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Functional Aspects of Peripheral and Spinal Cord Neurons Involved in Itch and Pain

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Abstract

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We have investigated the role of the metabotropic glutamate receptor 7 (mGluR7) and the gastrin releasing peptide receptor (Grpr) population that are involved at different levels of itch transmission. We found that *mGuR7* deficient mice displayed an anaphylaxis-like behavior when provoked with histamine. Analysis of blood revealed elevated plasma levels of histamine and mouse mast cell protease-1 (mMCP1), two indicators of anaphylaxis, in *mGluR7* deficient mice compared with control mice. Inhibition of the neurokinin 1 receptor, by preventing binding of the corresponding ligand substance P (SP), prior to provocation with histamine prevented the development of anaphylaxis in *mGluR7* deficient animals. However, blocking GRPR (gastrin releasing peptide receptor) only resulted in decreased itch levels in *mGluR7* deficient mice but did not prevent the systemic anaphylaxis-like behavior. Our findings indicate that mGluR7 normally functions as a brake on histaminergic itch that is mediated through GRPR as well as anaphylaxis through Substance P.

Grpr has previously been shown to mediate both histaminergic and non-histaminergic itch but little is known about the GRPR neuronal population. We used a BAC cloning strategy to construct a Grpr-Cre line, which we crossed with the reporter lines *tdTomato* and *Viaat*-egfp as well as with *Vglut2*-lox. We could conclude that Grpr-Cre neurons are mainly excitatory interneurons located in lamina II-IV, that convey itch using VGLUT2-mediated glutamatergic transmission to the next, currently unknown, step in the labeled line of chemical itch.

To eventually deduce the function of the endogenous opioids dynorphin and enkephalin, which are hypothesized to be involved in gating pain and itch in the spinal cord, we constructed two Cre lines using BAC cloning that targeted the precursor proteins preprodynorphin and preproenkephalin, respectively. Preprodynorphin-Cre neurons were mainly located in lamina II-IV and overlapped to 47% with *Vglut2* mRNA, while the co-expression with the inhibitory markers *Viaat*-egfp and PAX2 was 13% and 28% respectively in the spinal cord. Preproenkephalin neurons were more localized to lamina III in the dorsal horn, furthermore single cell analysis showed that they overlapped to 94% with *Vglut2* mRNA while 7% and 13% expressed *Viaat*-egfp and PAX2 respectively.

Keywords: mGluR7, anaphylaxis, Grpr, Penk, Pdyn, Cre line, BAC cloning, spinal cord, transgenic line

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"Science is simply the word we use to describe a method of organizing our curiosity"

Tim Minchin

List of Papers

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

I Rogoz K#, **Aresh B**#, Freitag FB, Pettersson H, Magnúsdóttir EI, Larsson Ingwall L, Andersen HH, Franck MCM, Nagaraja C, Kullander K and Lagerström MC. Identification of a Neuronal Receptor Controlling Anaphylaxis. *Cell Rep.* 2016 Jan 12;14(2):370-9. # shared

II **Aresh B**, Freitag FB, Perry S, Blümel E, Lau J and Lagerström MC. Spinal cord interneurons expressing the gastrin releasing peptide receptor convey itch through VGLUT2-mediated signaling. Manuscript.

III **Aresh B**, Stjärne L, Blümel E, Naga Maturi P and Lagerström MC. Characterization of preprodynorphin-expressing cells in the mouse nervous system. Manuscript.

IV **Aresh B**, Moelijker N, Blümel E and Lagerström MC. Characterization of preproenkephalin expressing neurons in the nervous system using a transgenic Cre line. Manuscript.

Contents

Abbreviations

AMN082	N,N-dibenzhydrylethane-1,2-diamine dihydrochloride
BAM8-22	bovine adrenal medulla 8-22
BAC	bacterial artificial chromosome
Bhlhb5	basic helix-loop-helix
CNS	central nervous system
CQ	chloroquine
DAG	diacylglycerol
DOR	delta opioid receptor
DRG	dorsal root ganglion
ET-1	endothelin 1
FcεRI	Fc epsilon receptor I
GPCR	G-protein coupled receptors
GRP	gastrin releasing peptide
GRPR	gastrin releasing peptide receptor
HR	histamine receptor
KOR	kappa opioid receptor
mGluRs	metabotropic glutamate receptors
MOR	mu opioid receptor
Mrgprs	Mas-related G protein-coupled receptors
Nppb	natriuretic polypeptide b
Npra	Nppb receptor
NK1R	neurokinin 1-receptor
PAR2	protease activated receptor 2
PCR	polymerase chain reaction
Penk	preproenkephalin
Pdyn	preprodynorphin
PIP_2	4,5-biphosphate
PKC	protein kinase C
PLCß	phospholipase Cß
SLIGRL	protease-activated receptor-2 activating peptide
SP	substance P
STT	spinothalamic tract neurons
TRPA1	transient receptor potential A1
TRPV1	transient receptor potential vanilloid receptor-1
ITAMs	immunoreceptor tyrosine-based activation motifs

Introduction

Itch and pain are multidimensional sensations that are part of our host defense system, but they also exists in chronic conditions, where itch for instance dominates in atopic dermatitis (Kiebert et al., 2002) and psoriasis (Krueger et al., 2001), and pain in rheumatoid arthritis (McInnes and Schett 2011) and fibromyalgia (Salaffi et al., 2009). Persistent itch or pain often leads to sleep deprivation, increased levels of anxiety and depression (Krueger et al., 2001; Kiebert et al., 2002; Salaffi et al., 2009; McInnes and Schett 2011), which emphasizes the importance of elucidating the neurobiological mechanisms involved these sensory modalities for improved drug targeting.

Glutamate and mGluR7

Glutamate is the most common excitatory neurotransmitter in the central nervous system (Brumovsky, 2013). Therefore there are a variety of glutamate receptors throughout the CNS (central nervous system) that participate in various functions (Niswender and Conn, 2010). Among these are the metabotropic glutamate receptors (mGluRs), which are members of the GPCRs (G-protein Coupled Receptors). Similar to other GPCRs, the mGluRs are membrane bound proteins which are coupled to a G-protein consisting of several domains; α, β and γ. Once the receptors are activated, they change conformation, which leads to activation of the G-protein that in the inactive state is linked to guanosine 5'-diphosphate (GDP) but now exchanges it for guanosine 5'-triphosphate (GTP). The different subunits then are freed and can interact with different second messenger systems including enzymes, transcription factors or ion channels and thereby affect many cellular functions (Niswender and Conn, 2010).

The mGluRs can further be divided into three subgroups depending on sequence homology, pharmacology and which second messenger system they are associated with (Niswender and Conn, 2010). mGluR7 belongs to subgroup III, is expressed presynaptically in the active zones of cells and has a low affinity for glutamate (Li et al., 1997; Shigemoto et al., 1997; Kinoshita et al., 1998). It is believed that mGluR7 functions as an autoreceptor and when an excessive amount of glutamate is released the

receptor is activated and thus prevents further release (Millan et al., 2002; Martin et al., 2007).

Studying mice lacking mGluR7 has made it possible to investigate the role of the receptor in the CNS. The mice do not have any acute sensory or motor difficulties (Masugi et al., 1999; Holscher et al., 2004), but they have difficulties completing tasks requiring short term memory (Holscher et al., 2004) as well as impaired fear response (Masugi et al., 1999). Both of these modalities are dependent on the amygdala and hippocampus, two structures in which the receptor is expressed (Kinzie et al., 1995; Shigemoto et al., 1997). These mice furthermore have reduced anxiety and are believed to display an antidepressant behavior (Cryan et al., 2003). These results have made the receptor interesting for targeting for psychiatric disorders that are triggered by stress (Mitsukawa et al., 2006; O'Connor and Cryan, 2013).

Mast cells and anaphylaxis

Anaphylaxis is defined as "a serious allergic reaction that is rapid in onset and may cause death" (Simons et al., 2012). Anaphylaxis can be triggered by many factors but commonly it is caused by food (different types of nuts, seafood, fish), medications (antibiotics, opiates, aspirin), venom (bee, snake) or by latex (Brown et al., 2001; Brown, 2004; Braganza et al., 2006; Metcalfe et al., 2009a). Anaphylaxis is not only dangerous because of the rapid onset but it can also be difficult to diagnose due to the diverse accompanying symptoms. The symptoms can affect the skin (swelling, hives, flushing or itching), the respiratory tract (cough, bronchospasm, chest tightness), the cardiovascular system (hypotension, dizziness) and the gastrointestinal tract (nausea, vomiting, abdominal pain) (Sampson et al.).

Mast cells are a type of white blood cells that are involved in the defense system (immune system) against pathogens as well as in allergy and anaphylaxis. They are present in many parts of the body, mainly in the skin, around blood vessels and nerves, in mouth and nose and in mucosa of lungs and digestive tract (Abraham and St John, 2010). Mast cells can be activated in several ways, for instance directly by pathogens or by immunological and non-immunological mediators (Marshall, 2004).The reason is that mast cells express several different receptors of which the most common are; Toll-like receptors (activated directly by pathogens) (Varadaradjalou et al., 2003; Trinchieri and Sher, 2007), Fc receptors (activated by antibodies conjugated with antigens) (Kinet, 1999), complement receptors (activated by complement components or complement split products) (el-Lati et al., 1994; Nilsson et al., 1996; Khodoun et al., 2009), cytokine receptors, chemokine receptors and different types of peptide receptors (Marshall, 2004). An anaphylactic shock in humans is commonly mediated by immunoglobulin E (IgE) activation of the epsilon receptor I (FcεRI) on mast cells (Blank and Rivera, 2004;

Kraft and Kinet, 2007). This receptor belongs to the multichain immune receptor family that consists of a α-subunit that serves as a binding site for the Fc part of IgE, a membrane-tetraspanning ß-subunit that acts as a stabilizing unit and a ɣ-homodimer that is responsible for initiating signaling (Repetto et al., 1996). At the cytoplasmic end of both the ß and the ɣ-subunits there are immunoreceptor tyrosine-based activation motifs (ITAMs), which serve as docking sites for Src family protein kinases (SFKs). After an exposure to an allergen, antibodies against it are produced that attach themselves to mast cells, after a repeated exposure to the same allergen a binding to the antigen on the mast cells occurs. This binding crosslinks (Metzger, 1992; Kinet, 1999) one or more FcεRI leading to the recruitment of the SFK, LYN (Lck/Yes novel tyrosine kinase), which phosphorylates the ITAMs on the ß and the ɣ-subunits. This consequently initiates the signaling cascade that leads to activation of several other SFKs as well as signaling molecules and second messengers ultimately causing calcium mobilization and finally release of preformed granules and new synthesis of many immune mediators (Kinet, 1999; Siraganian, 2003; Blank and Rivera, 2004; Kraft and Kinet, 2007). Within minutes of activation, mast cells release preformed granules, which contain many mast cell mediators including histamine (Riley and West, 1953; Hirasawa et al., 2002; Makabe-Kobayashi et al., 2002; Koarai et al., 2003), heparin (Oschatz et al., 2011), the proteases tryptase (Huang et al., 1998; Vitte, 2015), chymase (He and Walls, 1998; Tani et al., 2000) and carboxypeptidase A (Goldstein et al., 1989) , and proteoglycans (Ronnberg et al., 2012). Approximately 2-24h after exposure newly synthesized pro-inflammatory lipid mediators (prostaglandins, leukotrienes and platelete activating factor), growth factors, cytokines and chemokines are released. Each of these mediators contributes to the various symptoms seen during an anaphylactic shock (Metcalfe et al., 2009a). For instance, released histamine activates histamine receptors on pruriceptive receptors and hence contributes to the propagation of itch signaling (Kemp and Lockey, 2002). Histamine, heparin and lipid mediators (prostaglandins and leukotrienes) activate smooth muscles, which leads to contraction and constriction of airways (White; Oschatz et al., 2011), while PDs and LTs additionally stimulate mucous secretion, which can aid in removing parasites from the gastrointestinal tract (Bischoff, 2009). Furthermore histamine, heparin, prostaglandins and leukotrienes release results in vasodilation and increased vascular permeability of blood vessels so that immune cells can be recruited from the blood stream to the affected tissue (White; Marks and Greaves, 1977; Ogawa and Grant, 2007; Oschatz et al., 2011), and finally several cytokines, chemokines and platelete activating factor can stimulate synthesis, activate or attract effector cells such as macrophages, neutrophils, eosinophils and platelets (Ogawa and Grant, 2007; Metcalfe et al., 2009b).

It is estimated that the prevalence of anaphylaxis is between 0.05% to 2% (Clark and Camargo, 2007), and that in children it is mainly caused by food allergy while in adults it is generally related to venom or drugs (Brown et al., 2001; Brown, 2004; Braganza et al., 2006; Lieberman et al., 2010). Anaphylaxis is treated acutely with an intramuscular injection of epinephrine that reverses most of the physiological effects seen in anaphylaxis. It acts on α-adrenergic receptors located in most organs of the body, resulting in vasoconstriction and bronchodilatory effects that relieve laryngeal obstructions and mucosal edema (Lieberman, 2003). Epinephrine can also be combined with substances that reduce some of the experienced symptoms; examples include inhalation of a $ß_2$-agonist to alleviate the bronchospasm (Lieberman et al., 2010; Waserman et al., 2010), glucocorticosteriods, which have anti-inflammatory effects (Sampson et al.) or antihistamines (H1 and H2-antagonists) to alleviate the skin irritations (Lin et al., 2000).

Itch transmission from skin to DRG, spinal cord and brain

Most people associate itch (pruritus) with acute states, for instance after a mosquito bite. Itch is however multidimensional and can present with different severity. It can be classified into peripheral itch, neurogenic itch and neuropathic itch. Peripheral itch is experienced in atopic dermatitis, psoriasis, reactions to different drugs and inflammatory molecules. Neurogenic itch includes systemic states such as chronic liver disease, chronic liver failure and Human immunodeficiency virus (HIV) associated itch caused by immune failure. Lastly pruritus can be defined as neuropathic where the different neuronal itch pathways can be affected by different disease states like diabetes and multiple sclerosis causing itch as a side effect (Twycross et al., 2003; Stander et al., 2007).

Pruritus is generally also divided into histaminergic and non-histaminergic itch depending on whether histamine (Simone et al., 1987; Schmelz et al., 1997b) or other substances elicits itch. Most chronic states of itch cannot be treated with antihistamines and are therefore classified as non-histaminergic (Shim and Oh, 2008). Histamine induced itch is also characterized by a flare and wheal reaction around the inflicted area, which cannot be seen in non-histaminergic itch (Barnes et al., 1986; Wallengren and Hakanson, 1987; Petersen et al., 1997; Schmelz et al., 1997a).

The itch sensing neurons are slow conducting mechanically-insensitive C-fibers (histamine induced itch) (Schmelz et al., 1997b; Han et al., 2006; Imamachi et al., 2009), mechanically sensitive C-fibers (non-histaminergic itch, cowhage)(Namer et al., 2008) and thinly myelinated Aδ fibers(Ringkamp et al., 2011) with their cell bodies in the dorsal root gangli-

on (DRG) and free nerve endings innervating the skin. These fibers have receptors that are activated by a variety of endogenous and exogenous itch inducing agents. Once activated these DRG neurons relay the information to the dorsal horn of the spinal cord where it is either processed by local inter-neurons (Potenzieri and Undem, 2012; Han and Dong, 2014) or projected up to the brain (thalamus, somatosensory and cingulated cortex) through spinothalamic neurons or possibly other projection neurons (Figure 1) (Andrew and Craig, 2001; Simone et al., 2004; Davidson et al., 2007; Davidson et al., 2012).

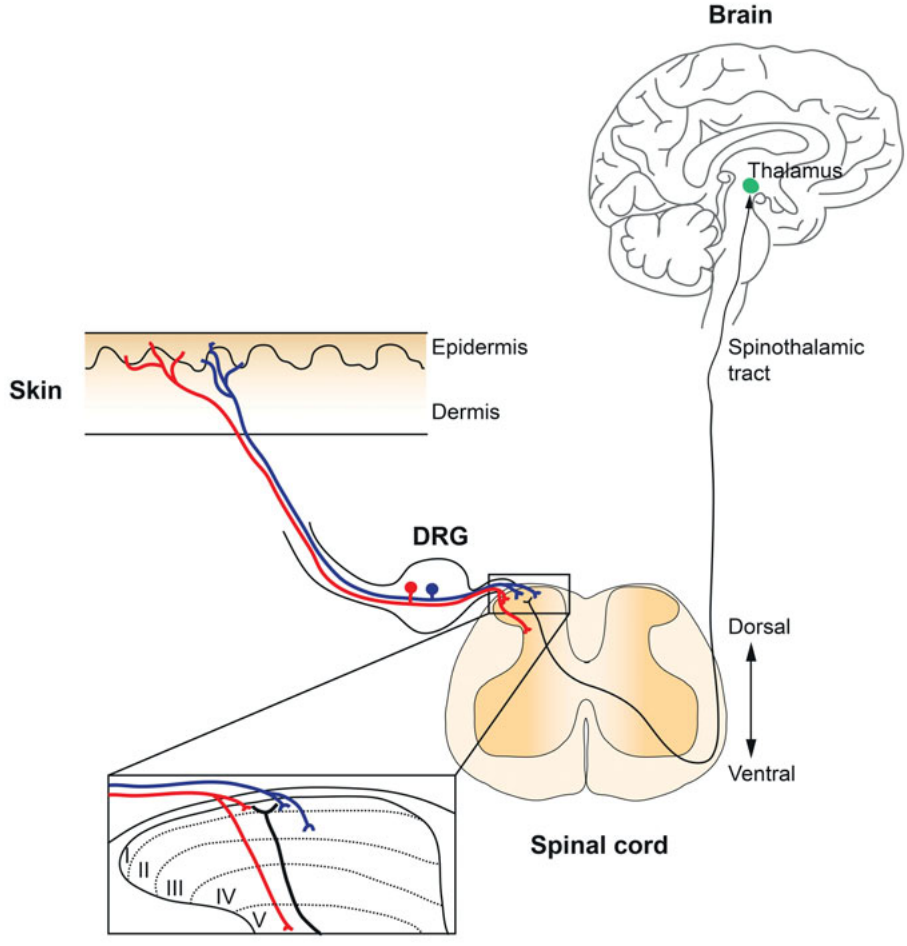

Figure 1. **Activation of free nerve endings in the skin.** Primary afferents have their cell bodies in the DRG with one fiber extending to the skin while the other one transmits the signal to the dorsal horn of the spinal cord. At spinal cord level the signal is further transmitted to the brain through projection neurons following the spinothalamic tract. Several regions in the brain are involved in itch processing, among these the thalamus (green). Adapted from (Braz et al., 2014).

I, II, II (marks lamina I, II and III).

Itch from the periphery to the spinal cord

Histamine-induced itch

Histamine-induced itch is mediated through histamine receptors 1,3 and 4 (HR1, HR3-4) (Bakker et al., 2002; Bell et al., 2004; Sugimoto et al., 2004) where HR1 has been suggested as the primary receptor involved in acute itch (Davies and Greaves, 1980). This receptor can either be activated directly by histamine or indirectly by for instance compound 48/80, which induces mast cells to release histamine (Inagaki et al.; Sugimoto et al., 1998). The HR1 belongs to the family of GPCRs that is coupled to Gq and upon conformational change in the HR1 this subunit (Bakker et al., 2002)activates C-fibers that are mechanically insensitive (Schmelz et al., 1997b; Schmelz et al., 2003). Data show that HR1 can activate sensory neurons via a downstream nonselective cation channel called transient receptor potential vanilloid receptor-1 (TRPV1), using two pathways. In the first pathway stimulation of HR1 leads to activation of intracellular mediator phospholipase Cß3 (PLCß3) that hydrolyze 4,5-biphosphate (PIP_2) to form the secondary messenger diacylglycerol (DAG) (Prescott and Julius, 2003; Han et al., 2006). DAG then binds to TRPV1 and induces an increased influx of sodium, potassium and calcium, which results in depolarization and thereby activation of voltage-gated sodium channels that drive the action potential along the axon (excite itch specific C-fibers) (Imamachi et al., 2009). In the second pathway, TRPV1 is linked to HR1 through a metabolic product of lipoxygenases. Once HR1 is stimulated, it activates phospholipase A2, which in turn, leads to an accumulation of arachidonic acid that is metabolized by LO to 12-hydroxyeicosatetraenoic acid. This secondary metabolite can bind to TRPV1 that is downstream of HR1 and thereby mediate histamine induced itch (Figure 2)(Shim et al., 2007).

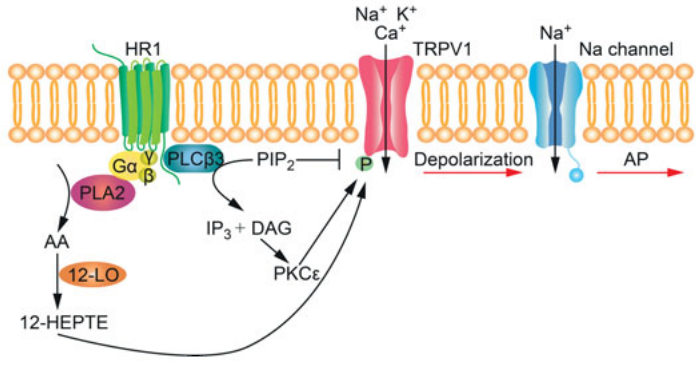

Figure 2. **Histamine induced itch transmission.** Activation of HR1 causes TRPV1 mediated Ca^+, K^+ and Na^+ influx by two pathways, either by PLCß3 or PLA2. Both induce phosphorylation of TRPV1 and later activation of voltage gated sodium channels that raises the intracellular concentration of Na^+ leading to an action potential. Adapted from (Kremer et al., 2014)

HR1 (histamine receptor 1), PLCß (phospholipase Cß), PIP_2 (4,5-biphosphate), DAG (diacylglycerol), LOs (lipoxygenases), PLA2 (phospholipase A2), 12-HPETE (12-hydroxyeicosatetraenoic acid), TRPV1 (transient receptor potential vanilloid receptor-1), PKC (proteinase kinase $C_ε$), IP3 (inositol trisphosphate), Gα, ɣ, ß (G-protein subunits).

Histamine-independent itch

None-histaminergic itch can be induced by chloroquine (CQ) (Liu et al., 2009; Roberson et al., 2013), protease activated receptor 2 (PAR2) agonist SLIGRL (Steinhoff et al., 2003), bovine adrenal medulla 8-22 (BAM8-22) (Sikand et al., 2011), α-methyl-serotonin (Yamaguchi et al., 1999), endothelin 1 (ET-1) (Gomes et al., 2012) and ß-Alanine (Liu et al., 2012). CQ is an anti-malaria drug that produces itch as a side effect in about 70% of the malaria patients(Aghahowa et al., 2010). The receptor for CQ is called MrgprA3 and is a member of a family of Mas-related G protein-coupled receptors (Mrgprs) (Dong et al., 2001), which includes several other known receptors including MrgprC11 and MrgprD that are receptors for BAM8-22 (Steinhoff et al., 2003; Roberson et al., 2013) and ß-Alanine (Liu et al., 2009; Liu et al., 2012), respectively. These receptors are expressed in small-diameter neurons in the DRG and trigeminal ganglia and the percentage of neurons in the DRG that is responsive to CQ is approximately 4%-5% (Imamachi et al., 2009). Both MrgprA3 and MrgprC11 require transient receptor potential A1 (TRPA1) for signaling, however they seem to use different secondary signaling molecules for opening of this channel (depolarization, sodium and calcium entry). CQ-induced activation of MrgprA3 releases Gßɣ, which activates TRPA1 directly or indirectly while BAM8-22-mediated activation of MrgprC11 activates the downstream ion channel TRPA1 through PLCß3 instead (Figure 3) (Wilson et al., 2011).

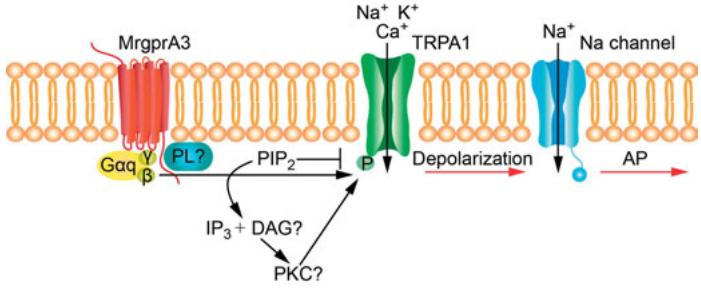

Figure 3. **Example of histamine-independent itch induced by chloroquine.** The full pathway is not completely known, except that activation of voltage gated sodium channels are mediated through TRPA1. Adapted from (Kremer et al., 2014).

MrgA3 (Mas-related G protein-coupled receptor A3), PL? (phospholipase?), PIP_2 (4,5-biphosphate), DAG (diacylglycerol), PKC (proteinase kinase C?), TRPA11 (transient receptor potential A1), Gα, γ, ß,q (G-protein subunits*).*

The amino acid ß-Alanine is a naturally occurring amino acid in humans and is often used as a supplement by bodybuilders to prevent muscle fatigue (Baguet et al., 2010). A common side effect of administration of this amino acid is itch and tingling in the skin (Decombaz et al., 2012). The neurons expressing the ß-Alanine receptor MrgprD have properties that are typical for that of nociceptive neurons and deleting this receptor in mice results in deficient responses to noxious mechanical stimuli but not normal responses to heat and cold stimuli(Cavanaugh et al., 2009). Although MrgprD was initially believed to be primarily involved in pain, recent data have shown that skin injections of ß-Alanine induced itch in human test subjects, and recombinant cell lines treated with this ligand induced activation of MrgprD as well as an increase in intracellular calcium concentrations (Liu et al., 2012).

The PARs are a family of protease receptors that belong to GPCRs and so far four PARs have been identified (Reddy et al.; Steinhoff et al., 2003). They are expressed in several areas in the body including keratinocytes, epidermal nerves, endothelia and DRG (Steinhoff et al., 2000). Characteristics for one of these receptors (PAR2) is that it has an extracellular NH_2, and when proteolytically cleaved by endogenous as well as exogenous proteases it acts as a tethered ligand and binds to the receptor (Shimada et al., 2006). When the PAR2 cleaved ligand SLIGRL binds, it activates phospholipase C, which cleaves PIP_2 into IP_3 and DAG, the latter activates PKC, which phosphorylates and activates TRPV1 (Amadesi et al., 2004; Dai et al., 2004). However another signaling mechanism has been proposed where MrgprC11 instead of PAR2 is activated by the ligand SLIGRL (Liu et al., 2011).

Endothelin-1 is a 21 amino acid neuropeptide that is produced and released by mast cells, endothelial cells and keratinocytes in the skin. It is upregulated in response to stress, hypoxia and inflammatory cytokines (Gandhi et al., 1994). Experiments in BALB/c mice have shown that ET-1 is a very potent itch inducer (Trentin et al., 2006; Liang et al., 2010; Gomes et al., 2012) with a delayed onset and that it induces scratching in a dose-dependent manner. It is also the endogenous ligand for endothelin A receptor, a GPCR that belong to the rhodopsin-type receptor superfamily (McQueen et al., 2007). The endothelin A receptor is coupled to adenylyl cyclase and protein kinase C (PKC) through a G-protein and it has been shown that the intracellular calcium concentration increases drastically following stimulation with ETs. However it is not determined through which mechanism this occurs since ET-1 mediated itch does not involve TRPV1, but interestingly activation of TRPA1 suppresses itch induced by ET-1 (Douglas and Ohlstein, 1997; Liang et al., 2011).

Itch signaling in the spinal cord

Grpr

It is currently still unclear which transmitters are involved in transmitting the sensory information of itch from the DRG to the dorsal horn of the spinal cord. Both gastrin releasing peptide (GRP), glutamate and natriuretic polypeptide b (Nppb) have been suggested as possible candidates (Sun and Chen, 2007; Andoh et al., 2011; Koga et al., 2011; Mishra and Hoon, 2013). Glutamate is the major excitatory neurotransmitter used in the central nervous system (Brumovsky, 2013), while GRP is a peptide belonging to the family of bombesin like peptides (Jensen et al., 2008; Takanami et al., 2014). It was suggested that GRP released from DRG neurons would activate gastrin releasing peptide receptor (GRPR) in the dorsal horn of the spinal cord. GRPR is a G-protein coupled receptor that is expressed in the outer layers of the dorsal horn of the spinal cord (Sun and Chen, 2007). Experiments with GRPR knockout mice have shown that this receptor is not involved in any pain states tested (thermal, mechanical, inflammatory and neuropathic pain) but mediates itch in the spinal cord. Both GRPR knockout and littermate wild type controls were injected intradermally with several histaminergic as well as non-histaminergic itch-inducing substances and a significant reduction of scratching was seen in the knockout compared to the wild type, indicating GRPR as a mediator of both histamine-dependent and independent itch (Sun et al., 2009). GRP was lately found to be mainly expressed in GRP spinal cord interneurons and not in DRG neurons (Solorzano et al., 2015). However , there remains some controversy, it has been suggested that opti-

mization of experimental procedures determines if GRP is detected in the DRG or not (Barry et al., 2016).

Nppb

The most recent model that involves itch transmission from the periphery to the spinal cord involves the neuropeptide natriuretic polypeptide b (Nppb) and the corresponding receptor Npra (Mishra and Hoon, 2013). Nppb is expressed in small diameter neurons in the DRG and all Nppb positive neurons overlap with TRPV1 and PLCß3 and almost completely with Mrgprs. The receptor Npra is expressed in the outer layer I of the dorsal horn spinal cord. Wildtype mice injected intradermally with several itch-inducing substances triggered excessive scratching, while the Nppb knockout mice had an almost complete loss of itch response to any of the itch-inducing substances tested. Intrathecal injection of Nppb in wildtype and *Nppb-/-* however induced scratching, suggesting that activation of primary afferent induces release of Nppb postsynaptically, which likely activated the receptor Npra. This model however questions GRP as the main neurotransmitter for transmitting peripheral itch signals. The group showed that GRP-induced scratching was normal in *Nppb-/-* mice as well as in mice ablated of Npra expressing neurons through Nppb-saporin (a cell targeting toxin). Furthermore, selective deletion of neurons expressing GRPR gave reduced scratching in mice not only in response to GRP and histamine but also to Nppb suggesting that the first step of itch transmission occurs via Nppb and its receptor and not through Grp and Grpr as previously indicated (Sun and Chen, 2007; Mishra and Hoon, 2013).

Bhlhb5

A neuronal population expressing the transcription factor basic helix-loop-helix (Bhlhb5) referred to as B5-I interneurons has gained interest (Ross et al., 2003). Approximately five percent of the neurons in the spinal cord express Bhlhb5 and its expression is restricted to lamina I and II of the dorsal horn. It appears that Bhlhb5 is required for the survival of a subset of inhibitory neurons and mice lacking the transcription factor have skin lesions that are caused by extensive scratching. These mice also have higher pain threshold than wild type littermates, which together with the increased itch phenotype indicates a neuronal population that in normal conditions functions to inhibit itch in the spinal cord (Ross et al., 2010). The suggested mechanisms is that in wildtype mice the itch signal induced from the periphery is relayed to the dorsal horn of the spinal cord where it can be subjected to modulatory signals from both inhibitory and excitatory neurons. In the Bhlhb5 knockout however the modulatory inhibitory neurons (i.e. Bhlhb5 expressing neurons) are missing and hence there is a reduced inhibitory input in the spinal cord,

which results in increased itch signaling to the brain (Kardon et al., 2014). Furthermore Bhlhb5 knockout mice had an almost complete loss of dynorphin and administration of a KOR (kappa opioiod receptor) agonist prior to a challenge with pruritic substances resulted in reduced scratching in these mice. Application of capsaicin (activates TRPV1), the active ingredient in mustard oil allyl isothiocyanate (activates TRPA1) and menthol (activates TRPM8) under electrophysiological conditions in Bhlhb5-Cre neurons concluded that this population receives input from primary afferents expressing the different ion channels providing a possible mechanism by which chemical counter stimuli can inhibit itch (Kardon et al., 2014).

Projection from the spinal cord to the brain

The itch signal from the spinal cord is relayed to the brain via projection neurons located in mainly lamina I through the spinothalamic pathway. The projection neurons are mechanically sensitive and cross the midline to the contralateral side before they project up to the major itch relay station, the thalamus, and are therefore referred to as spinothalamic neurons. From thalamus, the itch signal is further transmitted to the cortex giving the discriminative and affective part of itch. (Andrew and Craig, 2001; Davidson et al., 2007; Davidson et al., 2012).

Brain regions involved in itch processing

The neural processing of itch is still today not completely unraveled. Researchers have used several techniques such as positron emission topography and functional magnetic resonance imaging on human test subjects to get a better insight into this complex sensory modality (Crosson et al., 2010). Data from these analyses stress itch sensations as multidimensional, involving several nuclei in the brain instead of pinpointing a single brain region as an itch center. The itch-associated areas include prefrontal areas, supplementary motor areas, premotor cortex, anterior insular cortex, anterior midcingulate cortex, primary and secondary somatosensory corticles, thalamus, basal ganglia, cerebellum and claustrum. Each of these brain regions are responsible for different aspects of itch processing, including the sensory, emotional, cognitive and motivational aspects (Leknes et al., 2007; Mochizuki et al., 2009; Papoiu et al., 2012; Papoiu et al., 2013).

The endogenous opioids

The endogenous opioids are produced by proteolytic cleavage of three precursor proteins; proopiomelanocortin, which gives ß-endorphin; preproenkephalin (PENK) that encodes leucine (Leu)- and methionine (Met)- enkephalins and preprodynorphin (PDYN), which encodes dynorphin A, dynorphin B and neoendorphin (Hollt, 1992; Yaksh and Wallace, 2011; Feng et al., 2012) Each of these opioids has different affinities for the three opioid receptors: mu opioid receptor (MOR), kappa opioid receptor (KOR) and delta opioid receptor (DOR). For instance, enkephalins cross reacts with DOR and MOR (low affinity), while dynorphin primarily binds to KOR with negligible affinity for MOR and DOR (Corbett et al., 1982; Yaksh and Wallace, 2011; Chavkin, 2013). The three GPCR that are associated with Gi/o-proteins and upon conformational change attenuates the excitability of neurons by either inhibiting cyclic adenosine monophosphate production or direct G-protein subunit interaction with Ca^{2+}, TRPV1 or other ion channels, which reduces voltage activation of the channel (Al-Hasani and Bruchas, 2011). The opioids and the corresponding receptors are located in nerve fibers in the periphery, in the spinal cord and in the brain as well as in none-neuronal cells (Morris and Herz; Hökfelt et al., 1977; George et al., 1994; Wittert et al., 1996; Yaksh and Wallace, 2011).

In my thesis, I have focused on two of the opioids PDYN and PENK. They are expressed in the dorsal horn of the spinal cord, with PDYN primarily expressed in lamina I, II, III and V with high co-localization with GABA in laminas I and II and mainly with VGLUT2 in lamina III (Marvizon et al., 2009; Sardella et al., 2011). Dynorphin knockout mice do not show any evident difference from wildtype littermates in regard to fertility, growth rate, locomotion, cage behavior and body weight (Sharifi et al., 2001), but display a reduction in spinal analgesia induced by the cannabinoid delta-9-tetrahydrocannabinol (Zimmer et al., 2001) and while dynorphin is not required for initiation of neuropathic pain it seems to act pronociceptive in maintaining this state (Wang et al., 2001). Furthermore, as discussed previously, dynorphin overlaps with the Bhlhb5 spinal population and it has been suggested that once activated by counter stimuli such as menthol, capsaicin and mustard oil, these neurons inhibit itch by the release of dynorphin, which in turn inhibits either the Grpr expressing neurons or a population upstream of Grpr expressing cells (Kardon et al., 2014).

The enkephalins are also located in the dorsal horn of the spinal cord, mainly in lamina II (Huang et al., 2008; Hossaini et al., 2014) and co-expresses both GABA and glutamate (Huang et al., 2008). Enkephalin has been shown to have strong antinociceptive functions (Spanos et al., 1989; Kita et al., 1997; Noble et al., 1997) and mice lacking enkephalin has a decreased pain response at the supra spinal level and display increased anxiety and aggressiveness (Konig et al., 1996; Ragnauth et al., 2001).

Transgenic tools

Cre/lox system

The Cre system is a very convenient genetic tool that can for example be combined with either a reporter line to enable visualization or with a lox line to remove a specific gene of interest.

The Cre recombinase is an enzyme that originally comes from bacteriophage P1 and it recognizes two specific sequences (recognition sites) called loxP sites. The loxP sequence also originates from bacteriophage P1 and consists of an 8 base pair core long sequence that is flanked by two 13 base pair inverted repeats. When Cre and loxP are expressed together in a specific tissue, the Cre recombinase will catalyze the recombination between the two loxP sequences (the inverted repeats) and depending on which orientation the loxP sites have, the sequence between the loxP sites is either inverted or deleted (Sternberg and Hamilton, 1981; Argos et al., 1986).

This technique can be used to remove a gene of interest (conditional knockout), delete a population of interest (for instance using the diphtheria toxin-lox) or to visualize a population of interest (using a reporter line). When the Cre line is crossed with a reporter line such as *tdTomato* (Allen Brain Institute), the gene encoding the red flouroscence protein, which is located downstream of a stop codon flanked by two loxP sites, is expressed. In normal conditions, the stop codon prevents the expression of the reporter gene but when Cre recombinase is expressed in the same cell it promotes recombination between the loxP sites. This will subsequently remove the stop codon and allow transcription of the tomato gene enabling visualization (Figure 4) (Shaner et al.; Sauer, 1993).

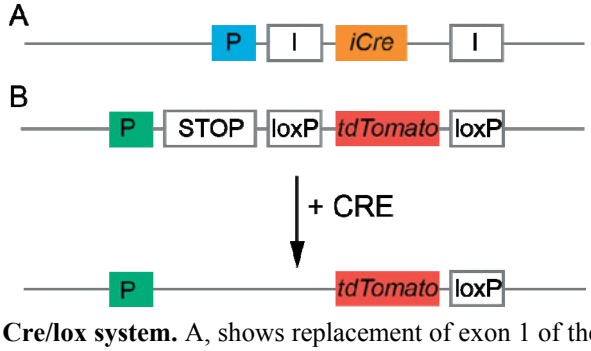

Figure 4. **The Cre/lox system.** A, shows replacement of exon 1 of the gene of interest with iCre during the BAC cloning step. The Cre recombinase is subsequently under the influence of the gene of interest's promoter. B, crossing a Cre line with a reporter line, here tdTomato, leads to a Cre-mediated recombination between the two loxP sites. This removes the stop codon and enables transcription of the fluorescent reporter.

P (promoter), I (intron), CRE (Cre recombinase).

Methodological considerations

Ethical considerations

All animal procedures were approved by the local ethical committee in Uppsala and followed the Directive 2010/63/EU of the European Parliament and of the Council, The Swedish Animal Welfare Act (Djurskyddslagen: SFS 1988:534), The Swedish Animal Welfare Ordinance (Djurskyddsförordningen: SFS 1988:539) and the provisions regarding the use of animals for scientific purposes: DFS 2004:15 and SJVFS 2012:26.

Animal housing

All behavior analyses were performed on adult (>7 weeks old) male and female mice in a controlled environment of 20-24°C, 45-65% humidity and 12 hours day/night cycle. The observers were blind to the genotype.

Animals used in the papers

Grpr-Cre, Pdyn-Cre and Penk-Cre were constructed using bacterial artificial cloning, see section below. Founders carrying Cre were crossed with the reporter lines *tdTomato* (Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$; Allen Brain Institute) and *Viaat-egfp* (GENSAT, MMRRC) or in the case of Grpr-Cre also *Vglut2*$^{f/f}$ mice (Wallen-Mackenzie et al., 2006). Offspring were genotyped for the presence of the *Grpr-Cre* allele, the *Pdyn-Cre* allele, the *Penk-Cre* allele, the *tdTomato* allele*, the Viaat-egfp* allele and the *Vglut2* allele.

The *Vglut2*$^{f/f}$;Trpv1-Cre mice were generated through a cross between Trpv1-Cre mice (C57BL/6NCrl*DBA/2) (Lagerstrom et al., 2010) and *Vglut2*$^{f/f}$ mice (Sv129/R1 * C57BL/6) (Wallen-Mackenzie et al., 2006). *mGluR7-/-* mice (129P2/OlaHsd * C57BL/6) were purchased from the MMRRC repository. Nc/Nga mice (Nc/NgaTndCrlj) were purchased from Charles River (Japan). C57BL/6 mice were bought from Taconic (Denmark).

Behavioral studies in mice

Spontaneous itch

The mice were placed in a transparent cage with bedding and recorded with a digital camera for 30 min. The scratching episodes were scored using the software AniTracker© v1.0 and the results were displayed as the mean number of scratching episodes per group in 30 min.

Chemically induced itch

Adult (>7 weeks) C57BL/6, knockouts or conditional knockouts and littermate controls were injected intradermally in the nape of the neck with saline and placed in a transparent cage with bedding and recorded for 1 hour. The subsequent experimental day the same animals were injected with an itch substance and recorded for another hour. The number of scratching episodes was scored using the software AniTracker© v1.0 and the data were presented as the mean number of scratching episodes for each group in 1 hour.

Pain

Von-Frey

The mice were placed in transparent plastic chambers that were placed upon an elevated metallic mesh floor for 90 min before the initiation of the experiment (acclimatization). Von Frey filaments (Scientific Marketing Associates) were applied to the planar surface of the hind paw of each mouse according to the Chaplan up-and-down paradigm (Chaplan et al., 1994). A lift of the treated paw was considered a reaction to the applied filament and a thinner filament was applied subsequently, while a lack of reaction resulted in application of a thicker filament. Every observation started with 0.6 g filament and the experiment was ended once six measurements were obtained around the 50% threshold. The Dixon Method (Dixon, 1965) was used to calculate the 50% threshold.

Randall-Selitto

The mice were restrained in a transparent cylinder with their tail hanging out and left to acclimatize for 15-20 min. The Randall-Selitto arm (Randall and Selitto, 1957) was placed on the tail and increasing pressure was applied until a reaction (tail-flick) was observed and the machine was stopped. The test was repeated at least three times with 10-15 min between each test. The cut off weight was 500 g and the results were expressed as mean withdrawal latency in grams (g) for each animal.

Hargreaves

The mice were placed in transparent plastic chambers that were placed on top of a glass floor for 90 min until no exploratory behavior was observed. A thermal laser beam (IITC Life Science) was directed towards their planar hind paws and the time until paw withdrawal was noted. The test had a cut-off time of 20 s and was repeated at least three times with 5 min between each observation. The result was presented as the mean withdrawal latency (s) for each animal and group.

Tail-withdrawal test

The mice were restrained in a plastic cylinder and left to acclimatize for 15-20 min before the tail was dipped halfway into -15°C ethanol. The tail was removed when a tail-flick was observed or the cutoff time of 30 s was reached. The test was repeated at least three times with 5-10 min between each test and the results were displayed as the mean withdrawal latency (s) for each animal and group.

Histological staining procedures in mouse

Immunohistochemistry

This technique was used to detect and quantify a specific protein in a tissue. The tissue was fixed in formaldehyde or paraformaldehyde, which ensures that the cells and tissue keeps their integrity and shape. A primary antibody (which is produced in animals) was incubated with the tissue and functions to recognize a specific site (epitope) on the desired protein to be detected. Subsequently the tissue was incubated with the secondary antibody which recognizes the primary antibody and is tagged with a fluorescent moiety that can be visualized under a microscope. The advantages of this technique are that several proteins can be detected simultaneously as long as the primary antibodies are produced in different animals and the secondary antibodies are tagged with different flourophores (Coons et al, 1942).

In situ hybridization

In situ hybridization is a technique to detect and visualize gene expression in a tissue (Gall and Pardue, 1969). Similar to immunohistochemistry, the tissue is fixed in formaldehyde or paraformaldehyde to maintain cell integrity. Thereafter the tissue is incubated with a digoxigenin (DIG)-labeled RNA probe that is complementary to the mRNA of the gene of interest. The DIG molecule can be recognized by an enzyme-linked antibody which stains positive cells upon substrate addition (Gall and Pardue, 1969; Rudkin and Stollar, 1977; Speicher and Carter, 2005).

Single cell analysis and laser capture microdissection

Using specific primers in polymerase chain reaction (PCR), the mRNA of genes of interest can be detected in individual cells. In paper I mouse DRG was dissected, dissociated and cultured where upon individual cells were picked using patch pipettes under RNAse free conditions.

In papers II, III and IV the spinal cords of *tdTomato* positive mice were snap frozen, sectioned and collected on membrane slides. Individual Cre positive cells were isolated using laser capture microdissection, a method where the membrane slides are placed under a microscope visualizing positive cells, which with the help of a laser are sectioned out and collected in small tubes located under the membrane.

Once the cells were collected, they were subjected to cDNA synthesis and used in two subsequent PCR rounds with proper primers to detect gene expression (Emmert-Buck et al., 1996; Kamme et al., 2004).

Bacterial artificial chromosome (BAC) cloning and pronuclear injection

The Cre lines in papers II, III and IV were constructed using BAC cloning described shortly below (http://recombineering.ncifcrf.gov/protocol/ Protocol1_DY380.pdf). BACs are DNA constructs that are derived from the F factor (fertility factor) of *E.coli* (Lederberg et al., 1952). The benefits of these constructs are that they have the capability to serve as vectors for large DNA fragments and can be transferred from one bacterial cell to another (Birren et al., 1999). We selected BACs (BACPAC resources) that contained our genes of interest; *Grpr*, *Pdyn* and *Penk*, cultured them and harvested the DNA (i.e. the plasmid) for transfer into electrocompetent bacterial cells EL250. These cells possess a recombination system that can be induced by heat, incubation with the DNA results in integration into the bacterial chromosome through homologous recombination (Yu et al., 2000). Subsequently we amplified the Cre sequence from a plasmid and replaced exon 1 of our genes of interest ultimately placing the Cre sequence under the influence of the promoters of *Grpr*, *Pdyn* and *Penk*. The bacterial chromosome was removed of any resistance cassette, purified and subjected to restriction enzymes before it was sent for pronuclear injection (Parkitna et al., 2008; Fu and Maye, 2011). Shortly, fertilized embryos are collected from a donor female that has been sacrificed and the DNA is injected into the pronucleus and transferred to the oviduct of a pseudopregnant female. The DNA is then hopefully incorporated into the genome through random integration and resulting in a founder carrying the Cre sequence (Ittner and Gotz, 2007).

Grpr-Cre was sent to Karolinska Center for Transgene Technologies (KCTT), while Pdyn-Cre and Penk-Cre were sent to the Uppsala University Transgenic Facility (UUTF) for pronuclear injection.

Aims

In large, the aims of these projects were to define the role of different subpopulations of dorsal spinal cord neurons and primary afferent neurons (DRG neurons) in the complex circuitry involved in itch and pain transmission. We have focused on the neuronal networks in the spinal cord that utilize the G-protein coupled receptors mGluR7 (metabotropic glutamate receptor 7) and Grpr (gastrin releasing peptide receptor), as well as the opioids dynorphin and enkephalin.

Paper I

The objective of this study was to investigate the involvement of mGluR7 in the regulation of histaminergic itch and anaphylaxis.

Paper II

The aim of this study was to construct a Cre line, using BAC (bacterial artificial chromosome) cloning, to target the Grpr population of spinal cord neurons. Furthermore, the study also aimed to characterize these Grpr-Cre neurons regarding their inherent properties and to determine their position in the labeled line of itch.

Paper III

The aim of this study was to improve our understanding of the population expressing preprodynorphin (PDYN) by constructing a Pdyn-Cre line using BAC cloning.

Paper IV

The purpose of the following study was to construct a transgenic line that targeted preproenkephalin expressing cells to enable further characterization of these cells in the peripheral and central nervous system.

Results and discussions

Paper I

Our group has previously shown that removing VGLUT2-mediated glutamatergic signaling from peripheral neurons expressing TRPV1 (transient receptor potential vanilloid 1) resulted in an increased spontaneous itch behavior (Lagerstrom et al., 2010). This prompted us to investigate how release of glutamate from TRPV1 neurons could be responsible for the regulation of histaminergic itch.

There are several glutamate receptors expressed in the CNS, among these, mGluR7, which is particularly interesting. It seems that this receptor is not only expressed in TRPV1 neurons but that it also acts in an auto-inhibitory manner. When a sufficient amount of glutamate is released from the presynaptic terminal (a threshold reached) mGluR7 is activated and thus prevents further release through reduced Ca^{2+} influx (Millan et al., 2002; Martin et al., 2007; Li et al., 2012). Using both immunohistochemistry and single cell analysis, we could confirm that mGluR7, TRPV1 and VGLUT2 are co-expressed in a subpopulation of primary afferent neurons. We were also able to show that intrathecal injection of the mGluR7 specific agonist AMN082 attenuated the spontaneous itch behavior displayed by the *Vglut2*-deficient mice. These data strongly suggests involvement of mGluR7 in itch regulation.

To further characterize the role of mGluR7 in itch, we injected wildtype mice with several itch inducing substances intradermally in combination with AMN082 intrathecally. Interestingly, a significant reduction of scratching could be seen for all of the substances that were involved in histaminergic transmission (histamine and compound 48/80) while the results for the non-histaminergic substances, except α-methylserotonin, were unchanged. Both histamine and α-methylserotonin are associated with the activation of the intracellular enzyme PLC (phospholipase C) β3, which is expressed in a subpopulation of TRPV1 neurons (Han et al., 2006; Imamachi et al., 2009). Our single cell analysis on *mGlur7* expressing primary afferents showed partial overlap with *Plcβ3* and *Hrh1*, hence our data indicates that mGluR7 selectively regulates histaminergic and PLCβ3-associated itch.

Given the implication of mGluR7 in itch regulation, it was of interest to look at itch responses in mice lacking *mGluR7*. These mice displayed normal responses to several pain stimuli, except for an attenuated response to nox-

ious mechanical pain and a significant increase in spontaneous itch compared to wildtype littermate controls. Injection of histamine not only resulted in increased levels of scratching in *mGluR7-/-* but also a drastic decrease in locomotor activity and drop in body temperature. These symptoms are reminiscent of those seen during an anaphylactic shock, which is caused by excessive histamine release from mast cells. Plasma levels of histamine and mouse mast cell protease-1 in the blood of the *mGluR7-/-* animals were increased compared with control mice after histamine provocation, which together with the behavioral findings suggests an anaphylaxis-like state in these mice.

We also found that *mGluR7-/-* mice injected with a NK1R (neurokinin 1 receptor) antagonist, prior to histamine injection, not only showed attenuated levels of itch behavior but also no longer displayed the drastic drop in body temperature. Our data indicate that peripheral release of SP (substance P) and activation of the NK1R provides the link between mGluR7, expressed on histaminergic primary afferents, with mast cells and anaphylaxis. Treatment of the *mGluR7 -/-* mice with a GRPR antagonist resulted in reduced itch levels but did not prevent anaphylaxis. Taken together these data suggest that mGluR7 regulated itch requires GRPR, while regulation of anaphylaxis is independent.

In conclusion, this paper identifies mGluR7 as an autoreceptor on a subpopulation of histamine sensing primary afferents that is activated during anaphylactic shock. Upon activation, this receptor prevents excessive itch and anaphylaxis that is mediated by SP and NK1R.

Paper II

The focus of this study was to investigate the role of the spinal cord population expressing Grpr in itch transmission. Previous data have shown that the Grpr receptor is involved in both histaminergic and non-histaminergic itch (Sun et al., 2009). Using BAC cloning we created a Grpr-Cre line, which provided us with a powerful transgenic tool to further study this population. Here we replaced exon 1 of the *Grpr* gene with a *Cre* recombinase gene, leaving Cre under the influence of the Grpr promoter. The line was crossed with the reporter line *tdTomato* to visualize the Cre expressing neurons whereby single cells were collected using laser dissection and analyzed for *Grpr* mRNA. The results showed that 32% of the Grpr-Cre cells actually comprised the *Grpr* population. Electrophysiological characterization showed that application of the specific agonist gastrin releasing peptide (GRP) induced spike responses in 43.3% of the patched Grpr-Cre neurons. The expression pattern as well as the neurotransmitter type was also analyzed in the spinal cord, here the majority of the Grpr-Cre positive cells were located in lamina II, III and IV. Furthermore, 67% of the *Grpr* mRNA posi-

tive neurons co-expressed *Vglut2* mRNA, while only 11% and 6% of the Grpr-Cre cells were co-localized with *Viaat-egfp* (vesicular inhibitory amino acid transporter) and PAX2 (paired box gene 2) respectively, which indicates that the Grpr-Cre population is mainly excitatory. We also analyzed brain sections, which showed Grpr-Cre expression in several nuclei, particularly in thalamic structures and midbrain structures were the expression was dense. Analysis of the DRG revealed a few Grpr-Cre positive cells/section, and we therefore also concluded that Grpr-Cre expression is mainly limited to the CNS.

To determine the termination of spinal cord Grpr-Cre neurons, tracing analyses was performed. The retrograde tracer fluorogold was injected to two sites in the brain, the ventral posterolateral thalamic/ventral posteromedial thalamic nucleus and lateral parabrachial nucleus. Subsequently several fluorogold positive neurons could be detected in the spinal cord, however only two overlapped with Grpr-Cre, which implies that the population mainly consists of interneurons.

Since the majority of the population was excitatory, we crossed Grpr-Cre with *Vglut2*-lox to remove VGLUT2-mediated glutamatergic signaling in these cells. The *Vglut2-lox*;Grpr-Cre mice displayed less spontaneous itch as well as attenuated responses to both histaminergic and non-histaminergic itch substances.

We can from these analyses conclude that Grpr-Cre cells are excitatory interneurons located in the dorsal spinal cord upstream of projection neurons and that they utilize VGLUT2-mediated signaling to transmit spontaneous and chemical itch.

Paper III

Preprodynorphin (PDYN) is a precursor peptide that is subsequently cleaved to dynorphin, a member of the endogenous opioid family (Akil et al., 1984), with several functions in the central nervous system, among these gating itch and mechanical pain, as well as being involved in maintaining neuropathic pain (Lai et al., 2006; Duan et al., 2014; Kardon et al., 2014). We therefore constructed a Pdyn-Cre line for characterization and to get a better understanding of the population of neurons expressing dynorphin. Pdyn-Cre was crossed with the reporter line *tdTomato* for visualization of the population in the spinal cord, which showed that the majority of the Pdyn-Cre expressing cells were found in lamina II-IV. Single cells analysis showed that 43% of the cells expressed *Pdyn* mRNA and that 47% of these co-expressed *Vglut2* mRNA, while only 13% and 28% overlapped with the inhibitory markers *Viaat-egfp* and PAX2 respectively. Furthermore Pdyn-Cre was expressed in many brain nuclei, including primary and secondary somatosensory

corticles, striatum, amygdala, insular cortex, raphe magnus nucleus and parts of hippocampus.

Hence our data shows that Pdyn-Cre marks *Pdyn* cells and is therefore a useful transgenic tool to further study this population.

Paper IV

Preproenkephalin (PENK) is an endogenous opioid that has been implicated in antinociception (Spanos et al., 1989) together with fear and anxiety (Konig et al., 1996; Ragnauth et al., 2001). We constructed a Penk-Cre line using BAC cloning targeting the PENK expressing neurons to get a better understanding of this population. The Penk-Cre line was crossed with the *tdTomato* reporter to enable visualization of Penk-Cre expressing cells. We found that a majority of the Penk-Cre cells were located in lamina III, furthermore 41% of the cells in the superficial laminas expressed *Penk* mRNA among these 94% also expressed *Vglut2* mRNA. Immunohistochemical analysis showed that only 7% and 13% expressed the inhibitory markers *Viaat-egfp* and PAX2 respectively, which indicates that the majority of the Penk-Cre cells are excitatory. Expression in the brain was seen in many nuclei, such as striatum, nucleus accumbens, insular cortex, cingulated cortex and several amygdaloid nuclei.

In conclusion the Penk-Cre line provides a useful technique to further study PENK expressing neurons in the nervous system.

Concluding remarks/future perspective

During the last few decades, transgenic techniques such as knockouts, reporters and Cre lines have emerged as very powerful tools and have therefore increased exponentially in publications. We have used an extensive amount of transgenic mouse lines in our studies to gain a better understanding of the mechanisms underlying itch and pain, with a main focus on the spinal cord and the primary afferent neurons. In paper I we chose to focus on the function of the mGluR7, were we used a knockout line targeting the receptor. We were able to show that a proper interaction between the immune system and nerve fibers (here with mGluR7) is essential for maintaining a normal state, where unbalance, results in the life threatening state of anaphylaxis.

Although the function of Grpr neurons in the spinal cord is well established as an itch mediating population, in paper II we were able to conclude firstly that Grpr-Cre neurons use glutamate for conveying chemical itch signals, and secondly that they are interneurons and therefore located upstream of projection neurons. However we still have no knowledge whether they contact projection neurons directly or if they relay the signaling to other interneurons before it reaches projection neurons (i.e. STT neurons) and finally the brain. It has been hypothesized that Grpr neurons express KOR since intrathecal injection of a KOR agonist attenuated itch signaling mediated by GRP. The most likely source of inhibitory input comes from populations expressing the transcription factor Bhlhb5 which has an almost complete overlap with dynorphin (Kardon et al., 2014). It has been shown that Bhlhb5 positive neurons receive input from primary afferents that respond to capsaicin, mustard oil and menthol, furthermore counter stimuli such as menthol reduced chloroquine-induced scratching in wildtype mice but not in *Bhlhb5* knockout mice suggesting that they function to gate itch. Most likely, these above mentioned counter stimuli leads to the release of dynorphin from this inhibitory population, which inhibits Grpr neurons or a population upstream of Grpr neurons (Kardon et al., 2014). To resolve this question we wish to perform single cell analysis on Grpr-Cre neurons for the KOR and apply dynorphin to Grpr-Cre tissue as we perform calcium imaging.

Papers III and IV are in the initial stage, we have just recently finished the neurochemical characterization of these lines, next step would be to functionally characterize them. We initially crossed the Cre lines with *Viaat*-lox to remove signaling via GABA/glycine and in that way determine the func-

tion of this small inhibitory subpopulation in the spinal cord, we however did not receive any conditional knockouts indicating that normal Viaat transporter function is too vital in the respective subpopulation of neurons. Since both Pdyn-Cre and Penk-Cre are extensively expressed in the brain (and most DRG neurons are excitatory) the lethal phenotype most likely originates from here. We are currently looking into other strategies to unravel the role of Pdyn and Penk neurons in the spinal cord.

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