig. 4.4C).

There was no significant effect on DDH WDR neuronal activity in response to von Frey stimulation of the ipsilateral paw in the CL group (repeated-measures ANOVA [group]: F_{1, 6} = 4.122, P = 0.089) (Fig. 4.4D). Responses to dynamic brushing, however, were increased after 20 µg of spinally-applied naloxone (paired t-test: P = 0.004) (Fig. 4.4E). Amongst electrically evoked parameters, only C-fibre responses were facilitated (P = 0.033), while the other remained unchanged: Aβ- (P = 0.858), Aδ- (P = 0.294) evoked activity and post-discharge (P = 0.131), input (P = 0.520) and wind-up (P = 0.557) (all: paired t-test) (Fig. 4.4F). This partly facilitatory response suggests the presence of a tonic opioidergic inhibition.

Descending noradrenergic modulation of DDH WDR neuronal activity may occur via actions on spinal interneurons. Descending NA can inhibit spinal neuronal responses either directly via ARs expressed on the cells that I am recording from (i.e. DDH WDR neurons), or indirectly by inhibition (via α2-ARs) or activation (via α1-ARs) of inhibitory or excitatory interneurons respectively, which in turn can ‘silence’ DDH WDR neurons. Indeed, the inability of atipamezole to reverse the inhibitory actions of tapentadol (Falk et al., 2015a), taken together with observed inhibition after atipamezole application in CIBP (Fig. 4.3), may suggest that in CIBP NA action is strengthened via actions at inhibitory interneurons expressing α1-ARs.

To reveal if such inhibitory interneurons are opioidergic, 100 µg of atipamezole was co-administered spinally with 20 µg of naloxone. A facilitatory trend of DDH WDR neuronal-evoked responses was observed after this spinal application of the drug mix, detailed as follows. In the SL group, naloxone-atipamezole spinal application had no effect on DDH WDR neuronal activity (n = 6) to mechanical (repeated-measures ANOVA
Amongst electrically evoked parameters, only Aβ-fibre responses were facilitated (P = 0.020), while others remained unchanged, Aδ- (P = 0.564), C-fibre (P = 0.175) evoked activity and post-discharge (P = 0.270), input (P = 0.163) and wind-up (P = 0.769) (all: paired t-test) (Fig. 4.4I). Interestingly, in the CL group (n = 6) the atipamezole-evoked inhibitions observed earlier (Fig. 4.3J-L) were fully reversed by the naloxone co-administration. No inhibitory effect on DDH WDR neuronal activity (n = 6) in response to von Frey stimulation (repeated-measures ANOVA [group]: F1, 5 = 6.326, P = 0.053) (Fig. 4.4J), dynamic brushing (paired t-test: P = 0.070) (Fig. 4.4K), or to any of the electrically evoked parameters (Aβ- (P = 0.358), Aδ- (P = 0.521) and C- fibre (P = 0.663) evoked activity and post-discharge (P = 0.263), input (P = 0.170) and wind-up (P = 0.252) (all: paired t-test) (Fig. 4.4L) was observed.

Figure 4.5. Pharmacological blockade of the locus coeruleus in the late stage CIBP rats abolishes spinal atipamezole-mediated inhibitions. Schematic representation of experimental approach (A). Following 3 stable baseline extracellular single-unit recordings (averaged) of deep dorsal horn lamina V/VI WDR neurons in late (day 14-16) CIBP stage rats, lidocaine (400 nl, 2%) was injected into ipsilateral locus coeruleus (LC). 10 minutes after lidocaine application, atipamezole (100 μg) was administered spinally and brush- and punctate mechanical-evoked action potentials were measured with 10-minute intervals (B). All the data represent the mean ± SEM and each dot represent one animal (n = 3). Statistical analysis not performed due to the small sample size. This is a pilot data only.

4.4.5 Pharmacological blockade of the locus coeruleus in the late stage CIBP rats abolishes spinal atipamezole-mediated inhibitions

The majority of descending NAergic projections to the dorsal horn of the spinal cord originate from the locus coereleus (LC) and were previously shown to synapse mainly within laminae I-III omitting LV, suggesting that interactions with spinal interneurons ultimately drive and/or influence deep dorsal horn-embedded neuronal activity (Kwiat et al., 1992). I wanted to check whether, in late stage bone cancer, the spinal inhibitory function of atipamezole depends on LC activity. To do this I performed
a simultaneous injection of 400 nl 2% lidocaine in the ipsilateral LC (10° rostral angulation from lambda: RC: -2.1 mm, ML: 1.3 mm, and -5.8-6.2 mm deep from the cerebellar surface) while measuring DDH WDR neuronal responses following intrathecal application of 100 µg atipamezole (n = 3) (Fig. 4.5A). Lidocaine block of the LC seemed to reverse the inhibitory action of spinal atipamezole previously observed in CL rats to all mechanically-evoked responses (Fig. 4.5B). These results need to be taken with care (pilot data).

Figure 4.6. Spinal noradrenaline originates from the locus coeruleus. Schematic representation of the optogenetic experimental setup. Two weeks before the experiment
channelrhodopsin-2 was expressed in the locus coeruleus (LC) utilising canine adenovirus (CAV-sPRShChR2(H134R)-mCherry). Simultaneous recordings of LC neurons and ipsilateral lamina V/VI WDR neurons were made and followed by optoactivation of the LC (A). Histological analysis of the transduced LC. First 4 panels represent individual confocal images taken of the transduced LC: DAPI (nuclei staining, blue), tyrosine hydroxylase (TH, green), virally-delivered construct with reporter (mCherry, red), dopamine-β-hydroxylase (DBH, magenta). Last panel represent the merged 4 images with an overlaid indication of the optrode’s position (B). Example electrophysiological recording from the transduced LC neurons. Light-evoked (top panel indicating blue diode current: 470 nm, 15 mW, 5 Hz) action potentials of the LC neurons (bottom). Optimal stimulus width was chosen experimentally (here 25 ms) induced as many action potentials as any higher widths (2 representative 25 ms light pulses with corresponding spikes are magnified for clarity) (C). Spike2 traces represent: top row – 470 nm diode on/off indication, second row – extracellular activity in the LC, third row – single-unit activity of WDR spinal neuron, bottom row – columns representing number of spikes per second (D). Punctate mechanically-evoked action potentials of deep dorsal horn WDR neurons in naïve rats (averaged 3 stable baselines) and effects of the LC optostimulation (3 averaged, with minimally 5 minutes breaks between tests). All the data represent the mean ± SEM and each dot represent one animal (n = 5). One-way RM-ANOVA with Greenhouse-Geisser correction and Bonferroni post hoc: *P < 0.05, **P < 0.01 vs. baseline, $P < 0.05, $$P < 0.01 vs. light ON (E). Effects of spinally administered atipamezole (100 µg) on the LC-mediated inhibition of von-Frey filaments-evoked deep dorsal horn WDR neuronal activity. Activity was measured with 10-minute intervals. All the data represent the mean ± SEM (n = 5 animals). One-way RM-ANOVA with Greenhouse-Geisser correction and Bonferroni post hoc: *P < 0.05 vs. baseline light OFF, $P < 0.05 vs. baseline light ON, #P < 0.05 vs. 0 min. (F).

4.4.6 Optoactivation of the locus coeruleus in naïve animals mimics the altered spinal noradrenergic-mediated actions observed in late stage CIBP rats

To check whether selective activation of the LC noradrenergic neurons was sufficient to induce the observed alterations in spinal atipamezole pharmacology, channelrhodopsin-2 (ChR2) controlled by a NA-specific synthetic promoter (PRS) was delivered in canine adenovirus (CAV-sPRShChR2(H134R)-mCherry) to the LC (Fig. 4.6A, B). A simultaneous recording from the transduced LC (with in-house manufactured optrode) and from the ipsilateral lumbar L3-4 DDH WDR neurons was performed (n = 5) (Fig. 4.6A, C, D). Notably, ventral LC (optrode with 10° angulation from lambda: RC: -2.1 mm, ML: 1.3 mm, -5.8-6.2 mm deep from the cerebellar surface) optoactivation resulted in a decreased firing rate of lumbar DDH WDR neurons to mechanical stimulation of the peripheral receptive field (hind paw) (Fig. 4.6D, E). The optimal optoactivation regimen was found experimentally (Fig. 4.6C). The decrease in DDH WDR neuronal firing upon LC optoactivation was significant for both innocuous and noxious stimuli (One-way RM-ANOVA with Greenhouse-Geisser correction [light]: 8 g von Frey: $F_{1,41,5.63} = 21.96, P = 0.003$, Bonferroni post hoc: $P < 0.05$ light on vs. baseline, $P < 0.01$ light off vs. light on; 15 g von Frey: $F_{1,27,5.10} = 25.23, P = 0.003$, Bonferroni post hoc: $P < 0.01$ light on vs. baseline, $P < 0.01$ light off vs. light on; 26 g von Frey: $F_{1,10,4.41} = 13.10, P = 0.02$, Bonferroni post hoc: $P < 0.01$ light on vs. baseline, $P < 0.05$ light off
vs. light on; 60 g von Frey: $F_{1,23,4.91} = 10.72$, $P = 0.02$, Bonferroni post hoc: $P < 0.05$ light on vs. baseline, $P < 0.05$ light off vs. light on), but not for dynamic brushing of the receptive field ($n = 5$ cells from 5 animals, One-way RM-ANOVA with Greenhouse-Geisser correction [light]: Brush: $F_{1,10, 4.40} = 6.57$, $P = 0.056$) (Fig. 4.6E). Finally, spinal application of 100 $\mu$g atipamezole did not reverse the inhibitory action of LC optoactivation. Light pulses were delivered 30 s before and throughout each series of tests (approximately 5 minutes per series) and minimally 5 minutes of the recovery time was allowed between the tests. The inhibition to all mechanically-evoked stimuli after atipamezole application was similar or even higher than for the light effect by itself (One-way RM-ANOVA with Greenhouse-Geisser correction [time]: 8 g von Frey: $F_{2,53, 10.13} = 5.525$, $P = 0.019$, Bonferroni post hoc: 0 min vs. baseline light ON: $P = 0.008$, rest: $P > 0.05$; 15 g von Frey: $F_{2,09, 8.37} = 15.275$, $P = 0.002$, Bonferroni post hoc: baseline light ON vs. baseline light OFF: $P = 0.026$, 0 min vs. baseline light ON: $P = 0.018$, rest: $P > 0.05$; 26 g von Frey: $F_{1,86, 7.45} = 13.926$, $P = 0.003$, Bonferroni post hoc: baseline light ON vs. baseline light OFF: $P = 0.037$, 0 min vs. baseline light ON: $P = 0.018$, 10 min. vs. 0 min.: $P = 0.020$, 20 min vs. 0 min.: $P = 0.016$, rest: $P > 0.05$; 60 g von Frey: $F_{1,86, 7.45} = 13.93$, $P = 0.003$, Bonferroni post hoc: all $P > 0.05$) (Fig. 4.6F).
Figure 4.7. Optoactivation of lamina I projection neurons inhibits deep dorsal horn wide dynamic range neurons. Schematic representation of experimental approach. Two weeks before the experiment channelrhodopsin-2 was expressed in the lamina I projection neurons (PNs) by injecting lateral parabrachial nucleus with AAV retrograde viral particles (pAAVrg-Syn-ChR2(H134R)-GFP) to retrogradely label superficial dorsal horn (SDH) PNs. Recordings lamina V/VI WDR neurons were made and followed by optoactivation of the ipsilateral lamina I cells, approximately 500 µm rostrally from the recording site (A). Histological confirmation of the transduced SDH-PNs. The microphotograph of PACT-cleared 1000 µm-thick cross-section of the lumbar spinal cord. Virally-delivered construct with reporter (eGFP, green), dopamine-β-hydroxylase (DBH, magenta). White arrows indicate lamina I and lateral spinal nuclei PNs. Note the stronger labelling on the contralateral side to the injected lPB area. (B). A microphotograph of PACT-cleared 500 µm-thick cross-section of the pontine lPB area.
injected with the AAVs. Note that AAVrg serotype labels cells also locally (C). Example electrophysiological recording from the transduced lamina I neurons. Light-evoked (top panel indicating blue diode current: 470 nm, 15 mW, 5 Hz, 25 ms pulse width) action potentials of the lamina I neurons (bottom) (D). Spike2 traces represent: top row – 472 nm diode on/off indication, middle row – single-unit activity of lamina V/VI WDR neuron, bottom row – columns representing number of spikes per second (E). Brush- and punctate mechanical-evoked action potentials of deep dorsal horn WDR neurons in naïve rats (averaged 3 stable baselines) and effects of the lamina I optostimulation (3 averaged, with minimally 5 minutes breaks between tests). All the data represent the mean ± SEM and each dot represent one animal (n = 5). One-way RM-ANOVA with Greenhouse-Geisser correction and Bonferroni post hoc: *P < 0.05, ****P < 0.0001 vs. baseline, ##P < 0.01 vs. light ON (F). Effects of spinally administered atipamezole (100 µg) on the lamina I-mediated inhibition of von-Frey filaments-evoked deep dorsal horn WDR neuronal activity. Activity was measured with 10-minute intervals. All the data represent the mean ± SEM (n = 2 animals) – statistical analysis not performed (G). Effects of spinally administered naloxone (20 µg) on the lamina I-mediated inhibition of von-Frey filaments-evoked deep dorsal horn WDR neuronal activity. Activity was measured with 10-minute intervals. All the data represent the mean ± SEM (n = 3 animals) – statistical analysis not performed (H).

4.4.7 Optoactivation of lamina I projection neurons inhibits deep dorsal horn wide dynamic range neurons

Next, I wanted to physiologically verify the origin of input evoking the LC hyperexcitability. Neurons from spinal lamina I (LI) project to the brainstem nuclei and the majority (up to 92%) of these projections are to the lateral parabrachial nuclei (lPB), with some projection to the LC (Kwiat et al., 1992). These SDH neurons have previously been reported to be hyperexcitable in the CIBP rat model (Urch et al., 2003). To test the hypothesis that selective activation of supraspinally projecting lamina I cells is sufficient to activate the LC and stimulate descending NA-mediated control of DDH WDR neuronal responses, ChR2 was selectively expressed in LI projecting cells in naïve animals. This was achieved by retrograde labelling of these cells from the contralateral to the recording site IPB nucleus. Retrograde serotype of adeno-associated virus (AAVrg) was injected to the IPB nucleus to express ChR2 under neuronal-specific promoter – synapsin (pAAVrg-Syn-ChR2(H134R)-GFP) in LI cells projecting to the IPB area (Fig. 4.7A, B, C).

After placing the bare optic fibre in contact with the spinal cord surface 0.5-1 cm rostrally from the DDH recording site, lamina I neurons were optoactivated while recording action potentials from the lumbar DDH WDR neurons (n = 7) (Fig. 4.7A). The optimal optoactivation regimen was found experimentally by recording light-evoked action potentials from SDH neurons (the recording electrode in this case was placed 50-150 µm below the cord surface and proximal to the optic fibre) (Fig. 4.7D). Mechanically-evoked activity of lumbar DDH WDR neurons was potently inhibited by the optoactivation of lamina I cells (Fig. 4.7E, F). Precisely, no direct light-evoked action potentials were fired when recording from DDH neurons proving poor 470 nm light penetration to deeper
cord areas. The decrease in DDH WDR neuronal firing upon LI optoactivation was significant for noxious but not non-noxious stimuli (One-way RM-ANOVA with Greenhouse-Geisser correction [light]: 8 g von Frey: $F_{1.57, 9.43} = 5.14, P = 0.037$, Bonferroni post hoc: $P > 0.05$ for all; 26 g von Frey: $F_{1.32, 7.89} = 9.42, P = 0.012$, Bonferroni post hoc: $P < 0.05$ light on vs. baseline; 60 g von Frey: $F_{1.15, 6.89} = 24.73, P = 0.001$, Bonferroni post hoc: $P < 0.0001$ light on vs. baseline, $P < 0.01$ light off vs. light on) (Fig. 4.7E, F), nor for dynamic brushing of the receptive field (One-way RM-ANOVA with Greenhouse-Geisser correction [light]: Brush: $F_{1.21, 7.28} = 5.37, P = 0.048$, Bonferroni post hoc: $P > 0.05$ for all) (Fig. 4.7E, F).

Finally, spinal application of 100 µg atipamezole ($n = 2$) seemed not to reverse the inhibitory action of LI optoactivation (Fig. 4.7G). Light pulses were delivered 30 s before and throughout each series of tests (approximately 5 minutes per series) and minimally 5 minutes of the recovery time was allowed between the tests. The inhibition to all mechanically-evoked stimuli after simultaneous atipamezole application and optoactivation was similar or even higher than for light action itself (Fig. 4.7G). Interestingly, spinal application of 20 µg naloxone ($n = 3$ cells) abolished light-evoked inhibitions of DDH WDR suggesting opioidergic mechanisms (Fig 4.7H). These provisional results studying atipamezole and naloxone pharmacology were not statistically analysed due to the small sample size.

### 4.5 Discussion

As described in the previous chapter, changes in peripheral nervous system activity, in particular the employment of silent nociceptors with bone cancer, was expected to have a large impact on the spinal processes. Indeed, the behavioural data discussed in this chapter demonstrates that animals with CIBP manifest features of secondary mechanical hypersensitivity. The behavioural changes develop after day 7 of cancer implantation and progress with time. Observed mechanical hyperalgesia in the early stage results in mechanical allodynia by the late cancer stage, suggesting progressive central sensitization. These results correspond to other studies using similar rodent models (Medhurst et al., 2002; Urch et al., 2003).

### 4.5.1 Deep dorsal horn wide-dynamic range neurons are not hyperexcitable in CIBP

The observed peripheral sensitization and recruitment of silent nociceptors with bone cancer, as described in Chapter III, were not directly reflected in the DDH WDR neuronal responses. Here, a large population of single unit recordings from DDH revealed a lack of modulation to electrically- and mechanically-evoked activity between
analysed groups. Interestingly, Urch et al. reported WDR neuronal excitability in the CIBP DDH, however this was restricted to the selected modalities tested (i.e. to heat, but not to mechanical), and the facilitation was far less noticeable than that observed for the SDH WDR cells (Urch et al., 2003). In contrast, the SDH WDR neurons have been consistently shown to be hyperexcitable (Donovan-Rodriguez et al., 2004, 2005, 2006; Urch et al., 2003, 2005).

This divergence in responsiveness of SDH and DDH neurons can be partly explained based on the anatomy of primary afferents. Lamina I neurons receive direct input from Aδ- and C-fibres as well as silent nociceptors (Prato et al., 2017). These afferents predominantly innervate tibiae. In contrast, DDH WDR neurons receive direct inputs from large Aβ- and small Aδ-myelinated fibres and indirect polysynaptic inputs from C-fibres from distal dendrites that extend into superficial laminae (Magerl et al., 2001). Here, responses of Aβ-fibres, which are seldom in bone, were unaffected (both after electrical and brush simulation).

4.5.2 Tonic descending noradrenergic controls are altered in CIBP

Since the superficial dorsal horn is also the origin of the spino-bulbo-spinal loop, an investigation of potential alterations in descending modulatory controls seemed vital. Rodriguez et al. showed that both SDH and DDH neurons are under ongoing facilitatory control, mediated by spinal 5-HT₃ receptors, suggestive of an enhanced descending serotoninergic drive (Donovan-Rodriguez et al., 2006). Unlike SDH WDR neurons that are hyperexcitable in the CIBP model, I showed that DDH WDR neurons were not, suggesting the presence of ongoing (tonic) inhibitory controls. Therefore, I focused here on the analysis of descending noradrenergic inhibitory controls mediated by spinal α₂-ARs. I used a selective (Table 1) α₂-AR antagonist, atipamezole, and expected to reveal a stronger inhibitory drive in the CIBP animals as compared with sham groups. In line with this hypothesis, antagonism of inhibitory α₂-ARs should classically result in facilitation, which should be more evident in CIBP conditions. Surprisingly, in the CIBP rats, spinal application of atipamezole significantly inhibited, rather than facilitated, neuronal responses to a range of innocuous and noxious mechanical, and electrical stimuli. The effect of atipamezole was dose-dependent and potentiated with disease progression. These rather unexpected results suggest significant alterations in descending controls plasticity in CIBP conditions. Additionally, in sham groups, spinally acting atipamezole did not evoke any changes (or sometimes demonstrated a slightly facilitatory tendency) in DDH WDR neuronal responses to almost all the analysed stimuli. This suggests the presence of another complementary inhibitory system in place of the α₂-AR-mediated inhibitions. Another interesting observation was that following electrical
stimulation of the receptive field, atipamezole application in late stage CIBP animals significantly decreased neuronal responses attributed to Aδ and C-fibre stimulation, confirming the anatomy of peripheral inputs, and highlighting the role of activated silent nociceptors in the CIBP. Observed changes could be explained based on the role of inhibitory interneurons in spinal neuronal network. Whether the atipamezole reached the DDH WDR neurons would need to be confirmed by for example comparison of the effects of the iontophoretic application directly to the recorded DDH neurons or binding of the radiolabelled atipamezole. One limitation of the first approach would be that the drug acts likely too locally, and considering significant level of arborisation of the spinal neurons the effect of locally released drug by the iontophoretic delivery may be not sufficient, therefore not comparable with in toto action of i.th. applied larger amounts of the agent. The latter, radiolabelling would be most informative, however it is challenging, and the resolution is often not satisfactory.

<table>
<thead>
<tr>
<th>NA</th>
<th>K_i [nM]</th>
<th>α_2a &gt; α_1a (6x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α_2a</td>
<td>56</td>
<td>O’Rourke et al., 1994</td>
</tr>
<tr>
<td>α_1a</td>
<td>330</td>
<td>Mohell et al., 1983</td>
</tr>
<tr>
<td>β_2</td>
<td>740</td>
<td>Pepperl and Regan, 1994</td>
</tr>
</tbody>
</table>

4.5.3 Inhibition of mechanosensation by spinal α_2-ARs block depends on a functioning opioid system in the late stage CIBP rats

Spinally released NA inhibits neurons via activation of α_2-ARs (Jones and Gebhart, 1986; Miller and Proudfit, 1990). The α_2a-ARs are mainly expressed on spinal nociceptive glutamatergic terminals (Stone et al., 1998), while α_2c-ARs are found on lamina II-III excitatory (Vglut2+) interneurons (89%), with the remaining 11% present on inhibitory (Gad+) interneurons (Huang et al., 2002; Olave and Maxwell, 2003a, 2004; Stone et al., 1998). Therefore, in the naive animals, spinal block of α_2-ARs should lead to increased facilitation of WDR neurons; however, for very selective α_2-ARs antagonists, this is rarely the case. Frequently, following α_2-AR antagonism in healthy animals, a small facilitation (restricted to non-noxious stimuli) or no effect (for noxious stimuli) was observed (Bannister et al., 2015; Rahman et al., 2008b, 2008a). NA is likely acting volumetrically via diffusion, rather than being restricted to synapses (Pertovaara, 2006; Rajaofetra et al., 1992; Zoli and Agnati, 1996). Therefore, by establishing a gradient, NA will also preferentially bind to receptors that have higher affinity for NA. α_2-AR affinity for NA is almost 6 times higher than that of α_1-ARs (Table 4.2). Thus, following spinal α_2-
AR antagonism, and upon simultaneous potent release of noradrenaline in the spinal cord, the inhibitory actions of NA may be instead mediated by inhibitory interneurons expressing $\alpha_1$-AR. Similarly, upon blockade of $\alpha_2$-ARs, the NA effects may be preferentially orchestrated via the $\alpha_1$-ARs. Due to the lack of selective antibodies the distribution of $\alpha_1$-ARs is less known than $\alpha_2$-ARs. Evidence from radioligand binding assays (Nalepa et al., 2005; Wada et al., 1997) and RNA in situ hybridisation suggests the presence of $\alpha_1$-ARs throughout the spinal cord (Atlas; Millan, 2002). Yet, physiological data suggests that $\alpha_1$-ARs are located preferentially on GABAergic interneurons, contributing to descending noradrenergic inhibitions and that can be potently activated by both NA and phenylephrine, an $\alpha_1$-AR agonist (Baba et al., 2000b, 2000a; Funai et al., 2014; Gassner et al., 2009).

![Figure 4.8. Schematic representation of the suggested mechanism.](image)

Increased peripheral nociceptive input facilitates superficial dorsal horn (SDH) projection neurons (PNs). Those in turn activate locus coeruleus (LC) to release noradrenaline from the spinal terminals (NA). Released NA acts via facilitatory ($\alpha_1$) and inhibitory ($\alpha_2$) adrenergic receptors, however its binding is preferential to $\alpha_2$ ($>6\times$ higher affinity). The $\alpha_2$ are located on primary afferents (PAFs), excitatory glutamatergic (Glu, red) and inhibitory GABA-ergic/enkephalinergic (GABA, ENK, blue) interneurons as well as on other neurons including LC terminals (autoreceptors). The $\alpha_1$ expression is less known, however, single cell RNAseq and physiological data suggests that $\alpha_1$ are mostly located on inhibitory interneurons. Upon strong stimulation to the LC, either via direct optoactivation to this structure or indirectly via lateral parabrachial (IPB) projecting SDH neurons activated by strong peripheral drive, spinal NA content is elevated but still buffers itself by activating autoreceptors. When in this system an antagonist of $\alpha_2$ (atipamezole) is introduced, it shifts the already elevated NA levels to extreme (by blocking autoreceptors). In the same time other spinal $\alpha_2$ receptors are also blocked, which allow for the competitive binding of the NA to $\alpha_1$ receptors. That in turn activates inhibitory interneurons expressing preferentially $\alpha_1$. In result, an inhibition of deep dorsal horn (DDH) wide-dynamic range (WDR) neurons, which are likely to have rich synaptic contacts with interneurons, is observed. References in the main text.
A large proportion of spinal inhibitory interneurons contain opioids (i.e. enkephalin, dynorphin etc.). To reveal if such inhibitory interneurons are involved in the descending noradrenergic signalling, I first wanted to confirm whether or not the spinal opioid system was functional in the late stage CIBP rats. A global blockade of spinal opioid receptors with intrathecally administrated naloxone resulted in a dose-dependent facilitation of DDH WDR neuronal responses in both SL and CL groups, confirming a proper functioning of the spinal opioid system. These results corroborate the previously reported effectiveness of morphine (Urch et al., 2005), and predominant role of MOR-mediated tapentadol analgesia (Falk et al., 2015a) in CIBP. Based on the hypothesis that NA-mediated activation of spinal $\alpha_1$-ARs expressed on inhibitory opioidergic interneurons could lead to the observed inhibitions of DDH WDR neurons upon block of $\alpha_2$-ARs, I next showed that atipamezole-evoked inhibition of DDH WDR neurons firing could be fully reversed by the co-administration of naloxone. These results highlight that the mechanism is likely opioidergic, however, whether Enk or Dyn or other opioids mediate this action needs to be further investigated.

Future studies utilising a co-administration of atipamezole and prazosin ($\alpha_1$-ARs antagonist) will reveal whether the postulated increase in the spinal NA could act via spinal $\alpha_1$-ARs activating inhibitory interneurons. Another possible mechanism via which atipamezole could increase the spinal NA content is via blocking NA autoreceptors ($\alpha_2$-ARs on the LC spinal terminals). Since the inhibitory actions of atipamezole are only recorded in advanced CIBP and upon potent LC optoactivation, an extra stimulation to the LC and a simultaneous block of the $\alpha_2$-ARs on other spinal neurons may be needed to shift the action of atipamezole to inhibitory (Fig. 4.8).

4.5.4 Pharmacological blockade of the locus coeruleus in the late stage CIBP rats abolished spinal atipamezole-mediated inhibition

A recent finding suggesting similar alterations in the atipamezole’s spinal pharmacology was reported by Hickey et al. The authors showed that upon selective optoactivation of LC neurons intrathecal administration of atipamezole had an inhibitory effect on the heat-evoked muscle reflexes (Hickey et al., 2014). These results suggested that the increased activity within the ventral LC could affect spinal pharmacology of adrenoceptors. To verify if activation of the LC can be responsible for my observed alterations in atipamezole pharmacology in late CIBP animals, I injected the ipsilateral LC with a general sodium channels blocker, lidocaine, while recording from DDH WDR neurons. The inhibitory effect of simultaneously applied atipamezole seemed to be abolished by the LC block, suggesting its involvement in the observed analgesic effect.
of spinal atipamezole. I would like to highlight that these results need to be considered with care as a higher number of cells needs to be analysed to confirm obtained data.

4.5.5 Optoactivation of the locus coeruleus in naïve animals mimics the altered spinal noradrenergic-mediated actions observed in late stage CIBP rats

In contrary to Hickey et al., who focused on reflex monitoring using electromyography, I have analysed the evoked activity of DDH WDR cells in response to mechanical forces following optoactivation of the LC. As expected the LC activation inhibited DDH WDR neuronal activity confirming previous findings (Hickey et al., 2014; West et al., 1993). Simultaneous spinal application of atipamezole not only maintained the LC-mediated inhibitory effects on the DDH WDR mechanically-evoked neuronal activity, but also apparently potentiated the observed inhibition. Therefore, it can be concluded that optoactivation of the LC in naïve animals mimics the altered spinal noradrenaline-mediated actions observed in late stage CIBP rats. This also suggests an ongoing hyperexcitability of the LC in the advanced bone cancer conditions.

A plethora studies indicate that the analgesic effect(s) of LC activation are mediated by spinal \( \alpha_2 \)-ARs (Jones and Gebhart, 1986; Jones SL, 1986; Miller and Proudfit, 1990; Pertovaara, 2006). A majority of these reports utilised less selective \( \alpha_2 \)-ARs antagonists including yohimbine and idazoxan (Table 4.1), and one study showed that the analgesic action of LC stimulation could not be abolished by spinally applied idazoxan (West et al., 1993). Since atipamezole has improved selectivity for \( \alpha_2 \)-ARs it suggests that the spinal actions of descending NA are likely to be mediated by \( \alpha_1 \)-ARs (Fig. 4.6F and (Hickey et al., 2014)).

Interestingly, one study reported that, following selective chemogenetic activation of retrogradely labelled spinally projecting NA-neurons, intrathecal atipamezole fully reversed the analgesic actions of the LC activation (Hirschberg et al., 2017). One possible explanation of these results is that other spinally projecting NA nuclei could have also been labelled; since a chemogenetic approach has lower spatial control than an optogenetic one, a possibility exists that other NAergic pathways were activated for which atipamezole would be effective. That would also impose the activation of more potent \( \alpha_2 \)-ARs-mediated NA descending controls impacting DDH WDR neuronal activity (see Chapter V for more).

4.5.6 Optoactivation of lamina I projection neurons inhibits deep dorsal horn wide dynamic range neurons

Next, I decided to check what drives the postulated increased activity of the LC in animals with advanced bone cancer. One investigative possibility arose from the fact that the SDH neurons are hyperexcitable in the CIBP model, which was previously
evident both in electrophysiological studies (Urch et al., 2003) and in c-Fos immunopositivity of these cells following gentle palpitation of the cancer-bearing leg (Doyle and Hunt, 1999; Schwei et al., 1999). Around 80% of SDH neurons are NK1 (a receptor for substance P) positive and around 90% of them decussate and project through the dorsolateral funiculus (DLF) to the lateral parabrachial nuclei (Hylden et al., 1989; Kitamura et al., 1993). There are other known bilateral projections of these cells to the brainstem centres, including the LC (Li et al., 1998; McMahon and Wall, 1988; Swett et al., 1985) and the anterior pretectal nucleus (Rees and Roberts, 1993). Majority of LI PNs have direct synaptic contacts with nociceptors and mediate painful signals to the supraspinal nuclei for further processing. Therefore, it is worth to ask whether the activity of these cells in CIBP conditions can influence the supraspinal activity, in particular: are they able to activate the LC? Apart from ascending fibres, the DLF also comprises descending inhibitory projections, originating mainly from the rostral ventromedial medulla (including primarily serotonergic nucleus raphe magnus and the nucleus paragigantocellularis) and the dorsolateral pontine tegmentum (including catecholaminergic LC) (Basbaum and Fields, 1984; Fields et al., 1991).

I showed that the selective optoactivation of LI cells potently inhibits mechanically-evoked DDH WDR neuronal activity in otherwise naïve animals. This suggests the activation of some kind of inhibitory mechanism. One possibility is that activated SDH neurons inhibit DDH neurons via a local spinal circuitry, for example engaging inhibitory interneurons. However, limited data supports this mechanism. Another possibility is that SDH projection neurons activate supraspinal nuclei by means of spino-bulbo-spinal loop, which in turn inhibit spinal neuronal activity. A confirmation for the latter was presented by McMahon and Wall (McMahon and Wall, 1988). The authors stimulated the DLF at the cervical level and recorded single units from both superficial and deep dorsal horn neurons in the lumbar enlargement. They showed that electrical activation of the DLF resulted in the expected antidromical facilitation of the SDH cells, but DDH neurons were unexpectedly inhibited (McMahon and Wall, 1988). This confirmed that DDH WDR neurons constitute a different pathway (STT), and also that SDH cells can modulate DDH responses via a relay in the brainstem centres. Another supporting piece of evidence came from data that showed that direct stimulation of the lateral parabrachial (IPB) area potently inhibited dorsal horn neuronal activity (Beitz et al., 1987; Chiang et al., 1994; Yoshida et al., 1997). Since the LPB area is in proximity to the LC, utilising electrical stimulation may have led to effects mediated by activity in both.

Interestingly, ablation of NK1+ neurons in the spinal cord results in potent reduction of DDH WDR neuronal firing. These initially counterintuitive results are
explained by the fact that LI PNs also drive descending facilitation, mediated by spinal 5-HT3 receptors (Suzuki et al., 2002). Therefore, a possible interplay exists between NAergic inhibitory and serotoninergic facilitatory mechanisms that, at least partly, share ascending circuity. Reduction of DDH WDR neuronal responsiveness as a result of NK1+ neurons ablation is not mediated by spinal \( \alpha_2 \)-ARs (Rahman et al., 2008a) but is to some extent by spinal GABA\( \Lambda \) (Rahman et al., 2007). The pilot pharmacological data that I present here suggest that LI-evoked inhibitions of DDH WDR neurons are mediated by spinal opioid receptors, but not by \( \alpha_2 \)-ARs. This further suggests activation of GABAergic/opioidergic inhibitory interneurons via \( \alpha_1 \)-ARs by descending noradrenaline.

4.5.7 Conclusions

I have presented data to show that CIBP animals exhibit mechanical hypersensitivity in behavioural testing, thus confirming a good development of rat CIBP model. Furthermore, I observed no changes in the basal firing rate of DDH WDR neurons from sham-operated or early or late stage CIBP groups. Spinal application of the selective \( \alpha_2 \)-AR antagonist atipamezole revealed an unexpected inhibitory effect on the evoked activity of DDH WDR neurons ipsilateral to the bone cancer. Equally, no changes were observed in DDH WDR neuronal activity after spinal application of atipamezole in the corresponding sham groups. Finally, the analgesic role for \( \alpha_2 \)-AR antagonists observed in a rodent model of CIBP may be explained on the basis of spinal inhibitory interneuron activity and potent overall-inhibitory supraspinal modulations.

I argue that the employment of silent nociceptors with bone cancer explicitly increases the nociceptive input to lamina I projection neurons, resulting in the hyperactivity of the SDH. Further, SDH projection neurons chiefly activate the pontine LC to release NA in the spinal dorsal horn (Fig. 4.8 and 4.9). This control acts tonically via \( \alpha_2 \)-ARs to inhibit DDH WDR neurons. Upon noxious stimulation, this priming in the CIBP rodent model further increases spinal NA levels to activate \( \alpha_1 \)-ARs located on inhibitory interneurons, thus reducing DDH WDR neuronal activity. This effect is more evident upon \( \alpha_2 \)-AR blockade. This can be explained based on the report from Eason and colleagues (Eason et al., 1992), which showed that with high agonist concentrations and high receptor expression, \( \alpha_2 \)-ARs couple to facilitatory G proteins. Alterations in protein levels are, however, unlikely as upon LC and/or LI optoactivation atipamezole pharmacology was changed instantaneously. It will be interesting to know whether an increase in NA levels is sufficient to switch \( \alpha_2 \)-ARs function from inhibitory to facilitatory. Similar alterations are expected in other pain models that show NGF-dependent recruitment of silent nociceptors.
Figure 4.9. Proposed mechanism of spino-pontine-spinal loop. Explanation in text.

4.6 Author Contributions

Dr Kirsty Bannister recorded from 5 late CIBP, 2 early CIBP (both for atipamezole) and 2 late sham (for atipamezole with naloxone) animals in order to verify the unexpected findings of the spinal atipamezole action. She was blinded for the experimental outcome. Dr Bannisters data is pooled with my data. I performed all surgical interventions (i.e. sham and cancer surgeries).
Chapter V
Chapter V. DNIC expression is dynamic in the progression of CIBP

5.1 Introduction

More than one form of inhibitory control that utilises descending noradrenergic circuitry exists. Diffuse Noxious Inhibitory Controls (DNIC) describes the phenomenon whereby application of noxious stimuli to one part of the body inhibits pain perception from another remote body region through descending systems, and these inhibitory controls are largely driven by $\alpha_2$-adrenergic receptor ($\alpha_2$-AR)-mediated responses (Bannister et al., 2015). Specifically, DNIC inhibit the activity of deep dorsal horn (DDH) wide-dynamic range (WDR) neurons. The pan-modality inhibitory function of DNIC on DDH WDR neurons suggests a postsynaptic mechanism, likely by direct inhibition of the neuronal cell body (Villanueva et al., 1984), and also suggests the expression of $\alpha_2$-AR on DDH WDR neurons. Condition pain modulation (CPM) is the presumed analogous process in humans (Yarnitsky, 2010; Yarnitsky et al., 2010).

Unsurprisingly, other pharmacological systems contribute to DNIC expression. In the spinal nerve ligation (SNL) model of neuropathy, an imbalance between NA-mediated inhibitions and 5-HT-mediated facilitations, in favour of facilitation via 5-HT$_3$ receptor-mediated activity, leads to a loss of DNIC expression (Bannister et al., 2015). A plethora of 5-HT receptors subtypes complicates the interpretation of serotonin actions, however 5-HT$_3$ ionotropic receptor-mediated activity predominates to mediate spinal facilitation (Bannister et al., 2017; Kayser et al., 2011).

Opioids have direct supraspinal interactions with descending modulatory systems (Helmstetter et al., 1993; Pavlovic et al., 1996), and as I showed in the previous chapter they contribute to spinal descending NA-mediated analgesia. Systemic administration of naloxone abolishes DNIC expression in naïve rats suggestive of the involvement of opioidergic mechanisms in DNIC expression (Le Bars et al., 1981). It is not known, however, if this effect is mediated by cerebral, spinal or maybe joint mechanisms, as systemic naloxone is easily distributed throughout the body within minutes.

Spinal transection abolishes DNIC expression, confirming its top-down action (Dickenson and Le Bars, 1983). The loss of CPM in patients with lateral medullary syndrome (Wallenberg syndrome) further highlights that brainstem-mediated mechanisms control this form of descending inhibitory modulation (De Broucker et al., 1990; Roby-Brami et al., 1987). Tracts within the dorsolateral funiculus (DLF) are crucial for DNIC expression (Okada-Ogawa et al., 2009), especially for ascending noxious
transmission (Lapirot et al., 2009). There are studies that have reported that, to evoke DNIC, NK1+ SDH projecting neurons are necessary (Bester et al., 2000; Lapirot et al., 2009; Suzuki et al., 2002). DNIC are anticipated to originate from the medullary subnucleus reticularis dorsalis (SRD, also abbreviated as medullary nucleus dorsalis – MdD) (Bouhassira et al., 1992a). Tracing studies from the SRD showed that direct projections ensue via the DLF to the DDH, entirely omitting the SDH (Villanueva et al., 1995). Interestingly, the SRD is modulated by higher brain centres including the neocortex (Youssef et al., 2016), linking the analgesic actions of distraction and pointing at the relationship with cognitive processes. The SRD has not yet been analysed for the presence of noradrenergic cells.

Until now there is no preclinical or clinical data available describing DNIC/CPM during the progression of metastatic disease. Therefore, this research is designed to answer if and how DNIC expression is altered in the progression of CIBP in well-validated rat model of the disease.
5.2 Rationale, hypothesis and aims

The functionality of DNIC in a rat model of CIBP has not yet been verified. Thus, my main goal was to investigate the presence or absence of DNIC, and to verify the spinal pharmacology of pathways that sub-serve it, in the CIBP model. In order to do this, in vivo spinal electrophysiological recordings of deep dorsal horn (DDH) wide dynamic range (WDR) neurons, crossed with spinal and brain pharmacology, was performed. In addition, I analysed the impact of CIBP on rodent sociability.

5.3 Materials and Methods

A detailed description of employed in this chapter procedures can be found in Chapter II. See pages referral below.

- Cell cultures and animals (detailed description, page 35)
- CIBP rat model (detailed description, page 35)
- In vivo spinal electrophysiology (detailed description, page 43-45)
  - Baseline responses (detailed description, page 43)
  - DNIC paradigm (detailed description, page 45)
  - Spinal pharmacology (detailed description, page 45)
  - Brain pharmacology (detailed description, page 46)
- Optogenetic experiments:
  - Viral delivery of opsins (detailed description, page 46)
  - Optoactivation with in vivo spinal electrophysiological recordings (detailed description, page 47)
- Quantification and statistical analysis (detailed description, page 48)
5.4 Results

Figure 5.1. DNIC expression in early and late stage of CIBP. Schematic representation of the experimental paradigm. In vivo single unit recordings of deep dorsal horn wide dynamic range (DDH WDR) neurons were performed under light isoflurane anaesthesia. To evoke DNIC, conditioning stimulus (noxious ear pinch) was applied ipsilaterally to the testing stimuli. (A). Magnitude of inhibition of mechanically-evoked DDH WDR neuronal responses following simultaneous noxious ear pinch in early (day 7-8) and late (day 14-16) stage CIBP and corresponding sham-operated rats (B). Deep dorsal horn lamina V/VI WDR neuronal responses to punctate mechanical stimulation of the receptive field in early (day 7-8) and late (day 14-16) stage CIBP and corresponding sham-operated rats before and after ear pinch (C, D, E, F). All the data represent the mean ± SEM from sham early (n = 6), CIBP early (n = 11), sham late (n = 31) and CIBP late (n = 31). Each dot represents one animal. 2-way RM-ANOVA with Bonferroni post-hoc: *P < 0.05, ****P < 0.0001 vs. corresponding baseline. Percentages reflect the effect magnitude as compared to the corresponding baseline.

5.4.1 DNIC expression in early and late stage of CIBP

DNIC expression was studied in sham early (SE, n = 6), CIBP early (CE, n = 11), sham late (SL, n = 31) and CIBP late (CL, n = 31) rats under light isoflurane/N2O/O2 anaesthesia (slight toe pinch reflex maintained). Terminal electrophysiological recordings of DDH WDR neurons were used to study the von Frey-evoked firing rate
changes upon simultaneous ipsilateral application of noxious conditioning stimulus (ear pinch) to evoke DNIC (Fig. 5.1A). One neuron was studied per animal. DNIC were expressed in SE, SL and CL animals, resulting in around 50%, 40% and 30% inhibition of the evoked action potentials to 8 g, 26 g and 60 g von Frey application, respectively (Fig. 5.1B). Interestingly, DNIC expression was impaired in CE rats (Fig. 5.1B).

In SE animals, DDH WDR neuronal activity was significantly inhibited when compared to baseline for all von Frey filaments (2-way RM-ANOVA: $F_{1,5} = 15.149, P < 0.05$; Bonferroni post hoc [8, 26, 60 g]: $P < 0.05$) (Fig. 5.1C). In contrary, DDH WDR neuronal responses in CE rats were not inhibited (2-way RM-ANOVA: $F_{1,10} = 8.461, P < 0.05$; Bonferroni post hoc: $P > 0.05$ for all tests) (Fig. 5.1D). In SL rats DNIC was expressed (2-way RM-ANOVA: $F_{1,30} = 113.523, P < 0.0001$; Bonferroni post hoc: $P < 0.0001$ for all tests) (Fig. 5.1E), as well as in CL animals (2-way RM-ANOVA: $F_{2,60} = 98.005, P < 0.0001$, Bonferroni post hoc: $P < 0.0001$ for all tests) (Fig. 5.1F).

Figure 5.2. Spinal $\alpha_2$-AR controls DNIC expression in early CIBP. Schematic representation of the experimental paradigm. In vivo single unit recordings of deep dorsal horn wide dynamic range (DDH WDR) neurons were performed under light isoflurane anaesthesia. To evoke DNIC, conditioning stimulus (noxious ear pinch) was applied ipsilaterally to the testing stimuli (A). Effects of 10 µg (B, C) and 100 µg (D, E) of atipamezole applied spinaly on the DNIC expression in early stage (day 7-8) CIBP and
corresponding sham-operated rats. All the data represent the mean ± SEM from sham early (n = 4), CIBP early (n = 6-7). Each dot represents one animal. 2-way RM-ANOVA. Percentages reflect the effect magnitude as compared to the corresponding baseline.

5.4.2 Spinal \(\alpha_2\)-AR controls DNIC expression in early CIBP

Having shown that DNIC are expressed in SE rats, a selective \(\alpha_2\)-AR blocker, atipamezole, was applied spinally in two doses: 10 and 100 \(\mu\)g. In the SE rats, atipamezole dose-dependently abolished DNIC expression (10 \(\mu\)g atipamezole: 2-way RM-ANOVA: \(F_{1,3} = 3.169, P = 0.173\), No post hoc; 100 \(\mu\)g atipamezole: 2-way RM-ANOVA: \(F_{1,3} = 0.375, P = 0.583\), No post hoc) (Fig. 5.2B, D). However, in the CE rats atipamezole had no effect at either dose (10 \(\mu\)g atipamezole: 2-way RM-ANOVA: \(F_{2,12} = 16.606, P < 0.0001\), Bonferroni post hoc: \(P > 0.05\) for all tests; 100 \(\mu\)g atipamezole: 2-way RM-ANOVA: \(F_{1,5} = 0.028, P = 0.875\), No post hoc) (Fig. 5.2C, E).

Figure 5.3. Spinal \(\alpha_2\)-AR controls DNIC expression in late CIBP. Schematic representation of the experimental paradigm. In vivo single unit recordings of deep dorsal horn wide dynamic range (DDH WDR) neurons were performed under light isoflurane anaesthesia. To evoke DNIC, conditioning stimulus (noxious ear pinch) was applied ipsilaterally to the testing stimuli (A). Effects of 10 \(\mu\)g (B, C) and 100 \(\mu\)g (D, E) of atipamezole applied spinally on the DNIC expression in late stage (day 14-16) CIBP and corresponding sham-operated rats. All the data represent the mean ± SEM from sham late (n = 4-11), CIBP late (n = 7-9). Each dot represents one animal. 2-way RM-ANOVA. Percentages reflect the effect magnitude as compared to the corresponding baseline.
5.4.3 Spinal α2-AR controls DNIC expression in late CIBP

In the presence of atipamezole, DNIC expression was completely abolished, confirming the existence of noradrenergic controls in both SL and CE/CL groups (Sham late: 10 µg atipamezole: 2-way RM-ANOVA: F_{1, 3} = 1.139, P = 0.364, No post hoc; 100 µg atipamezole: 2-way RM-ANOVA: F_{1, 10} = 0.062, P = 0.808, No post hoc, and CIBP late: 10 µg atipamezole: 2-way RM-ANOVA: F_{1, 6} = 1.214, P = 0.313, No post hoc; 100 µg atipamezole: 2-way RM-ANOVA: F_{1, 8} = 1.332, P = 0.282, No post hoc) (Late Sham: Fig. 5.3B, D and CIBP Fig. 5.3C, E).

Figure 5.4. Spinal opioid receptors do not control DNIC expression. Schematic representation of the experimental paradigm. In vivo single unit recordings of deep dorsal horn wide dynamic range (DDH WDR) neurons were performed under light isoflurane anaesthesia. To evoke DNIC, conditioning stimulus (noxious ear pinch) was applied ipsilaterally to the testing stimuli (A). Effects of 5 µg (B, C) and 20 µg (D, E) of atipamezole applied spinally on the DNIC expression in late stage (day 14-16) CIBP and corresponding sham-operated rats. All the data represent the mean ± SEM from sham late (n = 5-6), CIBP late (n = 5-7). Each dot represents one animal. 2-way RM-ANOVA with Bonferroni post-hoc: *P < 0.05, **P < 0.01 vs. corresponding baseline. Percentages reflect the effect magnitude as compared to the corresponding baseline.
5.4.4 Spinal opioid receptors do not control DNIC expression

Focusing on the late stage of CIBP I hypothesised whether spinal opioidergic mechanisms play a role in DNIC expression. Naloxone was applied topically on the spinal cord in order to block all the opioid receptors therein. Interestingly, naloxone did not abolished DNIC expression in both analysed groups, suggesting lack of spinal opioidergic control of DNIC (Sham late: 5 µg naloxone: 2-way RM-ANOVA: F\(_{1, 5}\) = 20.565, \(P < 0.01\), Bonferroni post hoc: [8, 26 g]: \(P < 0.01\) and [60 g]: \(P > 0.05\); 20 µg naloxone: 2-way RM-ANOVA: F\(_{1, 4}\) = 12.757, \(P < 0.05\), Bonferroni post hoc: [26 g]: \(P < 0.05\), and [8, 60 g]: \(P > 0.05\) for, and CIBP late: 5 µg naloxone: 2-way RM-ANOVA: F\(_{1, 4}\) = 6.138, \(P = 0.068\), No post hoc; 20 µg naloxone: 2-way RM-ANOVA: F\(_{1, 6}\) = 11.802, \(P < 0.05\), Bonferroni post hoc, [8 g]: \(P > 0.05\), [26 g]: \(P < 0.05\), [60 g]: \(P < 0.01\)) (Late Sham: Fig. 5.4B, D and CIBP Fig. 5.4C, E).

Figure 5.5. Spinal \(\alpha_2\)-AR control of DNIC do not depend on spinal opioids.
Schematic representation of the experimental paradigm. In vivo single unit recordings of deep dorsal horn wide dynamic range (DDH WDR) neurons were performed under light isoflurane anaesthesia. To evoke DNIC, conditioning stimulus (noxious ear pinch) was applied ipsilaterally to the testing stimuli (A). Effects of mix of 20 µg naloxone and 100 µg atipamezole applied spinally on the DNIC expression in late stage (day 14-16) CIBP (B) and corresponding sham-operated (C) rats. All the data represent the mean ± SEM from sham late (n = 6), CIBP late (n = 6). Each dot represents one animal. 2-way RM-ANOVA. Percentages reflect the effect magnitude as compared to the corresponding baseline.

5.4.5 Spinal \(\alpha_2\)-AR control of DNIC do not depend on spinal opioids

To assess whether or not \(\alpha_2\)-AR-mediated control of DNIC depends on spinal opioidergic neurons, atipamezole (100 µg) and naloxone (20 µg) were intrathecally co-administered in both late stage groups (SL and CL). Naloxone did not affect \(\alpha_2\)-AR control of DNIC in both late sham and CIBP animals, suggesting either direct inhibitory
action of DNIC-evoked noradrenaline on DDH WDR neurons or a different inhibitory system to opioidergic triggered to mediate DNIC (Sham late: 2-way RM-ANOVA: $F_{1, 5} = 0.241, p = 0.645$, No post hoc, and CIBP late: 2-way RM-ANOVA: $F_{1, 5} = 0.005, p = 0.946$, No post hoc) (Late Sham: Fig. 5.5B and CIBP Fig. 5.5C).

Figure 5.6. Locus coeruleus activity does not influence DNIC expression. Schematic representation of experimental approach (A). Following 3 stable baseline extracellular single-unit recordings (averaged) of deep dorsal horn lamina V/VI WDR neurons in late (day 14-16) CIBP stage rats, lidocaine (400 nl, 2%) was injected into ipsilateral locus coeruleus (LC) and punctate mechanical-evoked action potentials were
measured with 10-minute intervals. All the data represent the mean ± SEM and each dot represent one animal (n = 3). Statistical analysis not performed due to the small sample size (B, C, D). Schematic representation of the optogenetic experimental setup. Two weeks before the experiment channelrhodopsin-2 was expressed in the locus coeruleus utilising canine adenovirus (CAV-sPRS-hChR2(H134R)-mCherry). Simultaneous recordings of LC neurons and ipsilateral lamina V/VI WDR neurons were made and followed by optoactivation of the LC (E). Spike2 traces represent: top row – 472 nm diode on/off indication, second row – extracellular activity in the LC, third row – single-unit activity of WDR spinal neuron, bottom row – columns representing number of spikes per second (F). Punctate mechanically-evoked action potentials of deep dorsal horn WDR neurons in LC-ChR2 expressing rats (averaged 3 stable baselines) before and after ear pinch (DNIC) in the absence of light (G). Same conditions as in (G), but LC was optoactivated (3 averaged, with minimally 5 minutes breaks between tests) (H). All the data represent the mean ± SEM and each dot represent one animal (n = 5). One-way RM-ANOVA with Greenhouse-Geisser correction and Bonferroni post hoc: *P < 0.05, **P < 0.01 vs. baseline. Effects of spinally administered atipamezole (100 µg) on the LC-mediated inhibition of DNIC expression. Activity was measured with 10-minute intervals. All the data represent the mean ± SEM (n = 5 animals). One-way RM-ANOVA with Greenhouse-Geisser correction. (I).

5.4.6 Locus coeruleus does not mediate DNIC expression

To verify the potential contribution of LC activity in DNIC modulation, I injected a general sodium channel blocker, lidocaine (400 nl of 2% solution), into the ipsilateral LC, while simultaneously recording from DDH WDR neurons (Fig. 5.6A). These pilot experiments (n = 3 animals) suggested that LC block could abolish DNIC expression for non-noxious, but not for noxious, stimuli (Fig. 5.6B-D).

Optoactivation of noradrenergic cells expressing channelrhodopsin-2 (delivered in CAV-sPRS-hChR2(H134R)-mCherry) in the ipsilateral ventral LC (optrode with 10° angulation from lambda: RC: -2.1 mm, ML: 1.3 mm, and -5.8-6.2 mm deep from the cerebellar surface) (Fig. 5.6E) diminished DNIC expression to the range of innocuous and to less extend to not noxious mechanical testing stimuli ([Light OFF]: 2-way RM-ANOVA: F₁,₃ = 44.632, P = 0.007, Bonferroni post hoc: [8, 15, 60 g]: P < 0.05 and [26 g]: P < 0.01; [Light ON]: 2-way RM-ANOVA: F₁,₃ = 4.219, P = 0.132) (Fig. 5.6F, G, H, see also Fig. 4.6 for the histological and electrophysiological confirmation of the expressed and active opsin).

Finally, spinal application of 100 µg atipamezole reversed the inhibitory action of DNIC, and LC optoactivation seemed to potentiate this reversal (RM-ANOVA one-way ANOVA for matching samples: [8 g]: F₁.07, 4.28 = 1.636, P = 0.270; [15 g]: F₁.57, 4.71 = 3.467, P = 0.122; [26 g]: F₂.01, 8.02 = 2.655, P = 0.130; [60 g]: F₂.62, 10.49 = 6.422, P = 0.0133, Bonferroni post hoc: all P > 0.05) (Fig. 5.6I).
5.5 Discussion

Descending controls, both facilitatory (Donovan-Rodriguez et al., 2006) and inhibitory (previous chapter) are altered in CIBP. The presence or absence of DNIC in an animal model of CIBP has not previously been verified. Here, I investigated the spinal pharmacology of pathways that sub-serve DNIC in the CIBP model.

5.5.1 DNIC are dynamic throughout the course of the metastatic disease progression

DNIC expression was shown to be dynamic in the CIBP rat model. DNIC was abolished in the early cancer stage, and fully restored by the late stage of the metastatic disease. Interestingly, in the late cancer stage, the spinal α2-AR-mediated control of DNIC was maintained, similarly as in healthy rats. This implies that the expression of α2-ARs is unaltered on WDR neurons, as pharmacological blockade of this receptor was still able to abolish the manifestation of DNIC.

I hypothesise that in the early stage of the cancer development, tonic noradrenergic controls are reduced (see chapter IV) as the early increase in facilitatory serotoninergic drive occurs (Donovan-Rodriguez et al., 2006). The situation changes by the late cancer stage, as tonic noradrenergic controls become hyperactive (see chapter IV), restoring the balance of inhibitory (NA) and facilitatory (5-HT) controls and at the same time allowing DNIC to be fully expressed again. Therefore, I suggest that a lack of DNIC expression can serve as an early indicator of the bone cancer pain phenotype development.

The presence or absence of CPM is proposed to be a reliable, simple diagnostic measure in terms of personalised pain pharmacotherapies in particular pain types (Bannister and Dickenson, 2017). Recently, several clinical paradigms have been developed for a quantification of the inhibitory impact of CPM (Yarnitsky et al., 2010). Analogically to DNIC, CPM is pan-modal in the sense of requirement for a test and conditioning stimulus (Kosek and Ordeberg, 2000). The conditioning stimulus must be noxious, since ‘one pain inhibits another’ and typically it has been achieved by application of a painful cold stimulus to the foot, while testing with heat or mechanical stimuli on the arm (Lewis et al., 2012). Recently, a novel approach utilises two pressure cuffs controlled by a fully automated algometer (Skovbjerg et al., 2017).

What is more, pain therapy utilising noradrenaline-reuptake inhibitors in the early cancer phase, which has diminished DNIC expression, could be therapeutically advantageous. Interestingly, CIBP is not the only pain state where dynamics in descending controls sub-serving DNIC/CPM have been recorded. CPM is not expressed...
in patients with cluster headache in the active phase, and yet is restored in remission (Perrotta et al., 2013).

5.5.2 Spinal $\alpha_2$-ARs modulate DNIC expression

It is established that DNIC orchestrate their function via spinal $\alpha_2$-ARs. To check if such controls are maintained in the rat model of CIBP, I applied a selective $\alpha_2$-AR antagonist, atipamezole. Atipamezole, dose dependently abolished DNIC expression in all sham and late CIBP groups, highlighting a fully functional noradrenergic system sub-serving DNIC. This contrasts with the tonic NAergic descending controls, which were significantly altered by CIBP (Chapter IV) and as such further suggests separate descending pathways for DNIC and tonic NA-controls. As mentioned in the introduction to this chapter, DNIC were proposed to originate from the medullary SRD (Bouhassira et al., 1992a), whereas tonic NA controls have their origin within the pontine LC (see chapter IV and (Aston-Jones et al., 1986; Hickey et al., 2014; Jones and Gebhart, 1986)). A definite origin of the descending NA pathway that modulates DNIC expression remains to be established. This data also provides an indirect confirmation that atipamezole reached the DDH neurons since the postulated separate NA-controls underlaying DNIC could be successfully abolished by i.th. atipamezole application.

5.5.3 Spinal opioid receptors do not modulate DNIC expression

A study utilising systemically administered naloxone revealed that DNIC expression was abolished (Le Bars et al., 1981). I hypothesised that the spinal opioid system was involved in DNIC expression. Here I show that intrathecal naloxone is ineffective at blocking DNIC expression, thus revealing a lack of spinal opioidergic mechanisms that sub-serve this form of descending noradrenergic control. Furthermore, $\alpha_2$-AR-mediated control of DNIC was insusceptible to the co-administered naloxone, further suggesting separate noradrenergic pathways for DNIC and for tonic inhibitions of DDH WDR neurons.

5.5.4 Locus coeruleus output does not modulate DNIC expression

Since the main source of spinal NA is the LC (Hickey et al., 2014; Jones and Gebhart, 1986), and since LC output appears to be altered in CIBP (chapter IV), I tested its role in the modulation of DNIC. By pharmacologically blocking LC activity using micro-injection of lidocaine, I suspected that DNIC expression was intact. Interestingly the inhibitory actions of DNIC, when conditioning stimulus was applied in the presence of a non-noxious test stimulus, seemed to be abolished by the LC lidocaine. These results need to be taken with care, as low number of WDR neurons has been recorded.

The lack of involvement of the LC in the modulation of DNIC is not surprising. It was previously shown that ipsilateral, contralateral or bilateral chemical lesions of the LC
had no significant effect on DNIC expression (Bouhassira et al., 1992b). Interestingly however in CIBP rats DNIC expression appeared to be abolished for non-noxious stimuli following lidocaine block of the LC. This needs to be taken with care as low numbers of units were recorded and subsequently further studied in order to find a possible explanation. Previous research hinted to a similar action of spinal $\alpha_2$-ARs-antagonism resulting in facilitations of DDH WDR neurons restricted to non-noxious mechanical stimuli (Rahman et al., 2008b, 2008c).

Interestingly, upon LC optoactivation DNIC was abolished, particularly strongly for non-noxious stimuli. Since in this study no intersectional approach was employed, one possibility is off-target optoactivation of neurons close to or projecting to the LC, which could lead to the activation of other, remote nuclei directly involved in DNIC expression. Previous studies indirectly hint on that possibility. In one, intrathecal atipamezole fully reversed the analgesic actions of chemogenetically activated retrogradely labelled spinally projecting NA-neurons (Hirschberg et al., 2017). In another, however, authors by utilising selective optoactivation of NA-neurons in the LC revealed that spinally applied atipamezole does not reverse, but rather potentiates the inhibitory effect of LC optoactivation ((Hickey et al., 2014), confirmed in chapter IV). Based on these studies it can be assumed that Hirschberg and colleagues activated not only LC, but also another, more potent $\alpha_2$-ARs-mediated NA descending controls impacting DDH WDR neuronal activity.

Projections to the LC are reach (Kwiat et al., 1992), therefore it is possible that the activation of these neuronal terminals, rather than inhibition of the LC neurons itself (like in the case of lidocaine or LC lesions) can have a dominant impact on the DNIC expression. In that, lidocaine block of action potential propagation is fast and therefore local, in contrast LC optoactivation (after LC virus delivery, and without the intersectional approach e.g. involving two viruses and cre-lox recombination) could lead to the generation of action potentials in other, remote cells by activating their axons within the LC and propagation of impulses towards the cell body in the remote nuclei directly involved in DNIC. Since I utilised PRS promoter here, it is likely that these cells are also noradrenergic.

Next, when I simultaneously applied spinal atipamezole and activated the LC with blue light, I revealed that DNIC expression was further modulated by spinal $\alpha_2$-ARs, independent from LC activity. At the ‘peak’ of atipamezole action, DNIC expression in the presence of conditioning stimulus was not only completely abolished but actually produced a facilitatory effect to non-noxious stimuli. Therefore, the dialogue of the two parallel descending noradrenergic pathways, tonic NA originating from the LC and DNIC (presumably) originating from the SRD, needs to be further investigated.
5.5.5 Conclusions

I showed that DNIC are dynamic during the course of CIBP progression since DNIC are lost in early but restored in late cancer stages. When expressed, DNIC are predominantly orchestrated via spinal $\alpha_2$-AR-mediated actions. Spinal opioid mechanisms are not involved in DNIC expression, neither do they control DNIC-evoked inhibitions via spinal $\alpha_2$-ARs. A functional DNIC pathway does not seem to directly rely on the function of the locus coeruleus, but it is possible that this structure modulates DNIC expression. Overall my data suggests a separate descending noradrenergic pathway for DNIC and tonic noradrenergic inhibition. Lastly, I propose that DNIC/CPM expression can be a useful test for optimally prescribing analgesic pharmacotherapies during different stages of metastatic disease progression.

5.6 Author Contributions

Dr. Kirsty Bannister recorded from 5 late CIBP, 2 early CIBP (both for atipamezole) and 2 late sham (for atipamezole with naloxone) animals in order to verify the unexpected findings of the spinal atipamezole action. She was blinded to the experimental outcome. Dr Bannister's data is pooled with my data. I performed all surgical interventions (i.e. sham and cancer surgery).
Chapter VI
Chapter VI. Thermoception in CIBP

6.1 Introduction

Alterations in cold sensitivity are evident in a range of neuropathic conditions of peripheral and central origin (Maier et al., 2010). This is also true for disturbances in heat sensations (Baron et al., 2017). In general, changes in surface temperature are detected by specialised receptors, and the majority of these belong to the ionotropic transient receptor potential (Trp) superfamily. For example, Trpm8, a member of the melastatin (Trpm) family, is proposed to detect cold sensations and also responds to menthol (Ceko et al., 2014; Knowlton et al., 2013; McKemy et al., 2002; Peier et al., 2002b, 2002a; Pogorzala et al., 2013). The vanilloid family member one (Trpv1) mediates responses to heat and can be activated by capsaicin (hot compound from chilli pepper) (Caterina et al., 1997, 2000). Trpv1 is expressed by around 30% of DRG neurons, whereas Trpm8 is expressed by much smaller (c.a. 5-8%) population of the primary afferents (Pogorzala et al., 2013). Functional temperature assays performed on mice with selective diphtheria toxin-ablated Trpv1 or Trpm8 populations suggest that heat and cold are perceived by different neurons (Pogorzala et al., 2013). Similar results were obtained by in vivo calcium imaging of DRG neurons, where a small percentage of mechano- (10%) or heating-sensitive (18%) neurons responded to cooling stimuli. The same authors showed that the perception of these distinct sensations is encoded in a different fashion by DRG cells. Namely, they presented evidence that heat is encoded by the primary afferents in a graded fashion (how strongly each neuron is activated and how many neurons are activated), whereas cold is coded in the combinatorial way (all-or-none activation of different neurons) (Wang et al., 2018).

Central relay of these remote sensory modalities was challenging to study due to poor accessibility. In contrast, more accessible for optical tools are superficial dorsal horn (SDH) neurons, which receive significant thermal inputs from the periphery (Basbaum et al., 2009). By imaging this dorsal horn neuronal population (between 25 – 85 μm below the cord surface in mice) in response to thermal stimuli, researchers previously suggested that SDH neurons encode absolute temperatures in the heating range, but binary change in temperature within the cooling range (Ran et al., 2016). This tightly reflects the peripheral input patterns observed by others (Wang et al., 2018).

Studies of deep dorsal horn (DDH) neurons are classically performed utilising electrophysiological techniques which have previously suggested graded temperature coding for both heat and cooling ranges (Patel et al., 2015; Suzuki et al., 2005). Wide-dynamic range (WDR) neurons are amongst the most abundant within the DDH. Since
these convergent neurons receive multimodal polysynaptic inputs from all types of primary afferents, they are exceptional 'tools' that can be used to explain some of the paradoxical sensations associated with nerve traumas or inflammation. Typically, in the majority of neuropathies, cold detection thresholds are decreased (cold hypoaesthesia). There are few exceptions in which the cold thresholds are increased. The most common cold-induced dysesthesias and paraesthesias occur in chemotherapy patients treated with platinum-based compounds (over 90% of cases) (Argyriou et al., 2013). Also, following nerve injury, cold alldynia does not correlate with mechanical alldynia or ongoing pain (Kleggetveit and Jørum, 2010). Heat hyperalgesia is common in inflammatory and neuropathic pains (Cesare and McNaughton, 1996; Galoyan et al., 2003). Heat hypersensitivity also follows heat (Campbell and LaMotte, 1983; Meyer and Campbell, 1981; Torebjörk et al., 1984) and cold injury to the skin (Khasabov et al., 2001). Cisplatin treatment can also induce heat hyperalgesia (Ta et al., 2010). Mechanistically, it was shown that NGF-evoked heat hyperalgnesia depends on a rapid increase in Trpv1 on cellular membrane (Zhang et al., 2005).

Since cancer-induced bone pain (CIBP) is primarily mechanoceptive in nature, only a few previous studies have focused on responses to heat and cold stimuli. Animals with the advanced bone cancer experience features of cold alldynia (Donovan-Rodriguez et al., 2005, 2006; Urch et al., 2003) and heat hyperalgesia (Menéndez et al., 2003; Zheng et al., 2012). SDH WDR neuronal responses (evoked) to heat stimuli were significantly increased in CIBP animals, whereas DDH WDR neurons were only partly facilitated (Donovan-Rodriguez et al., 2005, 2006; Urch et al., 2003). Regarding the central site, it was suggested that the allodynia and hyperalgesia observed after CIBP reflects spinal hyperexcitability and ongoing central sensitization (Clohisy and Mantyh, 2003; Donovan-Rodriguez et al., 2004; Mantyh and Hunt, 2004; Urch et al., 2003). Interestingly, there are only three reports to directly support these claims for heat stimuli, and (to best of my knowledge) there is rather scarce evidence for cold modality. Treatment with anticonvulsant gabapentin was shown to reduce cold alldynia in CIBP rats (by behavioural measures only) while acute gabapentin treatment in CIBP had no effect on the SDH WDR neuronal responses to heat stimuli (Donovan-Rodriguez et al., 2005). The involvement of descending serotonergic modulation was demonstrated with a restriction to heat hyperalgesia; descending facilitations orchestrated via spinal 5-HT3 channels were shown to potently increase firing rates of SDH WDR neurons to both innocuous and noxious temperatures, while contrasting DDH WDR neurons were facilitated only after noxious heat (Donovan-Rodriguez et al., 2006).

Due to this limited data, I decided to analyse both peripheral and central effects of thermal stimuli in the progression of bone cancer. Peripherally, I analysed responses
to noxious cold stimuli on a large population of primary afferents. Centrally, I focused on the responses of DDH WDR neurons to both innocuous and noxious cold and heat. Finally, I tested the role of descending inhibitory controls in modulation of DDH WDR neuronal activity following peripheral thermal stimulation.

6.2 Rationale, hypothesis and aims

As I showed in Chapter III, there is an increase in peripheral responsiveness to mechanical forces in the late bone cancer stage. Since thermoception was poorly studied in the CIBP, I wondered whether cold and heat responses are also affected at both peripheral and central sites. Therefore, here I present results from a series of experiments analogical to those in Chapters III and IV, however utilising thermal rather than mechanical stimulation.

6.3 Materials and Methods

A detailed description of employed in this chapter procedures can be found in Chapter II. See pages referral below.

- Cell cultures and animals (detailed description, page 35)
- CIBP rat model (detailed description, page 35)
- Behaviour: acetone (detailed description, page 40)
- In vivo calcium imaging in DRG neurons (detailed description, page 36-39)
- In vivo spinal electrophysiology (detailed description, page 43-45)
  - Baseline responses (detailed description, page 43)
  - Spinal pharmacology (detailed description, page 45)
  - Brain pharmacology (detailed description, page 46)
- Quantification and statistical analysis (detailed description, page 48)
6.4 Results

Figure 6.1. The recruitment of cold-responding neurons is enhanced in bone cancer; Cold allodynia develops with the progression of bone metastatic disease. *In vivo* calcium imaging in sensory neurons innervating cancer-bearing limb. Percentage of responders to ethyl chloride (noxious cold) application on the leg surface in three selected areas. N = Sham knee: 306, calf: 567, ankle: 405, and CIBP knee: 824, calf: 769, ankle: 1024 cells from 5 (sham) and 6 (CIBP) animals, unpaired t-test: *P < 0.05, ****P < 0.0001 vs. respective sham control) (A). Response intensity of responders to ethyl chloride (noxious cold) application on the leg surface in three selected areas. N = Sham knee: 27, calf: 22, ankle: 24, and CIBP knee: 57, calf: 86, ankle: 100 cells from 5 (sham) and 6 (CIBP) animals, unpaired t-test: *P < 0.05 vs. respective sham control (B). Behavioural responses of sham and CIBP rats to cooling sensation of acetone application on the ipsilateral (C) and contralateral (D) leg. Data represent the mean ± SEM. N = 20 animals per group. Kruskal–Wallis for independent samples: Ipsi: overall Sham vs. CIBP P<0.01, F[1, 128] = 8.295, pairwise comparison Sham vs. CIBP: day 0–day 7: non-significant (N.S.), day 14: *P < 0.05; Contra: overall Sham vs. CIBP P = 0.225, F[1, 128] = 1.470.

6.4.1 The recruitment of cold-responding neurons is enhanced in bone cancer

Firstly, I decided to study how primary afferents respond to noxious cold stimulation in healthy and advanced bone cancer rats. Considering that there was a substantial increase in the number of responding cells to mechanical stimulation in CIBP rats, I wanted to check whether alterations were also present to different modalities. I
decided to study noxious cold, since it has not previously been extensively studied in the CIBP rat model. As described in detail in Chapter III, I utilised in vivo calcium imaging in rats transduced with AAV9 delivering GCaMP. To deliver noxious cold stimulation, I utilised topical application of 5 drops of ethyl chloride (EtCl) in three different areas overlaying the cancer-bearing tibia (i.e. knee, calf and ankle). This fast evaporating solvent substantially decreases surface temperature reaching noxious levels (Leith et al., 2010).

Rat with bone cancer had double the number of responding cells to noxious cold stimulation applied to the calf surface (unpaired t-test: \( P < 0.0001 \)) (Fig. 6.1A). Smaller yet significant increases in the number of responders in CIBP state, was also found following EtCl application on the ankle (unpaired t-test: \( P = 0.020 \)) (Fig. 6.1A). Intriguingly, no changes in the number of responding cells were noted after the cold knee stimulation (unpaired t-test: \( P = 0.278 \)) (Fig. 6.1A). Analysis of the response intensities of cold-sensitive cells revealed a decrease following stimulation of calf and ankle (unpaired t-test: [knee]: \( P = 0.169 \), [calf]: \( P = 0.041 \), [ankle]: \( P = 0.044 \)) (Fig. 6.1B).

6.4.2 Cold allodynia develops with the progression of bone metastatic disease

In order to behaviourally assess the presence of secondary cold sensitisation, both sham—operated and cancer-bearing animals were subjected to the acetone test, where the solvent was applied to the plantar surface of the paw. Acetone evaporation is slower than EtCl, therefore it represents innocuous cold stimulation (Leith et al., 2010). Significant differences were observed in acetone test in day 14 after surgery on the ipsilateral site in CIBP group, but not in sham-operated animals, indicating the presence of cold hypersensitivity (Kruskal–Wallis for independent samples: Ipsi: overall sham vs. CIBP \( P<0.01 \), \( F_{[1, 128]} = 8.295 \), pairwise comparison: day 0 – day 7: non-significant (N.S.), day 14: *\( P < 0.05 \)) (Fig. 6.1C). No differences were observed in control and CIBP groups in non-operated legs (Kruskal–Wallis for independent samples: Contra: overall sham vs. CIBP \( P = 0.225 \), \( F_{[1, 128]} = 1.470 \)) (Fig. 6.1D).
Figure 6.2. Thermally-evoked activity of DHW DDR neurons is not altered by bone cancer. Schematic representation of the in vivo electrophysiological experiment. WDR, wide-dynamic range neurons (A). Deep dorsal horn lamina V/VI WDR neuronal responses to non-noxious (acetone) and noxious (ethyl chloride) cold (B) and heat (C, D) stimulation of the receptive field in early (day 7-8) and late (day 14-16) stage CIBP and corresponding sham-operated rats. All the data represent the mean ± SEM from sham early (n = 6), CIBP early (n = 11), sham late (n = 31) and CIBP late (n = 31). Each dot represents one animal. Univariate (B, D) or multivariate (C) ANOVA.

6.4.3 Thermally-evoked activity of DHW DDR neurons is not altered by bone cancer

Since, cancer-bearing animals develop secondary cold hypersensitivity, I studied dorsal horn neuronal activity utilising in vivo electrophysiology. The activity of DHW DDR neurons was examined in sham early (SE, n = 6), CIBP early (CE, n = 11), sham late (SL, n = 31) and CIBP late (CL, N = 31). The basal firing rate of DHW DDR neurons was unchanged in animals with CIBP as compared to sham-operated. Acetone application to the receptive field had no effect on DHW DDR neuronal firing rates between all analysed groups (univariate ANOVA: [group]: F3, 71 = 0.174, P = 0.914) (Fig. 6.2B). Responses after ethyl chloride application were also not affected between all analysed groups (univariate ANOVA: [group]: F3, 71 = 0.857, P = 0.468) (Fig. 6.2B). Effects of heat stimulation did not reach statistical significance in a multivariate ANOVA test (heat: multivariate ANOVA; F3, 233 = 1.656, P = 0.177) (Fig. 6.2C) and a comparison between each group for individual temperatures revealed no significant differences (one-way ANOVA: [42°C]: F3, 78 = 0.512, P = 0.676, [45°C]: F3, 77 = 0.823, P = 0.485, [48°C]: F3, 78 = 0.486, P = 0.693) (Fig. 6.2D).
Figure 6.3. Tonic descending noradrenergic controls are altered in CIBP. Following spinally applied atipamezole (100 µg) recordings were made of deep dorsal horn lamina V/VI WDR neuronal responses to non-noxious (acetone) and noxious (ethyl chloride) cold (A, C, E, G) and heat (B, D, E, F) stimulation of the receptive field in early (day 7-8, top panels) and late (day 14-16, bottom panels) CIBP stage and corresponding sham-operated rats. All the data represent the mean ± SEM from sham early (both cold and heat: n = 4), CIBP early (cold: n = 4, heat n = 6), sham late (both cold and heat: n = 11) and CIBP late (cold: n = 4, heat: n = 9). Each dot represents one animal. RM-ANOVA
with Bonferroni post-hoc: *P < 0.05, **P < 0.01 vs. baseline (for punctate mechanical stimulation) and paired t-test: *P < 0.05 vs. baseline (for brush and electrical stimulation). See also Figure S3.1 for effects of 10 µg of atipamezole.

6.4.4 Tonic descending noradrenergic controls are altered in a rat model of CIBP

Similarly to mechanically and electrically-evoked stimuli (see Chapter IV), cold hypersensitivity was not reflected in the evoked activity of DDH WDR neurons. This suggests the presence of inhibitory modulation of DDH WDR neurons to thermal stimuli. To verify the role of descending noradrenergic inhibitory controls, a selective α2-AR blocker atipamezole (see Table 4.1) was applied spinally to sham-operated and CIBP rats in two doses: 10 and 100 µg. For clarity only results utilising the higher dose of atipamezole are shown throughout (10 µg is reported in the Appendix 3, Figure S3.1).

In the early sham (SE) group (n = 4), spinal application of atipamezole had no effect on the DDH WDR neuronal responses, to peripherally applied cold stimuli (paired t-test: [acetone]: P = 0.424, [EtCl]: P = 0.135) (Fig. 6.3A), nor to heat (repeated-measures ANOVA: [group]: F₁, 3 = 1.184, P = 0.356) (Fig. 6.3B).

Similarly, in the early CIBP (CE) group, atipamezole had no effect on the DDH WDR neuronal activity, neither to cold (paired t-test (n = 4): [acetone]: P = 0.849, [EtCl]: P = 0.436) (Fig. 6.3C), nor heat (repeated-measures ANOVA (n = 6): [group]: F₁, 5 = 0.352, P = 0.579) (Fig. 6.3D) stimuli applied to the receptive field.

As in SE, in the late sham-operated (SL) group (n = 11), atipamezole had no effect on DDH WDR neuronal activity in response to innocuous cold (paired t-test: [acetone]: P = 0.297) (Fig. 6.3E), or to heat stimulation (repeated-measures ANOVA: [group]: F₁, 10 = 0.528, P = 0.484) (Fig. 6.3F). Interestingly, atipamezole significantly increased DDH WDR neuronal responses to noxious cold (paired t-test: [EtCl]: P = 0.035) (Fig. 6.3E).

Contrasting, in the late cancer (CL) stage group, spinal application of atipamezole dose-dependently inhibited DDH WDR neuronal activity (similarly to actions on mechanical stimuli presented in Chapter IV). Precisely, responses to noxious cold stimuli (paired t-test (n = 4): [EtCl]: P = 0.045) (Fig. 6.3G) as well as to heat stimuli (repeated-measures ANOVA(n = 9): [group]: F₁, 8 = 20.359, P = 0.002, Bonferroni post hoc: [42°C]: P < 0.05, [45°C and 48°C]: P < 0.01) (Fig. 6.3H) were inhibited. Responses to innocuous cold applied to the receptive field, however, were not affected (paired t-test (n = 4): P = 0.262) (Fig. 6.3G).
Figure 6.4. A spinal opioidergic system modulates tonic noradrenergic actions in the late stage CIBP rats. Following spinally applied naloxone (20 µg) recordings were made of deep dorsal horn lamina V/VI WDR neuronal responses to non-noxious (acetone) and noxious (ethyl chloride) cold (A, C) and heat (B, D) stimulation of the receptive field in late (day 14-16) CIBP stage and corresponding sham-operated rats. All the data represent the mean ± SEM from sham (cold n = 5, heat: n = 4), CIBP (both cold and heat: n = 7). RM-ANOVA (heat) and paired t-test (cold). After spinally applied mix of 20 µg naloxone and 100 µg atipamezole, recordings were made of deep dorsal horn
6.4.5 Spinal opioid system mediates tonic noradrenergic actions in the late stage CIBP rats

Here I observed similar actions of spinally applied atipamezole in the late CIBP group to that described in Chapter IV. Accordingly, I verified the potential engagement of the spinal opioid system. Naloxone was applied directly onto the spinal cord following measurement of stable baseline WDR responses (concentrations of 5 μg [Appendix 3, Figure S3.2], and 20 μg was applied in 50 μl saline).

In the SL group, spinal naloxone had no effect on DDH WDR neuronal activity following both innocuous and noxious cold stimuli (n = 5, paired t-test: [acetone]: P = 0.191, [EtCl]: P = 0.703) (Fig. 6.4A), nor to heat stimuli (n = 4, repeated-measures ANOVA: [group]: F_{1, 3} = 9.349, P = 0.055) (Fig. 6.4B).

Similarly, there was no significant effect of naloxone on evoked DDH WDR neuronal activity in response to cold stimulation in the CL group (n = 7, paired t-test: [acetone]: P = 0.221, [EtCl]: P = 0.130) (Fig. 6.4C) or to heat stimulation (n = 7, repeated-measures ANOVA: [group]: F_{1, 6} = 2.338, P = 0.177) (Fig. 6.4D).

To check if the inhibitory action of spinally applied atipamezole was mediated by dorsal horn opioidergic interneurons, 100 μg of atipamezole was co-administered spinally with 20 μg of naloxone. In the SL group, naloxone-atipamezole spinal application had no effect on DDH WDR neuronal activity (n = 6) following both innocuous and noxious cold stimuli (paired t-test: [acetone]: P = 0.726, [EtCl]: P = 0.274) (Fig. 6.4E), nor to heat stimuli (repeated-measures ANOVA: [group]: F_{1, 5} = 5.728, P = 0.062) (Fig. 6.4F) applied to the receptive field.

Interestingly, in the CL group, the earlier observed atipamezole-evoked inhibitions (Fig. 6.3G, H) were fully reversed by the naloxone co-administration. There was no significant effect of atipamezole-naloxone mix on DDH WDR neuronal activity in response to cold stimulation in the CL group (n = 6, paired t-test: [acetone]: P = 0.354, [EtCl]: P = 0.203) (Fig. 6.4G). Upon heat stimulation, however, concomitant application of the two drugs resulted in a potent facilitatory effect (n = 6, repeated-measures ANOVA: [group]: F_{1, 5} = 24.994, P = 0.004, Bonferroni post hoc: [42°C]: P > 0.05, [45°C]: P < 0.05, [48°C]: P < 0.0001) (Fig. 6.4H).
6.5 Discussion

6.5.1 Bone cancer recruits more cold-responding neurons

In chapter III I showed that mechanical stimulation to the cancer-bearing leg engages previously silent nociceptive afferents. Since there is scarce data regarding afferent function in CIBP following cold stimulation, here I investigated the effects of noxious cold stimulus when applied to the cancer-bearing leg. I employed in vivo calcium imaging of DRG neurons to pursue a large populational study. CIBP rats showed an increase in the number of neurons that responded to the noxious cold stimuli as compared with sham-operated group, suggesting that the silent nociceptors are cold-sensitive. Interestingly, apart from the fact that there were more cold responding cells in bone cancer conditions, I noted that these cells responses are smaller than in the sham group. This may likely reflect cold hyposensitivity observed in other models of peripheral neuronal damage including bone cancer (Kleggetveit and Jørum, 2010; Menéndez et al., 2003).

Typically silent nociceptors are described as sensory afferents that are insensitive to mechanical stimuli but that fire action potentials upon electrical stimulation above C-fibre threshold (Gold and Gebhart, 2010; Michaelis et al., 1996). Upon chemical priming with capsaicin, some previously mechanically- and heat-insensitive fibres were found to be responsive to both mechanical and heat stimuli (Schmelz et al., 2000). The gain in responsiveness to noxious cold stimuli by originally cooling-unresponsive fibres was not analysed. The primary mechanism for the sensitization of silent nociceptors was via TrkA downstream signalling engaging Erk1/2 and as yet unknown secondary mediator(s) to sensitize Piezo2 channels (Prato et al., 2017). This would explain a gain in mechanical sensitivity by these previously silent nociceptive afferents, but not their responsiveness to noxious cold or heat stimuli. What the molecular mediators and targets for the observed sensitization of silent nociceptors to cold are remains an open question. Finally, here I applied ethyl chloride onto the leg’s surface, unlike the methodology presented in Chapter III which utilised compression-stimulus, evenly applied, to the whole leg. Therefore, it can be suggested that not only do cancer-bearing bone neurons but also others from the bone surroundings are sensitized by locally-released mediators (e.g. NGF). Confirmation for this is also presented in the Chapter III tracing studies, where muscle and periosteum afferents were particularly sensitized by bone cancer.

6.5.2 Cold allodynia develops with the progression of bone metastatic disease

The employment of cold-sensitive silent nociceptors in bone cancer is reflected in the spinal events. Cancer-bearing animals show symptoms of cold allodynia in areas
distant to the cancer-growth area, likely representing the development of secondary cold hypersensitivity, and further confirming ongoing central sensitization (Urch et al., 2003). Cold allodynia has been presented before in various animal models of skeletal metastatic disease (Donovan-Rodriguez et al., 2004, 2005; Urch et al., 2003).

6.5.3 Thermally-evoked activity of DDH WDR neurons is not altered by bone cancer

Analogous to mechanically and electrically-evoked stimuli (see Chapter IV), DDH WDR neurons did not differ in the number of action potentials fired following cold or heat stimulation between cancer and sham groups. These surprising results contrast with the presented increase in the number of cold responding afferents and progressive cold hypersensitivity (acetone-evoked allodynia). They are also divergent to the heat hyperalgesia observed in the advancement of metastatic disease (Zheng et al., 2012). What is more, unlike DDH, SDH WDR neurons were previously reported to be hyperactive in response to heat stimulation in the CIBP-model (Donovan-Rodriguez et al., 2004; Urch et al., 2003). Central processing of cold stimuli, however, has not knowingly been studied in the CIBP rat model before.

6.5.4 Tonic descending noradrenergic controls are altered in CIBP

It is likely that peripheral hyperactivity drives plasticity in central pain controls. Supporting that view is an evidence of an increase in descending facilitatory controls (orchestrated via spinal 5HT$_3$ receptors) in CIBP rat dorsal horn neuronal activity after heating stimuli. As for mechanically-evoked responses (see Chapter IV), descending facilitations were particularly potent at facilitating heat-evoked responses in SDH rather than DDH neurons (Donovan-Rodriguez et al., 2006). Strikingly, antagonism of spinal $\alpha_2$-ARs using atipamezole in the advanced bone cancer state resulted in strong inhibition of WDR neuronal responses to both noxious cold and heat stimuli. Interestingly, there seems to be a potent $\alpha_2$-AR-mediated tonic control of noxious cold in late sham animals, since after atipamezole application responses in this group were strongly facilitated.

Some clusters of primary afferents are modality specific, which superimposes the existence of different synapses on the central (spinal) sites (Abraira et al., 2017; Todd, 2010; Usoskin et al., 2014). Therefore, this further suggests that the action of descending monoamines (i.e. 5-HT or NA) in the cord is rather diffuse, allowing for a broad (inhibitory or excitatory) control of multiple modalities (Pertovaara, 2006; Rajaofetra et al., 1992; Zoli and Agnati, 1996).
6.5.5 Spinal opioid system mediates tonic noradrenergic actions in the late stage CIBP rats

I sought to verify whether the observed inhibitory action of atipamezole was mediated by spinal opioidergic interneurons. Utilising intrathecal application of naloxone, a pan-opioid receptors antagonist, I firstly verified whether a spinal opioidergic system was involved in regulation of the thermally-evoked responses of DDH WDR neurons in late stage healthy and diseased rats. The observed mild potentiation of DDH WDR neuronal responses after high (20 µg) dose of spinally applied naloxone was suggestive of only a slight tonic opioidergic control of cold and heat-evoked activity of DDH WDR neurons. This in contrast to the potent opioid control of mechanically-evoked WDR neuronal response, as measured in terms of action potentials fired (Chapter IV). Interestingly, spinal naloxone fully reversed the morphine-induced inhibitory effect of heat-evoked SDH and DDH WDR neuronal responses in both sham and CIBP rats (Urch et al., 2005). Again, cold stimuli were not tested. What is more, in the murine osteosarcoma model, thermal hypoalgesia (instead of hyperalgesia) with co-existing mechanical hyperalgesia was observed (Menéndez et al., 2003). The authors postulated an involvement of opioidergic antinociceptive mechanisms since naloxone suppressed the inhibition of the response to noxious thermal stimulation.

I went on to show that naloxone potently reversed atipamezole-evoked inhibition of DDH WDR neurons to noxious cooling and heating stimuli. This further supports the proposal in Chapter IV that peripheral inputs are increased in the advanced cancer conditions and that they in turn hyperactivate descending noradrenergic systems. In turn, following noxious stimulation, spinally released noradrenaline acts via adra1 residing on dorsal horn opioidergic interneurons to further inhibit DDH WDR neurons. My hypothesis is further supported by research reporting that virtually all of the cold-responsive lamina I projecting neurons also respond to noxious mechanical stimuli (Bester et al., 2000).

6.5.6 Conclusions

Data presented in this chapter supports the results presented in Chapters III and IV. I demonstrated that bone cancer recruits more cold-responding neurons. I also present evidence that CIBP animals exhibit cold allodynia. I observed no change in DDH WDR neurons baseline firing, suggesting engagement of descending controls. Finally, I evidenced similar alterations in the descending noradrenergic controls to those described in Chapter IV for mechanical stimuli; the multimodal inhibitory actions exerted by spinally acting descending noradrenaline suggests a diffuse mode of action. I suggest that an analogous mechanism may exist for the thermal pain controls in the advanced rat model of CIBP.
6.6 Author Contributions

Dr. Kirsty Bannister recorded from 5 late CIBP, 2 early CIBP (both for atipamezole) and 2 late sham (for atipamezole with naloxone) animals in order to verify the unexpected findings of the spinal atipamezole action. She was blinded to the experimental outcome. Dr Bannister’s data is pooled with my data. I performed all surgical interventions (i.e. sham and cancer surgery). I would like to also thank Dr. Kim Chisholm for her help regarding the GCaMP data acquisition and analysis.
Chapter VII
Chapter VII. General discussion

Within this thesis I have introduced the basic anatomy of bone afferents and characterized them functionally in health and in the context of bone cancer. Considering the pain neuroaxis, I characterized spinal processing of CIBP in its early and advanced stage. Further, I analysed top-down noradrenergic influences on spinal processing of somatosensory information. Finally, I evaluated expression of DNIC in the development of CIBP and suggested its diagnostic potential for mechanism-based pain pharmacotherapy.

My most clinically-significant conclusion stated within this thesis is that the pain associated with cancer-induced bone pain (CIBP) is extremely heterogenous, even within this one disease state. I demonstrated here that a rodent model of bone cancer has distinctive, progressive phases, and that the associated pain differs in its symptomatology and underlying causative mechanisms. This translates to a requirement for tailored (pharmaco)therapy. Therefore, in the context of such disease states, one should rather talk about pains, in its plural form.

In metastatic bone tumours, peripheral neurons have been shown to sprout around the cancer-bearing bone in a NGF–driven fashion to create neuroma-like pathological structures (Bloom et al., 2011; Jimenez-Andrade et al., 2010c; Mantyh et al., 2010b). These changes are plastic; in the early disease stages both the intraosseous and periosteum neurons sprout, but by the late stage the increased compression (tumour mass) and hypoxic, acidic and toxic conditions damage the newly sprouted afferents. Increased innervation may be now present only outside of the bone matrix. I demonstrated that the increase in the number of mechanically responding cells, rather than the increase in individual cell sensitivity, likely translates to the mechanical hypersensitivity observed in both CIBP rats. A probable mechanism is the activation of silent nociceptors surrounding the tumour. I show that primary afferents respond differently according to the mechanical stimulus, suggestive of specific differential coding of pressure and proprioception (graded vs. combinatorial) by the somatosensory neurons. Finally, in sham animals, pressure is likely encoded by means of the frequency change, whereas in advanced bone cancer conditions frequency coding is largely dominated by the population coding resulting from the robust recruitment of silent nociceptors.

Results from others point that anti-NGF therapy limit neuroma formation and decrease pain in multiple CIBP animal models (Halvorson et al., 2005; Jimenez-Andrade et al., 2010c; Mantyh et al., 2010c) and patients (Sopata et al., 2015). I suggest that the decrease in pain readouts following NGF targeting could be also due to the prevention
of deep body silent nociceptors sensitization, such that they remain dormant. Separately, CGRP-targeting strategies have been shown to not only prevent cancer pain, but also strongly contribute to the inhibition of neovascularization of the tumour, thus decreasing tumour size (Toda et al., 2008). Such strategies highlight the importance of tumour-nerve dialog (Boilly et al., 2017). Here, I showed that many bone afferents are CGRP and TrkA positive. What is surprising is that these afferents are histologically different from most of the body's sensory neurons; over 40% of them do not express Avil, a proposed marker for sensory cells. This also precludes the universal usage of many murine models based on the Avil-driven transgene expression if one wants to target deep body afferents.

Spinally released peptides (CGRP and SP) increase DH neuronal excitability. Under inflammatory conditions these peptides potently contribute to the development of central sensitization, hyperalgesia and allodynia. I demonstrated that behaviourally, CIBP animals develop symptoms of mechanical hypersensitivity and cold allodynia. Particularly pronounced were the rat's reactions to both evoked (von Frey) and static (weight bearing) mechanical stimuli. This nociceptive effect was strengthened with the disease progression and reflects the mechanoceptive phenotype of CIBP as frequently identified in patients with neoplastic bone lesions (Falk and Dickenson, 2014; Mantyh, 2006a; Mantyh et al., 2002; Mercadante, 1997, 2006; Mercadante et al., 2004). Independent research groups utilising similar rodent models of the disease have published data that is in agreement with my findings from von Frey (Donovan-Rodriguez et al., 2006; Falk et al., 2015b, 2015c; Guedon et al., 2016; Luger et al., 2002), acetone (Donovan-Rodriguez et al., 2006), and weight bearing (Falk et al., 2015b, 2015c; Hansen et al., 2012; Minett et al., 2014) tests. What is more, other authors showed that CIBP animals also develop ambulatory-evoked pain (rotarod) (Donovan-Rodriguez et al., 2006), as well a sensitivity to pressure applied to the tumour-bearing leg (evidenced by Randall Selitto test) (Falk et al., 2015d).

Since both cancer patients and animal models of CIBP often show clear symptoms of secondary sensitization to mechanical and thermal stimuli, I focused next on the central processing of CIBP. I found that the stimulus-evoked activity of DDH WDR neurons was not altered in the cancer-bearing animals, which corresponds with previous observations (Falk et al., 2015a). However, it is unlike the well-documented hyperactive state of SDH neurons (Donovan-Rodriguez et al., 2004; Urch et al., 2003), suggesting the presence of potent stimulus-evoked or tonic inhibitory controls in the DDH of CIBP rats.

Next, I found that intrathecal atipamezole, an α2-adrenergic receptor antagonist, had a pronounced inhibitory effect on DDH WDR neurons' activity in CIBP rats. This contrasted with the observed facilitatory effect of spinal atipamezole in control animals.
This unexpected result was suggestive of altered descending noradrenergic controls in the rat CIBP model and I investigated this further. Firstly, I found that spinal lamina I projection neurons, when optoactivated, potently inhibit deep dorsal horn WDR cells. This being in agreement with a previous report from McMahon and Wall, and suggesting that upon DLF electrical stimulation, DDH but not SDH neurons are inhibited (McMahon and Wall, 1983, 1988). Secondly, optoactivation of the LC in naïve animals mimicked CIBP in the sense of altered atipamezole pharmacology. Also, a co-administration of naloxone reversed the inhibitory effects of atipamezole in the CIBP model. Finally, a pilot experiment with lidocaine block of the LC in CIBP animals seemed to abolish the inhibitory action of spinally administered atipamezole, further suggesting a LC contribution.

Table 7.1. Analysis of public dataset of single cell RNA sequencing of spinal inhibitory (GABA) and excitatory (GLUT) neurons.

<table>
<thead>
<tr>
<th>Percent of:</th>
<th>Actb/Gapdh (ALL)</th>
<th>Ornb</th>
<th>Penk</th>
<th>Pdyn</th>
</tr>
</thead>
<tbody>
<tr>
<td>(number of cells)</td>
<td>GABA (765)</td>
<td>GLUT (735)</td>
<td>GABA (493)</td>
<td>GLUT (83)</td>
</tr>
<tr>
<td>Actb/Gapdh</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td>Ornb</td>
<td>64.44</td>
<td>8.57</td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td>Penk</td>
<td>56.06</td>
<td>52.52</td>
<td>49.29</td>
<td>41.27</td>
</tr>
<tr>
<td>Pdyn</td>
<td>22.75</td>
<td>1.22</td>
<td>26.37</td>
<td>6.36</td>
</tr>
<tr>
<td>Adra1a</td>
<td>6.01</td>
<td>0.82</td>
<td>6.29</td>
<td>1.59</td>
</tr>
<tr>
<td>Adra1b</td>
<td>7.58</td>
<td>3.95</td>
<td>6.90</td>
<td>1.59</td>
</tr>
<tr>
<td>Adra1d</td>
<td>0.39</td>
<td>0.14</td>
<td>0.20</td>
<td>0.00</td>
</tr>
<tr>
<td>Adra2a</td>
<td>7.58</td>
<td>21.90</td>
<td>6.90</td>
<td>12.70</td>
</tr>
<tr>
<td>Adra2b</td>
<td>5.62</td>
<td>1.09</td>
<td>7.91</td>
<td>0.00</td>
</tr>
<tr>
<td>Adra2c</td>
<td>9.41</td>
<td>6.39</td>
<td>11.36</td>
<td>7.94</td>
</tr>
<tr>
<td>Stc32a1</td>
<td>80.00</td>
<td>4.08</td>
<td>80.93</td>
<td>9.52</td>
</tr>
<tr>
<td>Stc17a6</td>
<td>4.71</td>
<td>94.15</td>
<td>4.06</td>
<td>95.24</td>
</tr>
</tbody>
</table>

I would like to propose the following mechanism that explains the obtained results:

It was previously observed that a peripheral nociceptive input activates SDH neurons leading to hyperactivity of the superficial laminae cells in the advanced CIBP state (Donovan-Rodriguez et al., 2004; Urch et al., 2003). Activated projection neurons from the SDH directly stimulate supraspinal centres including the IPB area and the LC. In turn, descending noradrenergic projections from the LC increase spinal NA levels. It remains to be established whether there are functional connections between the IPB area and the LC, or if LI/LIIo projection neurons collaterals are responsible for the activation of the LC descending inhibitions. Because spinally released NA acts in a volumetric fashion (by diffusion from descending synapses), there are at least 2 factors controlling its actions: (1) the affinity of NA to its receptors (linked to the amount of NA released and the resulting gradient) and (2) the expression pattern of its receptors. In normal conditions NA preferentially binds to the α2-ARs, whose affinity to NA is 6 times
higher than $\alpha_1$-ARs (Table 4.2). Since in the spinal DH $\alpha_2$-ARs are expressed on both inhibitory and excitatory neurons, the effects of NA via these receptors seem to be balanced upon its ‘tonic’ release from synaptic terminals (Table 7.1 and (Olave and Maxwell, 2003a, 2003b, 2004)). This allows the system to be ‘tuned’ for incoming stimuli. Therefore, selective block of spinal $\alpha_2$-ARs (for example by i.th. atipamezole) has hardly any impact on the evoked spinal neuronal firing (chapter IV, VI, and (Burnham and Dickenson, 2013; Hughes et al., 2013; Rahman et al., 2008b, 2008a)).

However, the situation seems to be entirely different when high levels of NA are released at the spinal sites. Following a potent noxious stimulation, lamina I projection neurons convey nociceptive impulses to activate supraspinal centres, including IPB area and the LC. In turn, descending efferents of the latter release large amounts of NA both within the brain structures (to increase arousal; ‘fight and flight’) and spinally (to mediate analgesia). After a strong painful stimulus reaches lamina I and activates this loop, a resulting increase in spinal NA means that $\alpha_1$-ARs are occupied. Unlike $\alpha_2$-ARs, $\alpha_1$-ARs are predominantly expressed on inhibitory neurons within the DH, of which great proportion is opioidergic and express Penk or Pdyn (Table 7.1). Interestingly, these opioidergic populations often express Ror$\beta$ (Rorb), a suggested marker for a cluster of spinal inhibitory interneurons responsible for the inhibition of spinal activity after mechanical stimulation (Abraira et al., 2017; Del Barrio et al., 2013; Koch et al., 2017). Activation of $\alpha_1$-ARs expressed on inhibitory interneurons leads to the release of longer acting mediators like Enk, Dyn and GABA, which can potently inhibit spinal neurons, including DDH WDR cells. These results are supported by observed spinal tapentadol (Falk et al., 2015a), naloxone (chapter IV and VI) and GABA (Gassner et al., 2009) actions on the DDH neuronal excitability. This mechanism works to decrease spinal excitability following acute painful stimuli (like breakthrough pain). In situations where the nociceptive input is multiplied, for example following engagement of silent nociceptors, the activation thresholds of this inhibitory loop could be lowered.

The observed analgesic action of spinal $\alpha_2$-ARs antagonism following application of atipamezole in advanced CIBP rats can be explained as follows. Upon strong activation of the LC there is a massive release of NA (for example following the LC optoactivation, or in CIBP), which allows for the activation of inhibitory interneurons following $\alpha_1$-ARs binding. Some of these interneurons also express $\alpha_2$-ARs (i.e. around 11% of all $\alpha_2$-ARs are expressed by inhibitory interneurons). Thus, antagonism of these receptors by atipamezole allows the $\alpha_1$-ARs-mediated action to be fully expressed. Antagonism of $\alpha_2$-ARs can expose the full inhibitory potential of $\alpha_1$-AR-mediated inhibition only when sufficient levels of $\alpha_1$-AR’s are occupied. The involvement of $\alpha_1$-AR’s will be a substrate for my future studies utilising selective antagonists.
Another possible (and complementary) explanation is based on a report from Eason and colleagues (Eason et al., 1992), who showed that in the presence of high agonist concentrations and high receptor expression levels, $\alpha_2$-ARs couple to facilitatory G proteins. Alterations in protein levels are, however, unlikely since, upon the LC and/or LI optoactivation, the pharmacological effect of atipamezole application was changed instantaneously. It would be interesting to know whether an increase in NA levels is sufficient to switch $\alpha_2$-AR function from inhibitory to facilitatory (without the need for an increase in receptor density also).

It has also been reported that in the spinal nerve ligation (SNL) model of rat neuropathy, spinal BDNF can induce sprouting of descending noradrenergic afferents to compensate for the loss of inhibitory drive (Hayashida et al., 2008). This mechanism is rather less probable in the LC optoactivation experiment; however it may contribute to the CIBP phenotype.

I conclude that in my rodent model of CIBP, a monoaminergic transmission mechanism within the spino-pontine-spinal loop is likely altered, especially in sense of the descending inhibitory controls. A novel mechanism controlling deep WDR neurons within the loop is suggested. These findings have the potential to translate to clinical practice by repurposing already approved drugs that pharmacologically manipulate the noradrenergic system to combat CIBP.

Is this mechanism common for all NGF-mediated pains?

In other inflammatory models with known actions of NGF, for example the moniodoacatate (MIA) model of OA, evoked activity of DDH WDR neurons is similarly not affected (Burnham and Dickenson, 2013; Rahman and Dickenson, 2015). Also, in the early stage of OA but not the late stage, atipamezole was shown to reverse the inhibitory effects of milnacipran (SNRI) (Burnham and Dickenson, 2013). This suggests that by the late stage of OA, a recruitment of silent nociceptors (due to locally released NGF) may occur, potentiating activation of the suggested loop, and shifting NA-mediated actions to $\alpha_1$-AR activation. Whether SDH cells are hyperactive in late OA remains to be verified.

In contrast, both SDH and DDH WDR neurons in the SNL model of neuropathy exhibit no changes in their basal excitability after peripheral application of innocuous or noxious mechanical, thermal and electrical stimuli as compared to sham animals (Patel et al., 2015). Hence, an increased basal firing of SDH neurons in CIBP may instead be due to peripheral overstimulation by chemical mediators (NGF) involved in inflammatory pain signalling and resulting recruitment of silent nociceptors (whose input is mainly to lamina I and II (Prato et al., 2017)), rather than other mechanisms (such as those underlying classic neuropathy resulting from primary neuronal damage). This may
represent a common mechanism in inflammatory disease states that involve NGF-sensitization of silent nociceptors, especially in deep-body inflammatory conditions since therein the incident of silent nociceptors is particularly frequent.

Since peripheral sensitization, including the activation of silent nociceptors in CIBP, is extreme, it is likely that both the input duration as well as its strength has a decisive role in the central plasticity. It is clear that the plasticity of descending controls during the progression of bone metastatic disease is time/stage-dependent.

Prato et al. showed that, under inflammatory conditions, there is NGF-mediated employment of silent nociceptors. I showed here that in the rat model of CIBP there is a three-fold increase in peripheral input due to the engagement of silent nociceptive afferents. Therefore, LC hyperactivity is associated with inflammatory pain types that engage silent nociceptors, rather than with chronic neuropathies, where the LC was previously shown underactive (Ito et al., 2018; Wakabayashi et al., 2018). A crucial component for this observation appears to be a specific increase in nociceptive input to lamina I projection neurons, for example by employment of silent nociceptors. This in turn activates brainstem NAergic centres to release NA in the spinal loci.

Characteristic for neuropathic pain models is activation of all fibres resulting in a distinct pain phenotype compared to CIBP and OA pains (where mainly small diameter fibres are activated). Also, NGF-mediates its function via the TrkA receptor, which is not present on Aβ-fibres. Thus, it is possible that Aβ-afferent damage is required for complete development of the ‘classic’ neuropathic pain phenotype (i.e. following nerve injury). The presence or absence of silent nociceptor activation by inflammatory mediators (especially NGF) could substantially contribute to the development of the CIBP phenotype. Therefore, the input characteristics considerably differ between, for example, classic neuropathic, inflammatory and cancer-induced pains (Table 7.2). This and the afferent termination sites within the spinal cord may explain why only upon CIBP conditions hyperexcitability of SDH WDR (but not DDH WDR) neurons was observed.

Thus, pains involving NGF-sensitization of silent nociceptors are likely to evoke a large increase in input to the SDH laminae. The phenotype of these pains may be therefore very similar, suggesting a common treatment approach, in as much as deep body pains should be particularly responsive for this therapy since silent nociceptors populate deep tissues whilst being rather scarce in the skin.
Table 7.2. Suggested differences between selected factors underlaying pains with different aetiology.

<table>
<thead>
<tr>
<th></th>
<th>Neuropathic pain</th>
<th>Inflammatory pain</th>
<th>CIBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibres activated</td>
<td>Aβ, Aδ, C</td>
<td>Aβ, Aδ, C</td>
<td>Aδ, C</td>
</tr>
<tr>
<td>Silent nociceptors</td>
<td>Not active</td>
<td>Active</td>
<td>Active</td>
</tr>
<tr>
<td>Spinal laminae input</td>
<td>LII, LV</td>
<td>LII, LV</td>
<td>Mainly LII</td>
</tr>
<tr>
<td>Hyperactivity NA</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Hyperactivity 5-HT</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Expected atipamezole effect</td>
<td>No change</td>
<td>Inhibits DDH WDR neurons</td>
<td>Inhibits DDH WDR neurons</td>
</tr>
<tr>
<td>DNIC</td>
<td>Tonic NA hypofunctional, Unbalanced</td>
<td>Tonic NA hyperfunctional balances SHT hyperfunctional</td>
<td>Tonic NA hyperfunctional balances SHT hyperfunctional</td>
</tr>
</tbody>
</table>

DNIC

There is a growing body of evidence to support the hypothesis for the existence of at least two separate descending noradrenergic pathways originating from the brainstem and terminating in the spinal cord. Namely, these are projections from the pontine LC and from the subnucelus reticularis dorsalis (SRD). Both pathways are bilateral, with some degree of lateralization. Over 30 years ago it was been reported that the SRD had a strong contribution to DNIC expression (Bouhassira et al., 1992c; Villanueva et al., 1996).

This thesis reports detailed DNIC expression analysis in the rat model of CIBP in early and late stages. DNIC were found dynamic in the CIBP rat model. Their expression was abolished in the early cancer stage, and fully restored by the late stage of the metastatic disease. Interestingly, in the late cancer stage the spinal α2-AR controls of DNIC were maintained, similarly as in healthy rats. I also show that spinal naloxone does not control DNIC expression, revealing a lack of spinal opioidergic mechanisms that may have sub-served DNIC. Also, α2-AR-mediated control of DNIC was insusceptible to co-administered naloxone, suggesting separate noradrenergic pathways that modulate DNIC versus tonic inhibition of WDR neurons. Supporting are my results from pharmacological block of the LC, where DNIC was still potently inhibiting WDR neurons to the range of noxious stimuli. However, the dialogue of these two parallel pathways needs to be further investigated.

I hypothesise that, in the early stage of the cancer development, tonic NA controls are reduced in favour of a facilitatory serotonergic (5-HT) drive. The scenario changes by the late cancer stage, where tonic NA controls are hyperactive, restoring the balance of facilitatory (5-HT) and inhibitory (NA) controls, at the same time allowing DNIC to be...
fully expressed again. I suggest that DNIC expression status can serve as an early indicator of the bone cancer pain phenotype development. What is more, it can help to suit pain therapy, for example with noradrenaline-reuptake inhibitors in this cancer phase.

Due to the multiple connections between the LC, IPB area and other brain regions responsible for stress reactions (i.e. amygdala, hypothalamus, frontal cortices) it is likely that the clinical picture of pain is even more convoluted due to the often-associated mood disorders including anxiety and depression. This highlights the multidimensional character of pain, and further complicates therapy due to the comorbidities. In some cases, however, it could be possible to ‘kill two birds with one stone’ through the application of NRI drugs for depression and ongoing pain, where DNIC expression is abolished.
References


104.


Li, Y., Hickey, L., Perrins, R., Werlen, E., Patel, A.A., Hirschberg, S., Jones, M.W., Salinas, S.,
sympat
Li, J.
Manag.
conditioned pain modulation paradigm to assess endogenous inhibitory pain pathways. Pain Res.
4933
lateral parabrachial activation in the male rat. Pain
Med.
Lane, N.E., Schnitzer, T.J., Birbara, C.A., Mokhtarani, M., Shelton, D.L., Smith, M.D., and Brown,
conditioning stimulation in patients with painful osteoarthritis before, but not following, surgical
Research The Origin of Brainstem Noradrenergic and Serotonergeric Projections to the Spinal Cord
Dorsal Horn in the Rat The Origin of Brainstem Noradrenergic and Serotonergeric Projections to the
Lane, N.E., Schnitzer, T.J., Birbara, C.A., Mokhtarani, M., Shelton, D.L., Smith, M.D., and Brown,
Med. 363, 1521–1531.
receptor-expressing spinoparabrachial neurons trigger diffuse noxious inhibitory controls through
conditioned pain modulation paradigm to assess endogenous inhibitory pain pathways. Pain Res.
Manag. 17, 98–102.
sympathetic sprouting, and glial activation are attenuated by local injection of corticosteroid near
Li, Y., Hickey, L., Perrins, R., Werlen, E., Patel, A.A., Hirschberg, S., Jones, M.W., Salinas, S.,

330.
Kaan, T.K.Y., Yip, P.K., Patel, S., Davies, M., Marchand, F., Cockeye, D. a., Nunn, P. a.,
P2X2/3 receptors attenuates bone cancer pain behaviour in rats. Brain 133, 2549–2564.
Katz, N., Borenstein, D.G., Birbara, C., Bramson, C., Nemeth, M.A., Smith, M.D., and Brown,
Kayser, V., Lattrémolière, A., Hamon, M., and Bourgoîn, S. (2011). N-methyl-D-aspartate receptor-
mediated modulations of the anti-allodynic effects of 5-HT1B/1D receptor stimulation in a rat
responses of spinal dorsal horn neurons to heat and cold stimuli following mild freeze injury to
Kleggetveit, I.P., and Jorum, E. (2010). Large and small fiber dysfunction in peripheral nerve
injuries with or without spontaneous pain. J. Pain 11, 1305–1310.
(2013). A sensory-labeled line for cold: TRPM8-expressing sensory neurons define the cellular
Koewler, N.J., Freeman, K.T., Buus, R.J., Herrera, M.B., Jimenez-Andrade, J.M., Ghilardi, J.R.,
antibody raised against nerve growth factor on skeletal pain and bone healing after fracture of the
C57BL/6J mouse femur. J. Bone Miner. Res. 22, 1732–1742.
Koltzenburg, M. (1999). Neutralization of endogenous NGF prevents the sensitization of
conditioning stimulation in patients with painful osteoarthritis before, but not following, surgical
Research The Origin of Brainstem Noradrenergic and Serotonergeric Projections to the Spinal Cord
Dorsal Horn in the Rat The Origin of Brainstem Noradrenergic and Serotonergeric Projections to the
neurofibromatosis. Cancer Res. 50, 5396–5402.


Appendix 1 (related to Chapter 3)

Figure S1.1. Related to Figure 3.1. Many deep body CGRP afferents express little or no Advillin. Representative images of lumbar 3 (L3) DRG expressing GCaMP immunostained for CGRP (A), IB4 (B), and Advillin (Avil) (C) in Fast Blue (FB) traced tibial afferents. Arrows indicate FB neurons. Scale bars, 100 µm. Representative images of L3 DRG expressing immunostained for TubulinβIII and Advillin (Avil) in Fast Blue (FB) traced tibial afferents (D). Arrows indicate FB neurons. Scale bars, 100 µm. Example of PACT-cleared L3 DRG from Avil/eGFP mice (green), immunolabelled for CGRP (magenta). Scale bars, 200 µm (E). See also Movie 1, Movie 2.
Figure S1.2. Related to Figure 3.1. Many deep body CGRP afferents express little or no Advillin. Single cell RNAseq data from Ernfors lab ([Usoskin et al., 2014]; GSE59739) analysed for the presence of selected transcripts: Advillin (Avil), CGRPα (Calca), CGRPβ (Calcb), Substance P (Tac1) and TrkA (Ntrk1) in the sensory neurons. Cells with zero values for Tubb3 and Actb were excluded from analysis (A). Single cell RNAseq data from Linnarsson lab database (Zeisel et al. 2018; mousebrain.org, accessed on 01/11/2018), representing differential expression of Advillin (Avil), CGRPα (Calca), CGRPβ (Calcb), Substance P (Tac1) and TrkA (Ntrk1) in the sensory neurons (B).

B

<table>
<thead>
<tr>
<th>Index</th>
<th>Name</th>
<th>Description</th>
<th>Avil</th>
<th>Calca</th>
<th>Calcb</th>
<th>Tac1</th>
<th>Ntrk1</th>
</tr>
</thead>
<tbody>
<tr>
<td>201</td>
<td>PSPEP5 Peptidergic (PEP1.2), DRG</td>
<td>0.27</td>
<td>11.30</td>
<td>2.40</td>
<td>10.80</td>
<td>1.53</td>
<td></td>
</tr>
<tr>
<td>199</td>
<td>PSPEP7 Peptidergic (TrpM8), DRG</td>
<td>0.36</td>
<td>0.32</td>
<td>0.55</td>
<td>6.99</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>PSPEP6 Peptidergic (TrpM8), DRG</td>
<td>0.59</td>
<td>4.04</td>
<td>2.11</td>
<td>28.40</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>203</td>
<td>PSPEP4 Peptidergic (PEP1.1), DRG</td>
<td>0.81</td>
<td>37.30</td>
<td>2.97</td>
<td>15.10</td>
<td>3.04</td>
<td></td>
</tr>
<tr>
<td>204</td>
<td>PSPEP3 Peptidergic (PEP1.1.4), DRG</td>
<td>1.27</td>
<td>52.30</td>
<td>6.02</td>
<td>29.00</td>
<td>5.58</td>
<td></td>
</tr>
<tr>
<td>202</td>
<td>PSPEP2 Peptidergic (PEP1.3), DRG</td>
<td>1.51</td>
<td>43.40</td>
<td>6.19</td>
<td>8.24</td>
<td>5.05</td>
<td></td>
</tr>
<tr>
<td>198</td>
<td>PSPEP8 Peptidergic (TrpM8), DRG</td>
<td>1.61</td>
<td>0.39</td>
<td>2.68</td>
<td>7.22</td>
<td>1.22</td>
<td></td>
</tr>
<tr>
<td>209</td>
<td>PSNP1 Non-peptidergic (TH), DRG</td>
<td>2.29</td>
<td>0.17</td>
<td>2.73</td>
<td>0.59</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>207</td>
<td>PSNF2 Neurofilament (NF4/5), DRG</td>
<td>2.72</td>
<td>0.07</td>
<td>0.63</td>
<td>0.59</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>205</td>
<td>PSPEP1 Peptidergicv (PEP2), DRG</td>
<td>3.15</td>
<td>61.68</td>
<td>11.00</td>
<td>3.56</td>
<td>8.24</td>
<td></td>
</tr>
<tr>
<td>206</td>
<td>PSNF3 Neurofilament (NF2/3), DRG</td>
<td>3.29</td>
<td>0.64</td>
<td>0.74</td>
<td>0.00</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>212</td>
<td>PSNP4 Non-peptidergic (NP2.1), DRG</td>
<td>3.42</td>
<td>11.10</td>
<td>21.40</td>
<td>0.51</td>
<td>4.22</td>
<td></td>
</tr>
<tr>
<td>213</td>
<td>PSNP5 Non-peptidergic (NP2.2), DRG</td>
<td>3.45</td>
<td>34.50</td>
<td>7.15</td>
<td>0.36</td>
<td>2.99</td>
<td></td>
</tr>
<tr>
<td>208</td>
<td>PSNF1 Neurofilament (NF1), DRG</td>
<td>3.52</td>
<td>0.07</td>
<td>0.56 &lt;0.0256</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>211</td>
<td>PSNP3 Non-peptidergic (NP1.2), DRG</td>
<td>3.63</td>
<td>3.23</td>
<td>20.90</td>
<td>1.69</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>210</td>
<td>PSNP2 Non-peptidergic (NP1.1), DRG</td>
<td>3.85</td>
<td>6.38</td>
<td>17.80</td>
<td>1.54</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>214</td>
<td>PSNP6 Non-peptidergic (NP3), DRG</td>
<td>5.74</td>
<td>0.74</td>
<td>0.38</td>
<td>4.92</td>
<td>1.90</td>
<td></td>
</tr>
</tbody>
</table>

Values are trinarization scores generated using Bayesian beta-binomial model to determine if gene is expressed, not expressed, or indeterminate (see Zeisel A. et al. 2018 for details). The Linnarsson lab recommend 0.95 as a cut-off below which genes can no longer be confidently called to be expressed in a given cluster.
Figure S1.3. Related to Figure 3.2. Cancer progression affects bone innervation. Example micro-computer tomography reconstructions of rat tibiae. Figure explaining finding reference point for bone mineral density quantification in rat’s tibia (See methods) (A). Quantification of all ATF3+ afferents in each L2-5 DRG analysed as a percentage of all tibial cavity neurons traced with FB. Early (E, day 7/8 post cancer cells implantation) and late (L, day 14/15 post cancer cells implantation) stage groups. Each dot represents an average count from 10-20 10 µm sections from separate animals (n = 3-6). Data represent the mean ± SEM (B). Quantification of all ATF3+ afferents in each L2-5 DRG analysed as a percentage of all neurons therein (TubulinβIII) excluding FB+. Data represent the mean ± SEM (C). Body mass gain in rats after cancer implantation to tibia. Within a timepoint, each dot represents a single animal (n = 13-20 per group). Data represent the mean ± SEM. Kruskal-Wallis H for independent samples (Cancer vs. Sham): day 0: χ²(1) = 0.188, p = 0.665, day 2: χ²(1) = 1.503, p = 0.220, day 7: χ²(1) = 2.945, p = 0.086, day 14: χ²(1) = 1.087, p=0.297. (D). See also Movie 3.
Figure S1.4. Related to Figure 3.3. DRG afferents encode mechanical pressure stimuli in a graded fashion. Percentage of responders during calf compression in Sham and Cancer animals. Pressure in the neonatal cuff overlaying calf was increased in 50 mmHg steps every 10 s (see methods for more). The denominator for the total number of cells was established at the end of the experiment as shown in Fig. 2C. Data represent the mean ± SEM (shaded areas) of n = 496 cells (sham) from 6 animals, and
n = 1748 cells from 10 animals (Cancer). RM-ANOVA: F1, 2242 = 4.664, p = 0.031 (vs. sham) (A). Percentage of responders during ankle compression in Sham and Cancer animals. Pressure in the neonatal cuff overlaying ankle joint was increased in 50 mmHg steps every 10 s (see methods for more). The denominator for the total number of cells was established at the end of the experiment as shown in Fig. 2C. Data represent the mean ± SEM (shaded areas) of n = 595 cells (sham) from 7 animals, and n = 1595 cells from 9 animals (Cancer). RM-ANOVA: F1, 2188 = 6.967, p = 0.008 (vs. sham) (B). Table representing numbers of responders after each stimulus in both analysed groups. Note that the compression of the tumour-bearing area ('knee') results in the highest number of responding cells (C). Left panels depict cell size distributions in sham (top) and CIBP (bottom) of all responders to different stimuli. Green lines highlight cell size separators used: <700 µm² (small cells), >700<1200 µm² (medium cells). Right panels show a summary count of responders to the increased knee compression within each cell size range. Sham (top), CIBP (bottom). Note that the number of small-diameter responders increase linearly with the pressure surge (D). Example of 5 individual fluorescence traces (5 selected ROIs) across the whole imaging session (E). See also Movie 4.
Figure S1.5. Related to Figure 3.4. Leg compression and position are differentially coded by DRG sensory neurons. A pipeline of the largely unsupervised Markov Clustering Analysis (MCA) of primary afferents responses. See the full description in the Material and Methods section (A). Principal component analysis (PCA) was used to compare neuronal responses in pre-defined clusters. 363 knee responders fluorescence values after MCA clustering in (A) each with a cluster’s tag were used for PCA. Note that only the two most distinctive clusters (‘low’ vs. ‘high’) could be revealed with this approach (blue and yellow ellipses have opposite directions of the eigenvectors). The longitudinal nature of the gathered data (pressure ramp) requires advanced clustering (B). Eigenvectors in the performed PCA showing the directions of the variables (C). Percentage of the explained variability by each principal component after the PCA performed on the fluorescence intensity values of the knee responders (D). See also Movie 4.

A. Calf compression

B. Ankle compression

C. Knee compression

D. Calf compression

E. Ankle compression

F. Movement

G. Feeding

H. Pulling

I. Pushing

J. Constancy

* Pearson coefficient, R=0.9
** p=1.8, Pre=1.8, Scheme=3, Min=5/CL and removal of ‘no class’
Figure S1.6. Related to Figure 3.5. Leg compression and position are differentially coded by DRG sensory neurons. Normalised fluorescence intensities of all responding neuronal cell bodies from L3 and L4 DRG to calf compression. Pressure was increased in 50 mmHg increments every 10 s. Data represent the mean ± SEM (shaded areas). N = 62 cells (sham) from 6 animals, and n = 388 cells from 10 animals (Cancer). RM-ANOVA: F1, 448 = 3.334, p = 0.069 (vs. sham) (A). Normalised fluorescence intensities of all responding neuronal cell bodies from L3 and L4 DRG to ankle compression. Pressure was increased in 50 mmHg increments every 10 s. Data represent the mean ± SEM (shaded areas). N = 52 cells (sham) from 7 animals, and n = 230 cells from 9 animals (Cancer). RM-ANOVA: F1, 280 = 0.005, p = 0.942 (vs. sham) (B).

‘Low’ cluster of neuronal responses to the knee compression revealed by Markov Cluster Analysis with respect to the treatment group. Data represent the mean ± SEM (shaded areas). N = 16 cells (sham) from 3 animals, and n = 40 cells from 10 animals (Cancer). RM-ANOVA: F1, 54 = 0.189, p = 0.665 (vs. sham) (C). ‘Mid’ cluster of neuronal responses to the knee compression revealed by Markov Cluster Analysis with respect to the treatment group. Data represent the mean ± SEM (shaded areas). N = 5 cells (sham) from 4 animals, and n = 77 cells from 10 animals (Cancer). RM-ANOVA: F1, 77 = 0.303, p = 0.581 (vs. sham) (D). ‘High’ cluster of neuronal responses to the knee compression revealed by Markov Cluster Analysis with respect to the treatment group. Data represent the mean ± SEM (shaded areas). N = 11 cells (sham) from 2 animals, and n = 77 cells from 10 animals (Cancer). RM-ANOVA: F1, 86 = 0.089, p = 0.766 (vs. sham) (E). ‘Ramp’ cluster of neuronal responses to the knee compression revealed by Markov Cluster Analysis with respect to the treatment group. Data represent the mean ± SEM (shaded areas). N = 27 cells (sham) from 3 animals, and n = 113 cells from 10 animals (Cancer). RM-ANOVA: F1, 138 = 0.003, p = 0.959 (vs. sham) (F). Summary table of all responders after each step of Markov Cluster Analysis (MCA) performed on with respect to different stimulus (See Fig. S3A). CL reflects number of clusters present at each step of the analysis before final manual merging (G). ‘Pull’ cluster of neuronal responses to the leg movement along the body axis revealed by Markov Cluster Analysis with respect to the treatment group. Data represent the mean ± SEM (shaded areas). N = 17 cells (sham) from 5 animals, and n = 102 cells from 7 animals (Cancer). RM-ANOVA: F1, 117 = 2.278, p = 0.134 (vs. sham) (H). ‘Push’ cluster of neuronal responses to the leg movement along the body axis revealed by Markov Cluster Analysis with respect to the treatment group. Data represent the mean ± SEM (shaded areas). N = 4 cells (sham) from 3 animals, and n = 33 cells from 6 animals (Cancer). RM-ANOVA: F1, 35 = 0.001, p = 0.970 (vs. sham) (I). ‘Const’ cluster of neuronal responses to the leg movement along the body axis revealed by Markov Cluster Analysis with respect to the treatment group. Data represent the mean ± SEM (shaded areas). N = 2 cells (sham) from 2 animals, and n = 25 cells from 5 animals (Cancer). Statistical comparison not performed due to the low cell numbers in sham group (J).
Figure S1.7. Related to Figure 3.5. Intra- and peri-tibial afferent function in health and bone cancer. Representative images of Fast Blue (FB) traced tibial afferents in L3 DRG collected after the GCaMP experiments. Sliced DRG were immunostained for Piezo2 (A) and TrkA (B). Representative images of AAVrg/tdTomato (tdTomato) traced muscle and periosteum (MP) afferents in L3 DRG collected after the GCaMP experiments. Sliced DRG were immunostained for Piezo2 (C) and TrkA (D). Scale bars: 50 µm.
Figure S2.1. Related to Figure 4.3. Tonic descending noradrenergic controls are altered in our rodent model of CIBP. Following spinally applied atipamezole (10 µg) recordings were made of deep dorsal horn lamina V/VI WDR neuronal responses to punctate mechanical (A, D, G, J), dynamic brushing (B, E, H, K) and electrical (C, F, I, L) stimulation of the receptive field in early (day 7-8, top panels) and late (day 14-16, bottom panels) CIBP stage and corresponding sham-operated rats. PD-post discharge. All the data represent the mean ± SEM from sham early (n = 4), CIBP early (n = 6), sham late (n = 11) and CIBP late (n = 9). Each dot represents one animal. RM-ANOVA with Bonferroni post-hoc: *P < 0.05 vs. baseline (for punctate mechanical stimulation) and paired t-test: *P < 0.05 vs. baseline (for brush and electrical stimulation).
Figure S2.2. Related to Figure 4.4. Inhibition of mechanosensation by spinal $\alpha_2$-ARs block depends on a functioning opioid system in the late stage CIBP rats. Following spinally applied naloxone (5 µg) recordings were made of deep dorsal horn lamina V/VI WDR neuronal responses to punctate mechanical (A, D), dynamic brushing (B, E) and electrical (C, F) stimulation of the receptive field in late (day 14-16) CIBP stage and corresponding sham-operated rats. All the data represent the mean ± SEM from sham (naloxone: n = 5), CIBP (naloxone: n = 6). Each dot represents one animal. RM-ANOVA with Bonferroni post-hoc.
Appendix 3 (related to Chapter 6)

Figure S3.1. Related to Figure 6.3. Tonic descending noradrenergic controls are altered in CIBP. Following spinally applied atipamezole (10 µg) recordings were made of deep dorsal horn lamina V/VI WDR neuronal responses to non-noxious (acetone) and noxious (ethyl chloride) cold (A, C, E, G) and heat (B, D, F) stimulation of the receptive field in early (day 7-8, top panels) and late (day 14-16, bottom panels) CIBP stage and corresponding sham-operated rats. All the data represent the mean ± SEM from sham early (both cold and heat: n = 4), CIBP early (cold: n = 4, heat n = 6), sham late (both
cold and heat: n = 11) and CIBP late (cold: n = 4, heat: n = 9). Each dot represents one animal. RM-ANOVA with Bonferroni post-hoc.

Figure S3.2. Related to Figure 6.4. A spinal opioidergic system modulates tonic noradrenergic actions in the late stage CIBP rats. Following spinally applied naloxone (5 µg) recordings were made of deep dorsal horn lamina V/VI WDR neuronal responses to non-noxious (acetone) and noxious (ethyl chloride) cold (A, C) and heat (B, D) stimulation of the receptive field in late (day 14-16) CIBP stage and corresponding sham-operated rats. All the data represent the mean ± SEM from sham (cold n = 5, heat: n = 4), CIBP (both cold and heat: n = 7). RM-ANOVA (heat) and paired t-test (cold).

Appendix 4 – on the CD (related to Chapter 3)

Movie 1. PACT-cleared DRG visualisation. Avil-GFP-green, CGRP-red.
Movie 3. Micro-CT of cancer-bearing and sham-control tibiae. Top panel-side view, bottom panel-orthogonal projections. From left: Sham, CIBP Early, CIBP Late.
Movie 4. Example recordings of GCaMP6s fluorescence expressed in DRG L3, upon pressure cuff stimulation to the knee in sham (top) and cancer bearing (bottom) rats. Intensity of responses is colour-coded (yellow=intense).