Targeting and driving somatosensory neurons

FABIO FREITAG
Abstract

Pain and itch are two distinct sensations, but the fundamental question of how our nervous system distinguishes the processing and encoding of their related information is still far to be clearly delineated. At the spinal cord level, evidences have pointed out specific groups of neurons expressing the gastrin releasing peptide (Grp) and its receptor (Grpr) as responsible for carrying specifically itch-related information. Such important findings suggest a labeled line for itch and hypothesize the existence of separate pathways transmitting different sensory modalities already at this stage. Aiming at digging further on the pain/itch dualism, the present thesis focused first in addressing the GRPR-expressing dorsal horn interneurons and its roles in itch transmission. In the paper I, we observed that this population is composed mainly by excitatory interneurons, transmits itch through glutamate, and is at least partly downstream to the natriuretic peptide b (NPPB) signaling. Interestingly, increasing amount of behavior evidences have suggested that itch-related information is under local inhibition in the dorsal horn, since decrease of the local inhibitory tone by the peptide somatostatin is able to potentiate itch sensation in mice. In the paper II we complement these findings by showing in vitro that the itch-related GRPR-expressing dorsal horn neurons are under local tonic and phasic inhibition, besides being partly activated by somatostatin, corroborating that this population is indeed part of the disinhibition-induced itch circuitry. In order to confirm the itch-specific phenotype related to GRPR-expressing dorsal horn neurons and extend this theory to the rodent orofacial area, in the paper III we showed a new method developed to target and manipulate the orofacial-related trigeminal neurons. By using this method, we unexpectedly observed a functional switch in the GRPR population, from itch-related in the spinal cord to pain-related in the trigeminal nucleus caudalis, suggesting a labeled line of orofacial pain in this brainstem nucleus. As in the trigeminal nuclei, neuronal circuitry formed by defined cell types transmitting pain- and itch-related information has not been addressed yet in the somatosensory cortex. In the paper IV, we offer a mouse genetic tool that enables the target of barrel field spiny stellate cells, opening for more detailed knowledge of cortical circuitry encoding somatosensory information. In summary, the present thesis brings both complementary findings and new intriguing insights on how our nervous system transmits somatosensory stimuli from different modalities, paving basic knowledge on the mechanisms that build pain and itch as distinct percepts.

Keywords: Pain, Itch, GRPR interneurons, dorsal horn, trigeminal nucleus caudalis, Barrel field

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To all men and women that left home for a better life
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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### Abbreviations

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<th>Abbreviation</th>
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<tbody>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
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<td>DRG</td>
<td>Dorsal root ganglia</td>
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<tr>
<td>GRP</td>
<td>Gastrin releasing peptide</td>
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<tr>
<td>GRPR</td>
<td>Gastrin releasing peptide receptor</td>
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<tr>
<td>H1R</td>
<td>Histamine receptor 1</td>
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<tr>
<td>IB4</td>
<td>Isolectin B4</td>
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<tr>
<td>Lpb</td>
<td>Lateral parabrachial nucleus</td>
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<tr>
<td>MrgprA3</td>
<td>Mas-related G-protein coupled receptor member A3</td>
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<tr>
<td>NPPB</td>
<td>Natriuretic polypeptide B</td>
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<tr>
<td>NPRA</td>
<td>Natriuretic peptide receptor type A</td>
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<td>NPY</td>
<td>Neuropeptide Y</td>
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<td>NK1</td>
<td>Neurokinin 1</td>
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<tr>
<td>SpVc</td>
<td>Trigeminal nucleus caudalis</td>
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<tr>
<td>S1</td>
<td>Primary somatosensory cortex</td>
</tr>
<tr>
<td>TRPA1</td>
<td>Transient receptor potential cation channel subfamily A member 1</td>
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<tr>
<td>TRPV1</td>
<td>Transient receptor potential cation channel subfamily V member 1</td>
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<tr>
<td>Vglut2</td>
<td>Vesicular glutamate transporter 2</td>
</tr>
<tr>
<td>VPL</td>
<td>Ventroposterior lateral thalamic nucleus</td>
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<tr>
<td>VPM</td>
<td>Ventroposterior medial thalamic nucleus</td>
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Defined early, in the 17th century, as an “unpleasant sensation that elicits the desire or reflex to scratch” (Ikoma et al., 2006), itch is part of our innate defense system to avoid the presence of parasites, but can also be clinically present in patients suffering from its chronic condition (e.g. in atopic dermatitis and psoriasis), affecting drastically their quality of life.

Acutely, itch can be induced by different pruritogenic agents. Histamine, an endogenous compound discovered in the beginning of the 19th century, was early reported as a potent vasodilator (Thorpe, 1928) and an important substance involved in allergic reactions (Katz and Cohen, 1941). However, the same compound was later also reported as an agent capable of inducing a pure sensation of itch (Heyer et al., 1989; Simone et al., 1987), acting on the histamine 1 receptor (H1R) located on the nerve endings of peripheral primary afferent terminals (Davies and Greaves, 1980). Besides through histamine, itch sensation can also be chemically induced by other compounds, such as the active agent mucunain present in the spicules of the cowhage bean (Shelley and Arthur, 1955), and the antimalarial drug chloroquine (Ajayi et al., 1989).

Closely related to itch, pain has been defined by the International Association for the Study of Pain (IASP) as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (1979). In its acute forms, potential harmful stimulus can arise from a broad modality spectrum, such as mechanically (e.g. pinch), chemically (e.g. capsaicin and mustard oil), and by noxious heat or cold temperatures.

Although perceived as distinct sensations, itch and pain share common anatomical features and the understanding of how our nervous system processes and encodes these different modalities have been a matter of scientific debate. In the next sections, I will focus on each step of the somatosensory neuronal information flow, from the periphery to the cortex, and emphasize the functional distinctions that build pain and itch as two different percepts.
Primary afferents transmission

Slowly conducting unmyelinated mechanically-insensitive C-fibers transmit itch information induced peripherally by histamine (histaminergic itch) (Schmelz et al., 1997) while mechanically-sensitive C-fibers conduct non-histaminergic itch information (e.g. cowhage) (Namer et al., 2008). Furthermore, since peripheral histamine-sensitive C-fibers can respond also to capsaicin (the active component of chili peppers, which is inducing a burning sensation) while cowhage-sensitive fibers can respond also to noxious mechanical and thermal stimuli (Namer et al., 2008), both classes of fibers can further be classified as nociceptors. Finally, nociceptive information can also be transmitted by other groups of myelinated fibers, such as Aβ (Willer and Albe-Fessard, 1983) and Aδ (Willer et al., 1978) fibers.

Primary afferents have their cell bodies located in the dorsal root ganglia (DRG), which send another branch of fibers to the dorsal horn of the spinal cord. At the DRG level, neurons that give rise to unmyelinated afferents (C-fibers) are subdivided in two classes regarding the expression of peptides and fiber termination in the dorsal horn: peptidergic neurons such as substance P- and/or calcitonin gene-related peptide (CGRP)-expressing cells send afferents that arborizes mainly in lamina I and outer part of II, while non-peptidergic primary afferents, marked by the isolectin IB4 and expressing mas-related G protein-coupled receptor MrgD, terminate in mid-lamina II (Todd, 2017).

Interestingly, Liu et al., 2009 identified a subpopulation of small diameter Mrgprs-expressing DRG neurons functioning as detectors for chloroquine and specifically mediating non-histaminergic itch. Using calcium imaging in vitro to identify responses in transfected cells to specific pruritogenic agents, the authors found that within the family of Mrgpr receptors only MrgprA3 was able to show strong responses to chloroquine but not to histamine (Liu et al., 2009). Furthermore, calcium imaging data from DRG cultured neurons revealed that a substantial proportion of histamine-responsive neurons were also sensitive to capsaicin, and all chloroquine-sensitive neurons also responded to both histamine and capsaicin (Liu et al., 2009). Thus, although specific receptors sensitive to different pruritogenic agents can be identified (e.g. H1R and MrgprA3) in primary afferent neurons, these cells can also co-express different nociceptive-related receptors (Figure 1).
Based on staining (i.e. immunohistochemistry and *in situ* hybridization) and *in vitro* functional studies (i.e. calcium imaging and patch clamp), an estimation of the proportion of DRG neurons sensitive to different agents can be done. Accordingly, histamine-sensitive neurons represent around 15% (Han et al., 2006), while around 5% of the DRG neurons are chloroquine-sensitive and express MrgprA3 (Liu et al., 2009). Thus, chloroquine-sensitive neurons form a subset of histamine-responsive neurons and both are part of a bigger population of neurons, the capsaicin-sensitive TRPV1 neurons. Supporting this, pruritogenic agents failed to induce itch in mice lacking TRPV1-expressing neurons (Mishra and Hoon, 2013; Rogoz et al., 2014), where thermosensation was also severely impaired (Mishra et al., 2011). Moreover, natriuretic polypeptide b (NPPB) was reported as being expressed in a subset of DRG neurons, and co-expressed with TRPV1 and MrgprA3 (Mishra and Hoon, 2013). Nppb/-/- mice also showed severe decrease in itch sensation induced by different pruritogenic agents, as histamine and chloroquine, but thermal and mechanical pain as touch sensations remained in normal levels (Mishra and Hoon, 2013). Thus, a specific neuronal population detecting pruritogenic agents could also be identified as a restricted population releasing a specific peptide (NPPB) that mediates itch information to the spinal cord dorsal horn.

Interestingly, NPPB-expressing DRG neurons were recently shown to co-express the itch-related peptide somatostatin (Huang et al., 2018). However, in the orofacial-related trigeminal ganglia NPPB-expressing neurons form a population that is separate from MrgA3 neurons (Huang et al., 2018; Nguyen et al., 2017), suggesting the existence of NPPB and MrgA3 parallel afferent itch-related pathways transmitting orofacial information. Furthermore, facial
itch could be induced by specific stimulation of either MrgprA3 (Han et al., 2013) or NPPB/somatostatin (Huang et al., 2018) trigeminal afferent neurons, confirming their role in transmitting itch-related information.

Signal processing in the spinal cord

Primary afferents transmitting pain- and itch-related information from the periphery terminate and make their first synapses in the dorsal horn of the spinal cord. The primary afferent terminals are organized at this level based on axonal diameter and sensory modality (Figure 2). While nociceptive primary afferents (in this case unmyelinated thin C-fibers) terminate superficially in the dorsal horn (laminae I and II), deeper laminae (III and IV) receive mainly touch-related low threshold inputs from myelinated thick Aβ mechanoreceptors (Takazawa and MacDermott, 2010). Furthermore, primary afferents can connect to either projection neurons (lamina I and V) (Todd, 2002), which transmit the information further to the brain, or local interneurons that modulate and/or relay sensory signals (Bardoni et al., 2019; Pagani et al., 2019).

Figure 2. Spatial organization of primary afferent fibers from different classes innervating different layers of the spinal cord dorsal horn. Original from Basbaum et al., 2009.

Dorsal horn interneurons can be classified as inhibitory (releasing GABA and/or glycine) and excitatory (releasing glutamate). In the superficial laminae (I and II), inhibitory interneurons represent around 26% of the whole neuronal population, while this number increases to 38% in lamina III (Pol-
gár et al., 2013), suggesting a high proportion of excitatory neurons in this area. Besides using GABA, glycine and glutamate as neurochemical markers to classify dorsal horn interneurons, neuropeptides, their respective receptors and calcium-binding proteins have been extensively used (Todd, 2017) to identify subpopulation of neurons devoted to process and/or modulate specific somatosensory information.

Among neuropeptide-expressing interneurons, neuropeptide Y (NPY)-, galanin-, nitric oxide synthase (nNOS)- and parvalbumin-expressing cells can be classified as inhibitory interneurons (Rowan et al., 1993; Simmons et al., 1995; Todd, 2017), while neurokinin B (NKB)-, neurotensin (NT)-, somatostatin-, gastrin releasing peptide (GRP)-, gastrin releasing peptide receptor (GRPR)- and substance P-expressing neurons are thought form different classes of excitatory interneurons (Aresh et al., 2017; Gutierrez-Mecinas et al., 2014; Todd, 2017). Thus, besides the fast ionotropic action of glutamate, GABA and glycine, the dense presence of a great variety of neuropeptides and respective receptors in the dorsal horn neurons and primary afferent terminals suggests a high complex circuit processing inputs from the different afferent fibers and sensory sources. Membrane hyperpolarization can be observed when somatostatin and NPY bind to the somatostatin receptor subtype 2A (Kardon et al., 2014) and NPY Y1 (Miyakawa et al., 2005) receptor, respectively. On the other hand, GRP and substance P cause depolarization when binding respectively to GRPR (Aresh et al., 2017) and NK1 (Lu and Perl, 2005) receptor. These examples illustrate how different peptides can have different effects when activating their receptors.

To understand the specific somatosensory function of different subpopulation of neurons, defined by specific molecular markers, behavior and in vitro research have been using different approaches to target and manipulate restricted groups of neurons. Congenital ablation (through knockout mouse lines), deletion through toxin injections (e.g. bombesin-saporin) and the more recent use of transgenic mouse lines through the use of the Cre/lox system have raised important conclusions regarding the functional importance of different neuronal populations and their position in the dorsal horn circuitry.

Dorsal horn signaling and the Grp/Grpr system

A subpopulation of DRG neurons transmitting specifically itch information can be identified by the expression of the peptide NPPB (Mishra and Hoon, 2013). This finding pointed out the importance of the release of the NPPB peptide by a group of primary afferent neurons involved in itch processing, which was confirmed in the same study by deletion of the NPPB receptor, NPRA, present in the lamina I of dorsal horn. Mice lacking the NPRA receptor also showed a significant decrease in scratching compared to control
animals when histamine and NPPB were administrated (Mishra and Hoon, 2013).

Before the NPPB/NPRA system was reported as part of an itch-specific labeled line, the gastrin releasing peptide (GRP) was suggested as the first candidate to carry itch information from primary afferents to the dorsal horn. Sun and Chen, 2007 first showed that the expression of the GRP receptor GRPR is restricted to the superficial lamina I and although GRPR mutant mice had similar responses as control animals to mechanical and thermal nociception, the knockout animals showed decreased scratch reactions to the pruritogenic 48/80 (compound acting to de-granulate mast cells with a consequent release of histamine, mast-cell specific proteases and other mast cell constituents) and the ligand GRP. The number of scratch episodes induced by 48/80 and chloroquine could also be decreased by a GRPR antagonist (Sun and Chen, 2007). A few years later the same group ablated GRPR neurons through intrathecal injection of the neurotoxin bombesin-saporin and also reported decreased effect of chloroquine, histamine and serotonin in scratch behavior with no changes in mechanical, thermal and chemical induced pain (Sun et al., 2009). Finally, contrary to what was proposed by Mishra and Hoon, 2013 (in this study GRP was shown to be expressed in the dorsal horn but not DRG) GRP was reported to be expressed in small unmyelinated DRG neurons where 80% of them co-expressed TRPV1 (Sun and Chen, 2007) and also 93% of the chloroquine-sensitive MrgprA3 neurons co-expressed GRP (Liu et al., 2009).

NPPB/NPRA and GRP/GRPR systems are both suggested to be part of a dedicated labeled line of itch. However, their position in the itch neuronal circuitry is still a matter of debate. Mishra and Hoon, 2013 first showed anatomically and pharmacologically that the GRP/GRPR system functions downstream to NPPB/NPRA (NPPB peptide released by primary afferents activates dorsal horn NPRA neurons that would release GRP and finally activate GRPR neurons). One year later, Liu et al., 2014 contested this finding by first showing that both GRP and NPRA are expressed in DRG neurons and showing that NPPB induced a scratch behavior that could not be decreased by blocking GRP-GRPR signaling. Recently, through the use of a transgenic mouse line expressing Cre under the GRP promoter bred with the tdTomato reporter, and another line expressing the green fluorescent protein (GFP) under the GRP promoter, Sun et al., 2017 showed, in agreement with Mishra and Hoon, 2013, GRP promoter activity in the superficial dorsal horn, but not in the DRG. These neurons were heavily innervated by and monosynaptically connected to MrgprA3 primary afferents and showed a strong co-expression with the vesicular glutamate transporter 2 (Vglut2), classifying them as excitatory interneurons. Unexpectedly however, GRP dorsal horn neurons responded in a more intense way to the nociceptive agent capsaicin compared to chloroquine and histamine. Additionally, when this population of neurons was specifically activated in vivo, both pain- and
itch-related behaviors were observed (Sun et al., 2017). Thus, the first conclusions about the GRP/GRPR system being devoted to carry only itch-related information and its precise position in the labeled line of itch is still far from a scientific consensus. In **study I**, we give new insights on the functional role of dorsal horn GRPR interneurons in itch transmission and its position in the spinal cord itch-related circuitry. Proposed models of how the itch-related information flows from the periphery to and in the spinal dorsal horn can be seen in the figure 3a.

*Figure 3. Itch-related information pathway models, from the periphery to and in the spinal cord dorsal horn. (a) Excitatory pathway models. (b) Inhibitory dorsal horn circuit model. Original from Bautista et al., 2014.*
As stated previously, pain information share anatomical similarities with itch transmission (i.e. both sensations can be transmitted by the same group of primary afferents and terminate in the same dorsal horn laminae). One of the standard methods to treat pain is still through the use of opioids and one of its most known side effects is the induction of itch. In connection with opioid-induced itch side effects, the interaction of GRPR neurons with opioids has also been investigated. Accordingly, the morphine receptor MOR1D was shown to be co-expressed with GRPR and morphine-induced scratching was strongly impaired in GRPR knockout mice (Liu et al., 2011). The authors further suggest a heterodimerization between these two receptors and a possible unidirectional activation of GRPR by MOR1D. On the other hand, dynorphin, another opioid that instead binds preferentially to the kappa opioid receptor (KOR), was later suggested as an important itch inhibitor. In a set of pharmacology experiments, Kardon et al., 2014 showed that scratch responses to chloroquine, histamine and serotonin were significantly decreased when the animals were pre-treated with the KOR agonists U-50,488 and nalfurafine. No differences were observed for capsaicin, indicating an effect of dynorphin specific for itch. Interestingly, nalfurafine was also able to strongly decrease scratch responses induced by GRP, raising a possible direct interaction between the dynorphin/KOR effects in the GRP/GRPR system.

Adding to the dorsal horn local inhibitory modulation on itch transmission, mutant mice lacking inhibitory interneurons marked by the transcription factor Bhlhb5 showed spontaneous scratch behavior and strong heightened responses to pruritic stimuli (Ross et al., 2010). Moreover, the inhibitory action of the somatostatin sst2A receptor, which is present in the dorsal horn mainly in inhibitory interneurons, agonist octreotide induced spontaneous scratch behavior (Kardon et al., 2014), possibly through a disinhibition process. Also, decrease in itch responses to histamine and chloroquine were observed when dorsal horn glycinergic neurons were activated (Foster et al., 2015). These findings could suggest that itch sensation is under a tonic inhibition by local dorsal horn interneurons. Figure 3b summarizes a proposed model of local inhibition on itch-transmitting GRPR dorsal horn interneuron. Complementing these behavior findings, in study II we address with an in vitro approach the role of local dorsal horn inhibition controlling GRPR excitability.

Besides being directly contacted by local (Freitag et al., 2019; Liu et al., 2019) and brain descending inhibitory inputs (Liu et al., 2019), GRPR dorsal horn neurons receive also direct excitatory inputs from both primary afferents (Bardoni et al., 2019; Koga et al., 2011) and local interneurons (Pagani et al., 2019). Also, electrophysiological (Mu et al., 2017) and anatomical (Bardoni et al., 2019) evidences have suggested downstream projection neurons being directly connected to GRPR interneurons. Thus, although far from having a clear overall picture of how the itch-circuitry is organized in
the dorsal horn level, increasing amount of findings from molecular to behavior scale have enabled the drawing of an increasingly more detailed circuit not only transmitting but also locally modulating itch-related information in the spinal cord, with the GRP/GRPR system being a central and significant part of it.

Contrary to what it is observed for the spinal transmission of itch, it is not so evident that pain-related information processing follows the same logic, with a dedicated circuit transmitting nociceptive signals in the spinal cord level and further up to the brain. Interestingly, a recent study (Smith et al., 2019) suggested that calretinin-expressing dorsal horn neurons are mainly excitatory and when selectively activated, trigger a robust targeted behavior that is gradually inhibited and finally abolished by different doses of the analgesic morphine (Smith et al., 2019). This suggests the link of the calretinin-positive dorsal horn neurons to pain but not itch.

Pain- and itch-related projections to the brain

After being processed by local interneurons or possibly by direct connection from primary afferents, itch- or pain-related information reaches output projection neurons. If dorsal horn projection neurons process and transmit the different somatosensory modalities separately is still unclear. Histamine-sensitive spinothalamic tract (STT) neurons were reported in primates to also respond to capsaicin (Davidson et al., 2009), as did cowhage-sensitive neurons (Davidson et al., 2007). Furthermore, the same group of STT neurons responded specifically to either one of these two different pruritogens (Davidson et al., 2007), indicating a specificity between pruritogens. However, STT neurons recorded in cats could show specific activation to nociceptive (noxious heat and pinch), thermal (innocuous cool and warm stimuli) and histamine stimulation (Andrew and Craig, 2001), indicating a possible separate and dedicated output system to different stimulus modality.

Dorsal horn projection neurons send somatosensory information further to specific brain areas such as ventral posterolateral (VPL), ventral posteromedial (VPM) and posterior thalamic nuclei (Po), periaqueductal grey matter (PAG), lateral parabrachial area (LPb) and caudal ventrolateral medulla (CVLM) (Gauriau and Bernard, 2004; Todd, 2010). This ascending system can be divided into two parallel components: the discriminative aspects (spatial, temporal and intensity values) of somatosensory information, that is transmitted through the spinal-thalamus-cortex system, while the affective aspects (aversion and anxiety) are transmitted through the spinal-parabrachial-amygdala system (Figure 4).
Figure 4. Ascending somatosensory pathways transmit sensory-information in separate anatomical pathways serving distinct functions. Original from Basbaum et al., 2009.

The pain and itch spinal-related behavior problem

To delineate how the spinal cord processes different somatosensations, rodent behavioral experiments targeting the dorsal horn circuitry using transgenic models and intrathecal injections (Sukhtankar and Ko, 2013; Sun et al., 2017), in combination with intradermal injections either in the hind limb calf (Akiyama et al., 2014; LaMotte et al., 2011), paw (Tsuda et al., 1999) or in the nape (Aresh et al., 2017; Green et al., 2006), have been used. Operational problems arise from these strategies, since the nape can be reached only by scratching behavior, masking nociceptive reactions, and hind limb calf licking (nociceptive behavior) and biting (pruriceptive behavior) cannot be easily distinguished and thus scored, resulting in unclear behavior discrimination.

Clearly defined behavioral responses to a pruritogen, such as histamine, and an algogen, such as capsaicin, were reported when these substances were intradermal injected in the mice cheek (Shimada and LaMotte, 2008). Ac-
Accordingly, histamine elicited hind limb facial scratching behavior, while capsaicin triggered facial fore limb wiping (Shimada and LaMotte, 2008), establishing the cheek model as a valid behavior strategy to discriminate different somatosensations. Figure 5 illustrates different rodent behavioral models for pain and itch using stimuli that can induce these two different sensations in humans. Furthermore, facial fore limb wiping behavior was also reported when formalin (Chen et al., 2014) and capsaicin (Rossi et al., 2016) were intradermally injected in the mice whisker pad, and facial hind limb scratching was observed when trigeminal ganglia somatostatin primary afferent neurons were optogenetically stimulated (Huang et al., 2018), extending this approach to the whole mice facial region.

Figure 5. Pain and itch sensations in humans and rodents. (A) Histamine (blue) is reported to induce itch sensation in humans, while capsaicin (red) triggers a burning pain sensation. (B) Using the mouse cheek model, itch sensation can be inferred by hind limb scratching behavior in mice, while pain induces fore paw wiping targeted behavior. (C) Itch-related biting and pain-related licking are present in the calf model. Original from LaMotte et al., 2011.

Besides the cheek model, tests that target other orofacial areas have also been developed (Luccarini et al., 2006). Algogens, capsaicin and formalin
have been shown to induce a fore paw face-rubbing behavior when injected into the rodents vibrissa pad (Holanda Pinto et al., 2008; Quintans-Júnior et al., 2010; Siqueira et al., 2010) and the upper lip (Guimarães et al., 2012; Luccarini et al., 2006). The same behavior was also reported when nociception was induced by inflammatory reaction from complete Freund’s adjuvant (CFA) injected into the masseter muscle (Romero-Reyes et al., 2013). Thus, mice fore paw mediated wiping and face-rubbing constitute stable and reproducible behavioral models for nociception in the orofacial area.

Orofacial somatosensation and the trigeminal nucleus caudalis

Equivalent of the spinal cord dorsal horn in the brainstem, the trigeminal nuclei are the first relay stations receiving orofacial somatosensory information through afferent trigeminal ganglia projections. The trigeminal nucleus caudalis (SpVc) is the most caudal part of the trigeminal nuclei and lies in a transition zone between the medulla and the first spinal cord cervical segments. Interestingly, this trigeminocervical complex is spatially organized following a somatotopic arrangement, where its most rostral areas receive trigeminal afferent inputs that innervate the rodent rostral orofacial areas (e.g. oral and whisker pad areas), while its caudal parts, including the early cervical segments, are innervated by afferent fibers with peripheral terminals present in the most facial caudal parts, such as cheek and nape of the neck (Panneton et al., 2017).

In attempt to understand how the trigeminocervical complex transmits facial somatosensory information, extracellular recordings have identified unspecific trigeminal neurons that in its majority respond to both pruriceptive and nociceptive stimuli (Akiyama et al., 2010). Nociceptive-specific (Jansen and Giesler, 2015; Moser and Giesler, 2014), innocuous-sensitive nociceptive and nociceptive/pruriceptive trigeminothalamic (Moser and Giesler, 2013) and trigeminoparabrachial (Jansen and Giesler, 2015) tract neurons were also reported. Thus, although there is no empirical evidence for pruritogen-specific trigeminal neurons, a trigeminal nociceptive dedicated pathway could be naturally expected, since both trigeminothalamic and trigeminoparabrachial nociceptive-specific projection neurons were identified (Jansen and Giesler, 2015; Moser and Giesler, 2014).

As stated in the previous sections, considerable efforts and attention have been given to understand how the somatosensory information is transmitted and modulated in the spinal dorsal horn level. The use of transgenic mouse lines enabling the Cre/lox system strategy to mark and manipulate specific neuronal populations have been widely used by different research groups and important findings have significantly contributed to the elucidation of the dorsal horn functional organization. It is important to note that such sci-
Scientific advanced findings and efforts in dissect the somatosensory circuitry in its first relay stages, have been so far based mainly in the dorsal horn of the spinal cord.

Anatomical, pharmacological, and electrophysiological approaches have paved the trigeminal nuclei field and the understanding of how facial somatosensory information is functionally organized. However, deeper knowledge and details on its circuitry organization and functions drawn by the contributions of specific neuronal populations genetically marked has so far not been reached. Strongly driven by the facts that the facial somatosensation has such well-established and clear behavioral phenotypes to distinguish itch and pain sensations in rodents, and that the orofacial-related trigeminal functional circuitry knowledge is not as well-known and studied as the body-related spinal dorsal horn, I was promptly conquered by the idea of exploring this brainstem area.

As previously mentioned, the trigeminal nucleus caudalis is located in a transition zone between the medulla and the early cervical spinal cord. My initial idea was to dedicate and invest a great part of my PhD time in developing an optimal methodological approach to target and manipulate SpVc neurons specifically defined by genetic markers. After reaching the first successful results, I focused on the trigeminal GRP/GRPR system and its importance in transmitting orofacial itch and/or pain sensations. The results are represented in study III and the main conclusions bring important and new contributions to the orofacial pain field, besides offering a novel strategy to explore how the SpVc neurons are functionally organized and processing orofacial itch and pain.

Thalamus and somatosensory cortex

After being locally processed either in the spinal cord dorsal horn or in the trigeminal nuclei of the brainstem, local projection neurons transmit ascending somatosensory information to the brain. In its discriminative aspect (i.e. modality, intensity and location of the stimulus), this information reaches first the thalamic nuclei known as VPM, VPL or POm. The thalamic precise destination of spinal or trigeminal somatosensory afferents depends on the anatomical peripheral origin of the stimulus. Here, I will focus on the mouse whiskers/barrel field complex.

The rodent whiskers area is innervated by trigeminal peripheral fibers that transmit somatosensory information through the brainstem trigeminal nuclei. Different pathways can carry this information, depending on the brainstem origin, the thalamic relay station and cortical targeted layers (Feldmeyer, 2012). Accordingly, the lemniscal pathway reaches mainly the cortical layer IV barrel fields, with inputs from the thalamic VPM nucleus, while in the paralemniscal pathway the main cortical target is the layer Va, receiving
inputs from the POm thalamic area (Feldmeyer, 2012). Interestingly, such anatomical segregation is suggested to follow a functional distinction in these pathways, where lemniscal pathway showed to be specifically activated by whiskers movement in exploratory behavior and paralemniscal upon noxious stimulation through capsaicin treatment in the whiskers pad (Frangeul et al., 2014).

Furthermore, in vivo whole cell patch clamp recorded neurons in S1 II/III, IV and V cortical layers have located nociceptive specific neurons, with clear evoked responses to noxious pinch and mustard oil treatment in the whiskers pad area (Takeda et al., 2010), opposing the idea that nociceptive stimulation in the rodent whisker area would be transmitted specifically by the paralemniscal pathway and thus activating mainly the cortical layer Va neurons. In summary, although evidences have suggested the presence of nociceptive-specific S1 cortical neurons (Takeda et al., 2010), little is known regarding itch-transmission and its functional circuitry in this area. Thus, as in the trigeminal and somatosensory thalamic nuclei, pain- and itch-related functional circuitry have not been extensively studied and dissected in the S1 cortex compared to the spinal cord dorsal horn.

On the other hand, S1 cortical neurons have been described in its molecular, electrophysiological and morphological levels (Scala et al., 2019), and distributions in the different cortical layers (Narayanan et al., 2017). In the barrel field thalamo-recipient layer IV, local excitatory interneurons are composed by three morphologically defined cell types: spiny stellate cells, star pyramidal cells and pyramidal cells (Staiger et al., 2004). Although it is not possible to distinguish these defined cell types based on its spike profile responses (Schubert et al., 2003), spiny stellate cells have a clear lack of apical dendrites, while pyramidal layer IV neurons have longer processes, reaching the most superficial cortical layers (Scala et al., 2019). Thus, although intra-cortical connectivity studies have cleared basic knowledge on information flow in the barrel field cortex (Feldmeyer, 2012), no evidences have so far linked genetically, molecularly and morphologically defined cell types with functional circuitry processing pain- and itch-related information in the rodent S1 cortex.

In an attempt to target and possibly manipulate a genetically and morphologically defined barrel field layer IV cortical population, in study IV we offer a mouse transgenic tool to study spiny stellate cells and their importance in transmitting somatosensory information in the S1 cortex, opening for new insights in how this circuit is functionally organized and encoding different percepts.
Results and Significance

Study I

The main goals of this study were to report the construction of a Grpr-Cre mouse line through the use of BAC cloning and investigate the major role of this neuronal population in the transmission of itch. Exon 1 of the Grpr gene was replaced by a DNA sequence coding for the Cre recombinase enzyme and the generated mouse line was further crossed with a tdTomato reporter line. With this strategy, only neurons expressing Cre recombinase could express the red fluorescent protein and Grpr-Cre neurons could then be identified. Single cells expressing Grpr-Cre (as indicated by the expression of Tomato) were collected using laser dissection and single cell PCR using Grpr-specific nested primers identified Grpr mRNA in 32% of the collected cells. Grpr mRNA was never identified in Grpr-Cre negative cells. When the Grpr agonist GRP was bath applied in whole cell patch clamped Tomato neurons, 43.3% of the neurons responded with induced spike responses and 17% of the remaining patched cells responded with subthreshold depolarization, whereas all the 9 AAV8/hSyn-DIO-mCherry-infected patched neurons responded (2 with induced spikes, 5 with subthreshold depolarization and 2 with hyperpolarization) to GRP, findings which collectively indicates that Grpr-Cre is active in GRPR/Grpr expressing cells. Anatomically, a majority of the Tomato-expressing cells in the spinal cord dorsal horn were located in lamina II, III and IV. Also, 67% of the Grpr mRNA positive neurons co-expressed Vglut2 mRNA, while only 11% of Tomato neurons co-localized with the vesicular inhibitory amino acid transporter (Viaat)-egfp, suggesting that Grpr-Cre comprises mainly excitatory neurons.

To investigate if dorsal horn Grpr-Cre neurons are formed only by local interneurons, the neuronal retrograde tracer Fluorogold was injected in the two main brain targets for dorsal horn projection neurons; ventral posterolateral/ventral posteromedial (VPL/VPM) thalamic nuclei and lateral parabrachial nucleus (LPb). From 203 retrogradely traced projection neurons, only 2 showed co-localization with Tomato, proving that Grpr-Cre neurons are predominantly interneurons.

Grpr-Cre mice were also bred with a Vglut2-lox line and thus VGLUT2-mediated glutamatergic signaling was removed from these cells. Vglut2-deficient mice showed decreased number of spontaneous scratches and also
less responses to histaminergic and non-histaminergic itch-inducing substances.

Finally, to test the hypothesis of GRPR neurons being downstream to the NPRA population, the NPRA agonist NPPB was bath applied in spinal cord slices of mice injected with the virus AA9/CAG-DIO-GCaMP6f-WPRE. Infected Grpr-Cre neurons expressed the calcium sensitive GCAMP6 and 23.1% of the imaged neurons responded to NPPB, suggesting that part of the GRPR population could be downstream to NPRA expressing neurons.

From these results we can conclude that Grpr-Cre neurons form a population of dorsal horn excitatory interneurons that signal itch using glutamate through the VGLUT2 transporter and that part of this population can be downstream to the NPPB/NPRA system.

Study II

Besides being transmitted in the dorsal horn by an excitatory pathway signaled by glutamate, NPPB and GRP, itch has also been shown to be triggered and potentiated by a decrease in the dorsal horn inhibitory tone (Foster et al., 2015; Ross et al., 2010). Having a central role in this effect, somatostatin is a peptide that has an inhibitory effect on local dorsal horn inhibitory interneurons (Kardon et al., 2014), and thus possibly releasing local excitatory interneurons from inhibition.

With the aim to complement the behavioral disinhibition-induced itch previous published findings, in this study we first confirmed that Grpr-Cre-expressing neurons are mainly excitatory and co-express excitatory markers such as TLX3 and EBF2. Bioinformatics analysis of single cell mRNA expression data revealed that this population expresses subunits for GABA, with the highest prevalence for \( \text{Gabara}3 \) (44.7%), and glycine, with \( \text{Glrb} \) having higher prevalence (78.6%), receptors. Furthermore, whole cell patch clamped Grpr-Cre;tdTomato dorsal horn neurons showed that this population receives both AMPA-mediated glutamatergic spontaneous inputs, and GABA/Glycine mediated spontaneous inhibitory inputs. Additionally, both glycine and GABA induced tonic inhibitory currents in the GRPR neurons, and the GABA and Glycine receptor antagonists strychnine and bicuculline increased GRPR excitability, revealed by the decrease in their Rheobase and increase in input resistance values.

Finally, GCaMP6-mediated calcium imaging showed an increase in spontaneous activity in the GRPR dorsal horn neurons in slice preparations compared to intact spinal cord, and somatostatin could induce activity in part of the GRPR imaged population.

In summary, in this study we provide in vitro evidences that the dorsal horn itch-related GRPR population is under local tonic and phasic inhibition and can be at least partly activated by the indirect disinhibition action of
somatostatin. Thus, we here corroborated that GRPR neurons is part of the disinhibition-induced itch mechanism.

Study III

To investigate the contribution of peripheral and central neuronal defined cell types and signaling in the transmission and modulation of pain- and itch-related information, researchers have focused mostly in the body-spinal cord system, with targeted behaviors to rodents paw, calf and nape of the neck. By using these strategies, the behavioral output cannot be easily distinguished between nociceptive or pruriceptive. Accordingly, rodents can only reach the nape of the neck by hind paw scratching, masking nociceptive reactions, and algogenic stimuli applied to the rodent paw or calf induces licking reactions, while pruritogenic stimulation triggers biting responses, behaviors that are not easily differentiated while scored.

To overcome this important interpretation problem, a mouse behavior “cheek model” was proposed (Shimada and LaMotte, 2008). When mice were injected with histamine in the cheek area, scratching reactions using the hind limb could be observed, while cheek capsaicin treatment induced fore limb wiping behavior. Thus, pain and itch targeted behaviors to the facial area showed to be more reliably distinguishable when compared to the body-spinal system.

In this study we developed a new method to target the orofacial-related brainstem trigeminal nucleus caudalis (SpVc). With the goal to confirm the role of the GRP/GRPR system in transmitting itch but not pain information, we first infused this area with the peptide GRP. As expected, triggered scratching behavior targeted to the cheek area could be observed. Interestingly however, mice also showed vigorous wiping behavior towards the whiskers pad area, indicating that GRP was inducing mix sensations of rostral facial pain and caudal facial itch.

We next used the same angled approach to inject Cre-dependent virus in Grpr-Cre mice and successfully marked SpVc Grpr-Cre neurons could be detected with GFP. The analysis revealed a lateral and superficial expression of this population in the nucleus. Furthermore, FISH analysis confirmed the expression of Grpr mRNA in 88% of Grpr-Cre marked neurons and co-expression with the excitatory marker Vglut2, defining this population as excitatory.

To confirm the functional role of this SpVc population, we performed optogenetic stimulation in Grpr-Cre neurons expressing the light-sensitive channel-rhodopsin (ChR2). Interestingly, light activation triggered wiping behavior targeted to the whiskers pad area with events often following each pulse of light, reproducing the whiskers pad targeted behavior induced by GRP infusion.
Intrigued by the finding that SpVc GRPR neurons are pain-but not itch-related, we next activated this population by using the Cre-dependent excitatory DREADD system, delivered by virus injections. Here we showed again that SpVc GRPR neurons induced vigorous wiping behavior targeted to the whisker pad area, while activation of GRPR neurons from the C2-C4 cervical segments induced robust scratching behavior targeted to the cheek area. Finally, facial wiping behavior induced by selective activation of SpVc GRPR neurons was morphine-sensitive, confirming the nociceptive nature of this behavior phenotype.

In conclusion, in this study a new method was implemented to target and manipulate trigeminal brainstem neurons. Using this strategy, SpVc GRPR neurons showed to be nociceptive-related, while specific activation of the same population in the cervical segments confirmed its role in being itch-related in the spinal level. We here showed for the first time a functional switch in a somatosensory-related defined neuronal population, giving further strength to a potential labeled line of pain in the orofacial-related trigeminal nucleus caudalis.

Study IV

As previously mentioned, the Cre-lox system developed to target specific population of neurons is now widely used in neuroscience research, and many mouse lines expressing the Cre recombinase enzyme under specific promoters, marking defined cell types, are commercially available. Thus, it is logical to pursue the use of such strategy to investigate the contributions of different cell types in processing and encoding somatosensory stimuli.

In the present study, we took advantage of this transgenic system to screen for the expression of the vesicular monoaminergic transporter 2 (Vmat2) in sensory-related areas of the mouse central nervous system. First, we bred the Vmat2-Cre line with tdTomato mice. By doing so, we expected to see tdTomato expression in all neurons that were expressing the Vmat2 gene and thus the Cre enzyme. In adult mice, tdTomato was indeed heavily expressed in monoaminergic- (i.e. serotonin, dopamine, noradrenaline, histamine) rich areas, such as VTA, striatum, dorsal raphe and locus coeruleus. However, to our surprise, the S1 cortex also showed selective expression of tdTomato in layer IV, which was not expected since Vmat2 cortical expression has been reported to be present latest in the 10th postnatal day in mice.

Intrigued by this finding, we first aimed at confirming the anatomical position of these cells. Cortical layer IV is known to host the thalamic-recipient neurons. Thus, Chr2/GFP virus was injected into VPL/VPM thalamic nucleus and projections could be seen surrounding the Vmat2.Cre;tdTomato neurons. Additionally, when whole cell patch clamped, these neurons showed evoked excitatory postsynaptic potentials (EPSPs) upon light activation of
thalamocortical projections expressing Chr2, confirming that they are located in the S1 cortical layer IV.

Interestingly, when the same recorded cells were held in current clamp mode and given current in increasing steps, evoked action potentials were shown to be following the intrinsically bursting profile, a spike signature of cortical excitatory neurons, in every cell recorded, suggesting that this population is composed of excitatory neurons.

As stated earlier, three main classes of excitatory neurons are present in cortical layer IV: spiny stellate, star pyramidal and pyramidal cells. Although it is not possible to distinguish these cells based on spike profile, spiny stellate cells show a clear lack of apical dendrites compared to pyramidal neurons. Histological analysis of neurobiotin filled and virus infected Vmat2-Cre;tdTomato revealed that these neurons had processes located within the layer IV and thus did not show apical projections to the more superficial layers, suggesting that they were formed by spiny stellate cells. Importantly, when adult mice were injected with Cre-dependent virus, the Vmat2-Cre cells showed spread expression of the mCherry marker, confirming that the Cre enzyme was still present in this specific population of adult mice.

Finally, to molecularly characterize this population, we performed single cell mRNA sequencing of S1 cortical dissociated tdTomato neurons. This analysis revealed a high prevalent expression (92.7%) of the layer IV marker Rorb, lack of expression of the inhibitory marker Slc32a1 (Viat), and low levels of inhibitory markers, such as Gad1 (3.3%), somatostatin (2%), parvalbumin (3.3%), Npy (0%) and VIP (0%), indicating an excitatory nature and anatomical position of these neurons.

Furthermore, Vmat2.Cre;tdTomato neurons expressed glutamatergic (Gria2: 93.38 %, Grin2c: 93.38%, Grik5: 54.3 %) and GABAergic (Gabrb1: 86.09 %, Gabrg1: 66.89 %) receptor-related genes, and GPCRs including neurotensin receptor 2 (98.7%), endothelin receptor (84.8%) and histamine receptor 1 (47.7%). Thus, besides receiving excitatory inputs, this excitatory layer IV population is suggested to be also modulated by local interneurons using either fast inhibitory ionotropic- or slow GPCRs-mediated neurotransmission.

In summary, in this study we offer a genetic tool to target, in a specific way, S1 layer IV spiny stellate cells. By using this method, the contribution of this defined population in transmitting distinct somatosensations in the cortex can be achieved, hopefully helping to draw the functional circuitry encoding somatosensation in higher brain areas.
Summary

The main goal of the present thesis was to contribute to the understanding of how the central nervous system distinguishes somatosensory information from different modalities, such as pain and itch. In this work, I initially focused on the studies of the spinal dorsal horn GRPR-expressing neurons, a population that had previously been reported as itch- but not pain-related. We first showed that this population is composed by mainly excitatory inter-neurons (paper I and II), transmits itch-related information through glutamate signaling (paper I), and is at least partly downstream to the NPPB pathway (paper I). Regarding its intrinsic connectivity, we further reported that this population was under local tonic and phasic inhibition, and could be partly activated by the action of somatostatin (paper II), a peptide known to inhibit the action of local dorsal horn inhibitory interneurons. Thus, blockage of the local inhibitory tone could both increase the excitability and induce activity in dorsal horn GRPR-expressing neurons (paper II), corroborating behavioral findings that pointed the GRP/GRPR system as part of the disinhibition-induced itch effect. To be able to reproduce the importance of this population in transmitting itch- but not pain-related information also from the mouse orofacial area, I developed a new method to target the brainstem orofacial-related trigeminal nucleus caudalis. Infusion of GRP peptide, and optogenetic or chemogenetic selective activation of this population in this area, showed vigorous wiping behavior targeted to the whiskers pad area, while chemogenetic activation of the same population in the early cervical spinal cord triggered scratching behavior targeted to the most caudal facial areas (paper III). Thus, in this study we showed for the first time a functional switch in the same population from itch to pain in the spino-trigeminal axis. Finally, in paper IV we found that in the adult S1 cortex of the Vmat2-Cre mouse line, the Cre enzyme is selectively expressed in layer IV spiny stellate cells. With that, we offer a new mouse genetic tool to target a defined cortical population enabling for functional studies and elucidating its importance in the processing and encoding mechanisms of somatosensory information. In summary, the work presented in this thesis gives strength to a labeled line of itch in the spinal cord. Also, suggests GRPR neurons as being part of a potential labeled line of pain from the orofacial area. Finally, offers a new tool to target a defined cell type in the S1 cortex, extending our understanding on how somatosensory stimuli from different modalities are distinguished in our central nervous system.
Acknowledgments

If you think about this final work as a bowl of fresh fruits carefully selected to keep a fair combination of sweet and citric taste, kept and delivered in the right conditions, and finally consumed in the right environment and mindset, a great amount of time was spent on choosing, washing, peeling, cutting, tasting and mixing the right ingredients. As one can see such tasks are not necessarily ones of high technical demand. But instead, good amount of patience to overcome frustrations that insisted in cohabit with the cooker was necessary to be developed.

Being of extreme importance to me during this process, were people that I met here in this city, in different times and under different circumstances. Malin Lagerstöm brought me to the kitchen, proposed a menu and pointed out the cutlery. Kasia and Bejan were the first ones sharing the cut board with me. Stefano, Sanja and Julia formed a trio that truly inspired me on looking at the right fruits. Meanwhile Ernesto was the one suggesting me to look at the fruits from different angles, complaining that the current one was wrong, over and over. This promptly would make Pavol put the most contagious laugh, making my day, again.

And as you might know, an enormous variety of fruits can be found and are originally from different parts of our world. On that regard, I feel myself a real privileged person as I had the great chance to meet another trio formed by Petar, Özden and Umut, giving me through these years Balkan spices and different points of view based on respectful knowledge about how the fruits work, and also beers. Literally. Joining us, always contributing with her beautiful and pure soul, besides spicing the environment with high pitch spikes of also highly contagious laugh, was Ninnie.

It is also worth it to mention here Milos, Samer, Ana I., Ana P., Harmen, Hannah and Akram, people that shared not only working space with me but also some time outside the lab, often contributing to enjoyable moments.

Considering that I was born and raised in the northeast cost of Brasil, in a city where you have to clean the ground from excessive amounts of sweet mangos and cashews in certain parts of the year, it probably gets understandable the use of the present metaphor in this acknowledgment section. To keep feeding my nostalgia and home sickness, it is of fundamental importance to name the ground of my existence. My mom, Maria Salete, consistently brought to me a pretty consolidated and practical definition of persistence, symbolizing a true warrior, but at the same time always being the
kindest and expressing the most pure and empathetic soul I ever had the chance to meet. Importantly, she gave me the first models of a tasty fruit salad. A kind of ritual that would persists over time. On that task, my sister Patricia was always of crucial importance, making sure with her kindness that we were enjoying the final results as much as we could, revealing her generosity as one of her best qualities. Together with her, Thiago and João form now a beautiful team that I am sure will keep this tradition alive.

And interestingly, if I was not in Brasil during my PhD, my home country came close to me through people that I truly enjoyed being close to. Stefano brought joy and intellectual inspiration; Igor shone light with his delightful smile and care; Luimar, Felipe and Juliana formed another trio of always enjoyable laughs and conversations; Marina, Adriano, Diana, Eduardo, Jessica, Paula, Marcia, Lyvia and Gerra came later, but all brought together an impressive boost of genuine happiness always triggered when I could be part of their reunions. Finally, not Brazilians but almost, Marco and Leonor were also part of interesting and fun times during my PhD.

Such list of important names illustrates a good proof of my greatest luck during my PhD. Each year defined very well the different steps in this journey, with very intense experiences, mind changing. And what changed not only my mind, but also the incredible emotional states derived from it, in its most colorful perspectives, started to be drawn in the night I saw a gift personalized in a human body, named Heidur. Having the chance to know her, getting close to her, sharing uncountable easy laughs, talks, interests, frustrations, relieves, happiness, and… a daughter, was and it is what gave me the foundations and pillars to redefine what I consider to be the meaning of love. And with love the fruits were and are touched, selected, peeled, cut, mixed, and tasted. And in this last case, literally. For Ela, Ella and Myself.
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)