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XXV Ciclo

A subpopulation of itch receptors marked by Ret expression

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PhD Thesis

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PART I

Abstract

Sensory neurons are a heterogeneous group of cells that are specialized to detect stimuli acting on the skin such as touch, temperature, pain and itch. A major challenge in the sensory biology field is to isolate and characterize specific functional subsets of neurons as an exhaustive knowledge of many of them is still lacking.

In my PhD project I established a protocol to analyse and sort by Fluorescently Activated Cell Sorting (FACS) peripheral sensory neurons, in order to isolate specific subsets and further perform gene expression profiling.

In particular I applied these techniques to Avil-Cre::Ret^{eGFP} mice, where Ret-positive neurons express eGFP specifically in Dorsal Root Ganglia (DRG) neurons after Cre recombination. Ret is a receptor for the GDNF family expressed in at least 3 sensory neuron subsets: non-peptidergic nociceptors, Rapidly Adapting (RA) mechanoreceptors and C-fiber low-threshold mechanoreceptors. However, our immunohistochemical analysis suggested that there might be more Ret-positive subsets.

I analysed Ret-positive neurons by FACS, combining the analysis of the pattern of endogenous eGFP expression with IB4-binding arrangement and I identified 5 diverse Ret-eGFP-positive subsets. I focused on two of them, Ret-$eGFP^{Lo}$:$IB4^{Neg}$ and Ret-$eGFP^{Hi}$:$IB4^{Neg}$ that did not bind to IB4 and expressed low and high levels of eGFP, respectively. Their expression profiles suggested that Ret-$eGFP^{Hi}$:$IB4^{Neg}$ neurons represent the previously described RA Mechanoreceptors, while the Ret-$eGFP^{Lo}$:$IB4^{Neg}$ subset constitutes a new Ret-positive class of sensory neurons involved in itch perception.

To verify this assumption, we functionally characterized Ret-$eGFP^{Lo}$:$IB4^{Neg}$ neurons. We focused on three molecules whose receptors we found enriched in the subset: histamine, a well known pruritogen, IL-31, a cytokine that has been linked to the pathology of Atopic Dermatitis, and LY344864, a serotonin agonist whose receptors, $5\text{-}HT_{1f}$, was among the most expressed genes within the Ret-$eGFP^{Lo}$:$IB4^{Neg}$ subset. By calcium imaging we demonstrated that these substances are able to elicit a neuronal response mainly in Ret-eGFP/IB4 negative cells. Moreover, when injected in the nape of the neck of mice, they all cause scratching, substantiating a putative role of this population as itch receptors..

My data indicate that we have discovered a new Ret-positive subset of sensory neurons involved in itch perception. A more extensive characterization of these cells, for example with a Sst-*ires*-Cre line which we found to specifically mark Ret-$eGFP^{Lo}$:$IB4^{Neg}$ subset, will further clarify their role within the DRG in vivo.

1. Introduction

1.1.The somatosensory system: a historic overview

In our everyday life we experience a myriad of somatic sensations that can be as different as a caress and a painful slap or a cold wind and a steaming sauna.
Somatic sensibility allows us to perceive stimuli originating from the external world, so permitting our interaction with it and also fulfilling a very important protective role as, for example, feeling the heat of a boiling pan can prevent our hand from burning and perceiving the tickle caused by an insect can avoid dangerous stings. Moreover, the somatosensory system has an interoceptive and a proprioceptive function for the sensing of stimuli coming form inside of the body and the perception and balance of our limbs and trunk, respectively.
Conventionally, there are four major sensory modalities: touch, pain, warm and cool.
This classification comes from studies in the 1880s in which was shown, independently by Blix, Goldscheider and Donaldson, that specific skin spots for touch, warm and cold exist (reviewed in Norrsell et al, 1999). Whether pain could be considered a specific sensation has been for a long time on a debate. Max von Frey, in 1896, was able to provoke pain without evoking touch sensation by employing thin and sharp plant spicules. He proposed the "specificity theory" in which he claimed that pain is a distinct sensation. However Goldscheider came to different conclusion by using the same kind of stimulators but inserting the spicules slantwise. He formulated an "intensity hypothesis" of pain: a stimulus of weak intensity always evokes touch-like sensations and only when it becomes more intense the sensation can turn into pain (Goldscheider, 1898).
During the last years a similar debate has been ongoing about the existence of itch as a separate sensation. Although itch is now mostly listed among the sensory modalities, many aspects about its physiology and its relation to pain are still enigmatic, as it will be discussed in the next chapter.
All the findings in the late 1880's supported the idea that specific neural circuits convey the information from the skin to the brain, as proposed by Mueller in 1837 in his "law of specific nerve energies" in which he states that morphologically distinct receptors convey information to the brain through nerve fibers specific for that modality (Mueller, 1837).
However, nowadays we know the situation to be much more complex. For example, the same perception could be evoked by different stimuli or a similar stimulus can be perceived

differently in a normal versus pathological condition (Ma, 2012). Most likely the specific labeled lines do exist, but they also crosstalk among each other, as several recent studies have shown (Lagerstrom et al, 2010; McCoy et al, 2013; Roberson et al, 2013).

1.2. Peripheral sensory neurons

The components of the above-mentioned "lines" that detect the external and internal stimuli are the primary sensory neurons whose cell bodies lie in the Trigeminal Ganglia (TG) and in the Dorsal Root Ganglia (DRG), innervating the cranial structures and the limbs and trunk, respectively.

Primary sensory neurons have a unique morphology: they are pseudounipolar, that is their central and peripheral teminals originate from a common axis and, unlike the other types of neurons, are not biochemically distinct. Although the peripheral afferent is the only one that can perceive the stimulus, axon and dendrite of the same neuron share the majority of proteins synthetized by the cell and can be both targeted by endogenous molecules that regulate neuronal sensitivity (Basbaum et al, 2009).

The peripheral branches reach the skin or the internal organs where the sensory signal is transduced into an electrical signal, involving the opening of ion channels in the membrane following a mechanical deformation, a change in the temperature or the contact with molecules released from damaged tissue. The central afferents end up in the dorsal horn of the spinal cord, which is organized into anatomically and electrophysiologically distinct laminae. This neuronal structure it is suitable to their role in stimulus transduction and its transmission to the central nervous system.

Primary sensory neurons detecting different stimuli can be quite diverse in their morphology, electrophysiological characteristics and molecular signature. They can be classified as Aβ, Aδ or C type depending on their cell body size, their axonal diameter and its level of myelination and conduction velocity. Aβ sensory neurons have large cell bodies and heavily myelinated afferents, thereby displaying very rapid conduction properties, the velocity varying from 16 to 100 m/s.

Aδ subtype exhibits medium size cell bodies and lightly myelinated fibers, which lead to intermediate conduction velocities, ranging from 5 to 30 m/s. The smallest and the most abundant sensory neurons are the C-type, They have unmyelinated processes and the lowest conduction velocities, from 0,2 to 2 m/s.

Sensory neurons also differ in their firing patterns, as distinct subtypes fire with different velocities and for a variable time. In fact the thresholds of activation and the duration of the sensation can be variable as distinct receptors can adapt slowly or rapidly to sustained stimuli.
It is clear that all the characteristics mentioned above exist because of a differential expression of genes within different sensory neuron subpopulations. Many of these molecules, however, are still unknown, and this lack of information makes our understanding of the physiology of the peripheral nervous system still puzzling.
Nevertheless, we can broadly distinguish some classes of primary sensory neurons according to their function.
The sensory neurons that detect discriminative, innocuous touch are called mechanoreceptors. They all have low thresholds of activation (LTMR- low threshold mechanoreceptors), being activated by weak, innocuous mechanical force applied to the skin. Their firing patterns, on the other hand, can be quite different as they can range from slow (SA) to intermediate (IA) and to rapidly adapting (RA).
Even though most of them are Aβ neurons, a subset of neurons sensitive to down hair movement, known as D-hair, have Aδ fibers and recently several subsets of C-type LTMR have been described ((Li et al, 2011; Vrontou et al, 2013).
Mechanoreceptors can be further distinguished based on the cutaneous end organs with which they associate, which is also directly linked to the type of stimulus they are tuned by. Mammalian skin can be distinguished into 2 types, glabrous and hairy. There are 4 major end organs present in the glabrous skin: Merkel cells, which respond to indentation (and are also present in hairy skin), Meissner corpuscles which are associated to fibers that detect skin movement, Pacinian corpuscles that fire upon vibration and Ruffini organs, involved in stretch perception.
In hairy skin, LTMR are associated with hair follicles, which are specialized mechanosensory organs. There are 3 hair types that differ in abundance, length and pattern of LTMR subtype: zigzag, awl/auchene and guard hair. The zigzag type is the most abundant (76%) and receives C and Aδ-LTMR lanceolate endings, Awl/auchene hair follicles (23%) are innervated by endings of Aβ-RA-LTMR, Aδ-LTMR and C-LTMR, while the guard hair, which represent only 1% of hair follicles, are innervated by Aβ-RA-LTMR and are associated with Aβ-SA-LTMR (Li et al, 2011)..
Centrally, the mechanosensory afferents innervate the deeper laminae of the dorsal horn of the spinal cord, namely from lamina III to V.

From a molecular point of view, mechanoreceptors are associated by the expression of the gene Nefh, which encodes for the heavy protein neurofilament subunit (NF200) and which is largely used as a marker for this class of sensory neurons. It is also known that Rapidly Adapting mechanoreceptors express the tyrosine kinase receptor Ret (Bourane et al, 2009; Luo et al, 2009), but a complete molecular signature of the different functional subsets of mechanoreceptors, as well as of other sensory neuron subtypes, is still missing.

Regarding their physiology, for many years scientists have been seeking for a channel responsible for mechanical sensitivity transduction. Recently a good candidate has been identified: it is called Piezo 2 (Coste et al, 2012). However, its role as mechanotransducer is still under examination and it is likely that other channels could also be involved in the physiology of touch sensation.

Intense thermal, mechanical or chemical stimuli are detected by nociceptors, the primary sensory neurons involved in pain perception. They usually have high threshold of activation and Aδ or C fibers which all end in the skin as free nerve endings. It is possible to distinguish 2 classes of nociceptors according to the expression of neuropeptides. The so-called "peptidergic" nociceptors express neuropeptides such as substance P and CGRP (calcitonin-gene related protein) and innervate the deeper region of the skin, the dermis. They also express the neurotrophic receptor TrkA.

The "non-peptidergic" nociceptors are characterized by their ability to bind to isolectin B4 (IB4) and by expression of the neurotrophic receptor c-Ret. Their peripheral afferents reach a more superficial part of the skin, the epidermis.

These classes of nociceptors also reach different dorsal horn laminae: the peptidergic subtype innervates the most superficial lamina 1, while the non-peptidergic nociceptors reach the outer part of lamina II.

Nociceptors are usually polymodal, that is, they are able to respond to more than one stimulus. For example, there is a subset of Aδ nociceptors that can be activated by mechanical and chemical stimuli and can further respond to heat with a high threshold of activation. If the heat stimulus is maintained they will start responding to lower temperature and also become more sensitive in case of tissue injury. Another class of Aδ nociceptors, on the contrary, have lower heat thresholds and a much higher mechanical thresholds (Basbaum et al, 2009)

The different response of nociceptors to painful stimuli depends on a differential expression of channels that confer sensitivity to heat, cold, and chemical irritants. Transient receptor potential (TRP) ion channel family has a major role in pain perception: TRPV1 channel, for example, the receptor for the red hot chili pepper ingredient capsaicin, is the main endogenous

transducer for noxious heat (Caterina et al, 1997), while TRPM8, the menthol receptor, is the principal detector of enviromental cold (Bautista et al, 2007). Chemical irritants activate the TRPA1 channel, which may also be involved in cold perception (Bandell et al, 2004; Peier et al, 2002).

1.3. Itch

1.3.1. General aspects and the link with pain

Itch, or pruritus, is an "unpleasant sensation that elicits the desire or reflex to scratch", as defined by the German physician Samuel Haffenreffer 350 years ago. (Lavinka & Dong, 2013). The scratching provoked by pruritus has a protective role, as it results in the physical removal of a potentially harmful stimulus, and also helps suppressing the irritating itchy sensation by causing mechanical pain, which has been reported to inhibit itch in both humans and mice (Akiyama et al, 2011; Yosipovitch et al, 2007).

However, itch can be also associated to pathological conditions and become chronic, so seriously affecting upon the quality of life. Chronic itch is a complex sensory experience with sensory discriminative, cognitive, evaluative and motivational components (Ikoma et al, 2006), although the pathophysiology of most clinical itch conditions is unknown.

Chronic itch can arise for several reasons: it can be caused by skin diseases such as psoriasis, atopic dermatitis, urticaria, etc (pruriceptive itch), it can be centrally induced due to systemic disorders such as chronic liver disease, chronic renal failure and thyroid disfunction (neurogenic itch) or to neurological disorders of the central or peripheral nervous system like brain tumor, nerve compression, etc (neuropathic itch) or it can even be related to psychological and psychiatric disorders such as parasitophobia and obsessive-compulsive disorder (psychogenic itch) (Biro et al, 2007).

For a long time itch an pain have been considered as mediated by the same neurons: according to the "intensity theory" of itch proposed by von Frey in 1922, itch was caused by a weak activation of nociceptors, whereas a stronger stimulation was supposed to be responsible for the painful sensation (Biro et al, 2007).

According to that theory, application of low concentration of algogens on the skin should cause itch instead of pain and varying the stimulation frequency should lead to a transition from one sensory modality to the other one. However, none of these characteristics has been

observed as varying the concentration of the applied algogen just causes pain of different intensity and a different frequency of stimulation produces more intense itch or pain but never a transition from the former to the latter (Stander & Schmelz, 2006).

Recently it has become evident that a pruriceptive system exists, that is, there are primary sensory neurons that respond specifically and exclusively to pruritogenic stimuli as well as dedicated central itch processing. For example, it is well assessed that opioids produce analgesia but induce itch, probably by acting on distinct dorsal horn neurons (Ballantyne et al, 1988; Liu et al, 2011). Moreover, when ablating spinal cord lamina I neurons expressing the gastrin-releasing peptide receptor, mice showed a decrease in scratching response while no change in pain behaviour was observed (Sun et al, 2009). When central signalling of nociceptors is affected, mice show deficits in pain behaviours but also greatly enhanced itching (Liu et al, 2010b).

Despite the evidence that pain and itch are mediated through different primary afferent pathways, the link between the 2 sensations is unquestionable. Pruriceptors and nociceptors, in fact, share mediators and mechanisms and are very similar in terms of both morphology and processing.

In fact, pruriceptors express TRP channels, such as TRPV1 and TRPA1, that have a dual role in itch and pain and confer them the ability to respond also to stimuli that normally generate pain. Several theories have been proposed about how different stimuli can generate distinct itch or pain behaviours by activating the same channel. One is based on population coding: if a stimulus is purely pruritogenic will activate only itch-sensitive neurons, but if a stimulus activates both pruriceptors and nociceptors and it is strong enough to suppress itch at spinal level, a pain sensation will emerge. If the amount of pain is not sufficient to mask itch, then both itch and pain will be perceived. The “spatial contrast theory”, in alternative, states that the sensation that is evoked depends on the amount of activated neurons: if a small number is responding to a stimulus, itch sensation will be triggered, if more fibers are activated, then pain will be initiated.

Despite the fact that during the last years many researches are focusing on the role of itch in somatosensation, its relationship with pain is still under debate and many aspects of its physiology are still unkown.

1.3.2. Pruriceptors

Itch sensation can be distinguished into submodalities, which are likely to be perceived by different subsets of pruriceptors. Some of these subsets have been identified but several aspects of the pruriceptive system remain unclear.

Sensory neurons mediating itch sensation, likewise nociceptors, can have either Aδ and C fibers and it is considered that the former encode for the pricking/stinging sensation (Graham et al, 1951; Ringkamp et al, 2011) and the latter for the burning perception.

At least 2 Aδ subsets have been described in human and non-human primates: one is mechanosensitive and activated by the pruritogen cowhage (Mucuna pruriens), and the other one is mechanoinsensitive and responsive to histamine (Ringkamp et al, 2011).

However, the majority of itch receptors have unmyelinated afferents in both human and mice. It is possible to further classify them according to the type of pruritogen they are activated by. In fact, not all the pruriceptors respond equally to the various stimuli that cause itch: for example, a subset of mechano- and heat-responsive C fibers respond to cowhage and histamine (Ma et al, 2012) while there are some mechanoinsensitive C-type neurons that respond to histamine but not cowhage (Namer et al, 2008).

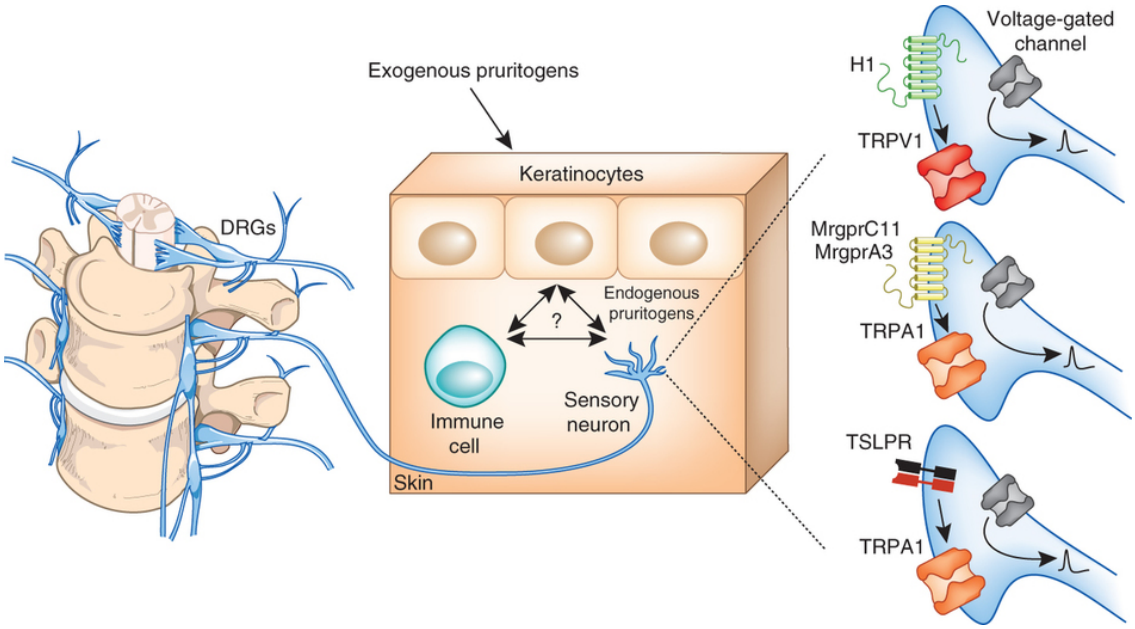

Figure 1. Pruriceptors innervate the skin where they can be activated by endogenous and exogenous itch-inducing substances, in a process that involves also immune system cells and keratinocytes. There are different subsets of pruriceptors that are activated by different pruritogens and whose activation follows different pathways. Taken from a recent review on itch receptors (Bautista et al, 2014)

1.3.3. Itch mediators and receptors

There are a variety of substances that can cause pruritus through activation of different receptors expressed by primary sensory neurons.

Histamine is naturally produced by mast cells when tissues are inflamed or stimulated by allergens, and it is also the main pruriceptive agent used to experimentally induce itch in both human and non-human primates and mice.

There are 4 subtypes of histamine receptors, named H1-H4, which are G-protein coupled receptors (GPCR). Only H1 and H4 have a role in itch sensation as shown by the observation that agonists specific for those subtypes evoke itch in mice, while antagonists suppress it (Davies & Greaves, 1980; Dunford et al, 2007).

Histamine signal transduction in primary sensory neurons has been shown to be mediated by TRPV1 (Imamachi et al, 2009), a member of TRP family channels. Many of those channels play a role in numerous sensory transduction pathways, such as thermosensation, mechanosensation, taste, etc.

TRPV1 is a heat- and ligand-activated cation channel, first identified as a receptor for the active ingredient of red hot chili peppers, capsaicin. Activation by heat and a variety of endogenous ligands leads to the influx of cations in the nerve terminal, depolarization and action potential propagation.

However, histamine cannot account for all the types of itch perceived. Anti-histamine treatments are indeed ineffective in some chronic itch diseases such as atopic dermatitis (Yosipovitch & Papoiu, 2008).

There is emerging information about compounds that drive itch sensation without the involvement of histamine and a growing evidence suggests an important role of another TRP channel family member, TRPA1, in these histamine-independent pathways. TRPA1 can be activated by a myriad of irritants, both endogenous and exogenous, and acts as a general mediator of inflammation. The most well known exogenous substance that activates TRPA1 is allyl isothiocyanate (AITC, or mustard oil), the pungent compound found in wasabi or mustard, but also cinnameldehyde (from cinnamon) and allicin (fron garlic extracts) can target the channel. Moreover, several endogenous inflammatory agents and reactive oxygen species can directly activate TRPA1.

Its role in itch emerged when the members of the Mas-related G-protein coupled receptor family, MrgprA3 and MrgprC11, were studied. These receptors are activated by Chloroquine

(CQ) and BAM8-22, respectively, which evoke calcium signals in a histamine-independent pathway, through activation of TRPA1(Wilson et al, 2011).

While it is evident that histamine-dependent and histamine-independent itch signal through 2 different molecular mechanisms (TRPV1 and TRPA1 channels, respectively), it is still under debate whether this activation can occur within the same neuronal subsets (Han et al, 2013) or whether each pathway of activation has also a dedicated subgroup of primary sensory neurons (Roberson et al, 2013).

Another endogenous substance that can cause pruritus is serotonin.

Reaction to serotonin differs between rodents and human. In rodents, mast cells are the primary source of serotonin while in humans that is not the case, even though human mast cells can synthetize and secrete it. Moreover, rats and mice scratch when the molecule is injected in their faces or backs (Akiyama et al, 2009; Klein et al, 2011), while in humans serotonin injections cause only weak itch or mixed feeling of itch and pain, through activation of a subset of cutaneous c fibers (Schmelz et al, 2003). The pruritus caused by serotonin is not suppressed by anti-histamines, thus suggesting a histamine-independent pathway (Hosogi et al, 2006). In mice, serotonin acts on several receptors but to date only the 5-HT_2 receptor subtype has been shown to be involved in itch-sensation (Yamaguchi et al, 1999).

Upon activation, pruriceptors release substance P (SP), which can indirectly cause itch by stimulating mast cells to produce and release histamine and other pruritogens.

Application of SP on human skin does cause pruritus that is prevented by subministration of antihistamines, pointing towards a histamine-dependent pathway (Hosogi et al, 2006). Interestingly, although SP induces scratching behaviour in mice, antagonists of histamine receptors does not prevent pruritus (Andoh & Kuraishi, 1998).

SP preferentially activates neurokinin 1 (NK1) receptors. Their antagonist, indeed, attenuated itch severity in both human and mouse model of chronic itch (Ohmura et al, 2004; Stander et al, 2010).

Endogenous serine proteases secreted by mast cells, such as tyrosine, kallikreins or tryptase, and exogenous ones released by a plant that has been extensively used in the sensory neuron field, Mucuna pruriens (or Cowhage) induce itch in both humans and mice through activation of a GPCR family, the Protease Activated Receptor (PAR) family. There are four PARs, three out of which have been shown to cause scratching in mice: Cowhage activates PAR2 and PAR4 which act through an histamine-independent pathway (Reddy et al, 2008), even though histamine-independence of PAR4 is somewhat controversial (Tsujii et al, 2008). Exogenous

proteases such as the active component of cowhage and the PAR agonist SLIGRL are also able to induce itch and are in fact widely used to assess scratching behaviour in mice.

Increased tryptase serum levels and PAR2 expression in keratinocytes and nerve fibers have been described in patients with atopic dermatitis (Steinhoff et al, 2003).

Itch can be also driven by cytokines.

Cytokines can communicate with sensory neurons indirectly, by activating immune and resident skin cells and thus provoking a release of mediators that can bind to sensory fibers, but also directly, by activation of specific receptors on the neuronal surface.(Saika et al, 2012)

Some members of the inflammatory immune-cell derived interleukins are involved in the pathogenesis of itch. In particular such a role has been shown for IL-2 (Martin & Murphy, 1995), IL-4 (Chan et al, 2001) and in particular for one of the components of the IL-6 family of cytokines, namely IL-31 (Dillon, Sprecher et al. 2004; Sonkoly, Muller et al. 2006),

An involvement of IL-31 in the pathogenesis of atopic dermatitis has been found in both human and mice (Dillon et al, 2004; Sonkoly et al, 2006).

IL-31 is produced by T helper type 2 cells and acts on heterodimeric receptors consisting of Interleukin 31 receptor α and Oncostatin receptor M β (Zhang et al, 2008). These receptors have been localized in a small subset of murine TRPV1+/TRPA1+ dorsal root ganglia neurons, and a direct action of IL-31 on nerve fibers has been recently described (Cevikbas et al, 2013).

Another cytokine with a role in atopic dermatitis has been recently discovered: it is an epithelial cell-derived cytokine called thymic stromal lymphopoietin (TSLP). It probably acts on both primary sensory neurons and immune cells to evoke itch behaviours. The neuronal targets express the ion channel TRPA1, suggesting a histamine-independent pathway for triggering itch.

Recently, much attention has been focused on a new family of receptors, Mrgpr (Mas-related G-protein coupled receptor family).

The Mrgpr family of receptors was discovered in 2001 (Dong et al, 2001). Their expression pattern suggests a specific role in somatosensation as many members are exclusively expressed in dorsal root ganglia.

In mice, the receptors can be grouped into 3 subfamilies: MrgprA, MrgprB and MrgprC, for a total of 50 genes and pseudogenes. In humans there are only 18 members and their similarity to mouse sequences is fairly low.

A role in itch has been assessed for MrgprA3 and MrgprC11. The former is the receptor for Chloroquine (CQ) an antimalarial drug that has as a side effect a strong pruritus, the latter is

activated by a Bovine Adrenal Medulla peptide (BAM8-22). They both activate histamine-independent pathways of itch sensation and require TRPA1 for the insurgence of action potential (Wilson et al, 2011). However their pathways are different as MrgprA3 signalling requires an active Gbeta/gamma complex while MrgprC11 requires phospholipase C as an intermediate (Wilson et al, 2011).

Interestingly, MrgprA3 and MrgprC11 largely overlap in DRG neurons (Zylka et al, 2003). Moreover, TRPV1 is present in this subset, but is not required for signalling (Wilson et al, 2011) and these neurons are also activated by histamine (Liu et al, 2009), pointing towards a possible presence within the same cells of histamine-dependent and histamine-independent pathways.

Other receptors that have been involved in the detection of pruritus are the Toll-like receptors. They have an important role in controlling innate immunity (Akira et al, 2006), but they are also expressed in neurons in both central and peripheral nervous system (Liu et al, 2012b; Okun et al, 2011), being in the latter involved in acute and chronic itch (Liu et al, 2012b; Liu et al, 2010a).

Such a role has been assessed for TLR3 and TLR7, which are expressed in subsets of small-sized, TRPV1-positive sensory neurons and they are considered to drive itch sensation associated with skin infection and tissue injury. These neurons also express GRP, a neuropeptide that elicits itch (Sun & Chen, 2007).

TLR7 recognizes some drugs such as imidazoquinoline derivatives and guanine analogs. Its ablation leads to reduction of scratching behaviour in response to non-histaminergic pruritogens while leaves unaltered the responses to histaminergic substances (Liu et al, 2010a). TLR3 ablation affects both histamine-dependent and histamine-independent itch (Liu et al, 2012a).

1.3.4. Spinal coding of itch

Many recent studies have focused on the spinal trasmission of itch, leading to the discovery of several neurotransmitters responsible for it, in particular the Gastrin-releasing peptide (GRP), Substance P (SP) and glutamate.

SP and GRP have been involved in itch sensation because neurotoxic ablation of their receptors in the dorsal horn of the spinal cord, Neurokinin-1 receptor and GRP-receptor, respectively, reduced scratching behaviour in response to Chloroquine. They did not,

however, affect histamine response, suggesting a role of these neurons in histamine-independent itch (Akiyama et al, 2013). Notably, the ablation didn't affect pain behaviour.

Regarding glutamate, it might be involved in the sensing of histamine-dependent itch as antagonists acting on its spinal receptor AMPA/kinate abolished neuronal activity of histamine-responsive spinal neurons (Koga et al, 2011).

Another molecule that has been found to have an important role in spinal itch transmission is the Natriuretic Polypeptide B (nppb) (Mishra & Hoon, 2013). Nppb is expressed in a subset of small unmyelinated, TRPV1-, MrgprA3- and PLCβ-positive sensory neurons, and its receptor Npra is found in Lamina I spinal neurons. Nppb knockout mice exhibit reduced scratching behaviour in response to many pruritogenic compounds but pain responses were not altered (Mishra & Hoon, 2013).

Following their observations and the report that most of the GRP is espressed by spinal rather than sensory neurons (Fleming et al, 2012), Mishra and Hoon (Mishra & Hoon, 2013) propose a theory in which nppb is released by pruriceptors and excites Npra-positive neurons in the spinal cord, which in turn activate GRPR-positive downstream spinal neurons that are then responsible for the transmission of itch to the higher centres.

It has also been shown that itch can be inhibited by different types of pain (thermal, mechanical, chemical or electrical) at spinal level. The normal reaction to itch itself, which is scratching, inhibits the pruriceptor firing (Akiyama et al, 2012) .

It is believed that there are interneurons in the spinal cord that release inhibitory neurotransmitters such as glycine and GABA that mediates the above mentioned pruriceptive inhibition (Akiyama et al, 2011).

Ablation of the transcription factor Bhlhb5 causes a selective loss of a subset of inhibitory neurons in the spinal cord that results in enhanced scratching response to pruritic agents (Ross et al, 2010).

Excessive scratching behaviour was also observed when knocking out the glutamate transporter VGLUT2 in a class of nociceptors (Lagerstrom et al, 2010; Liu et al, 2010b).

These findings point toward the idea that inhibitory interneurons are tonically active and only when their activity is reduced or abolished the itch sensation is unmasked.

1.4. The tyrosine kinase RET

1.4.1 Ret function and distribution: an overview

The development of many primary sensory neurons depends on growth factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), neurotrophin 4 (NT4) and the glial cell-line derived neurotrophic factor (GDNF) family.
Different subsets of sensory neurons usually express receptors specific for one or few neurotrophic factors, so that each of them exerts its function on a specific subpopulation.
Around 60% of DRG neurons express Ret, the signalling receptor for the GDNF family (Molliver et al, 1997), which has four members: GDNF, Neurturin, artemin and persephin. RET was first described as a proto-oncogene and it has been implicated in the progression of several types of tumors. It is a single-pass transmembrane tyrosine kinase that is required for normal development, maturation and maintenance of several tissues and cell types and is a highly conserved protein, from Drosophila through human (Mulligan, 2014). Its important role within the organism it's underlined by the fact that mutations associated with loss or reduction of RET function cause pathologies in humans and RET null mice die soon after birth (Schuchardt et al, 1994).
It is mostly expressed during embryogenesis and it has been demonstrated to be important for the development of the kidney, for spermatogonial stem cell maintenance, and it has multiple functions in the neuroendocrine and nervous systems. It is expressed in neural crest cells and it has an essential role for migration, development and maintenance of neurons of the central and peripheral nervous system (Mulligan, 2014).
In vertebrates, RET acts in concert with the glycosyl-phosphatidyl inositol-linked (GPI-linked) co-receptors called Gfrα1-4 that determine the ligand specifity of the receptor complex. GDNF binds to Gfrα1, Neurturin to Gfrα2, Artemin to Gfrα3 and Persephin to Gfrα4s.
The ligand binds first to its specific Gfrα receptor and then interacts with RET, recruiting it into cholesterol-rich membrane subdomains (lipid rafts) where the complex dimerizes and tyrosine phosphorylation and subsequent downstream pathways of the signalling occur. (Mulligan, 2014; Tansey et al, 2000).

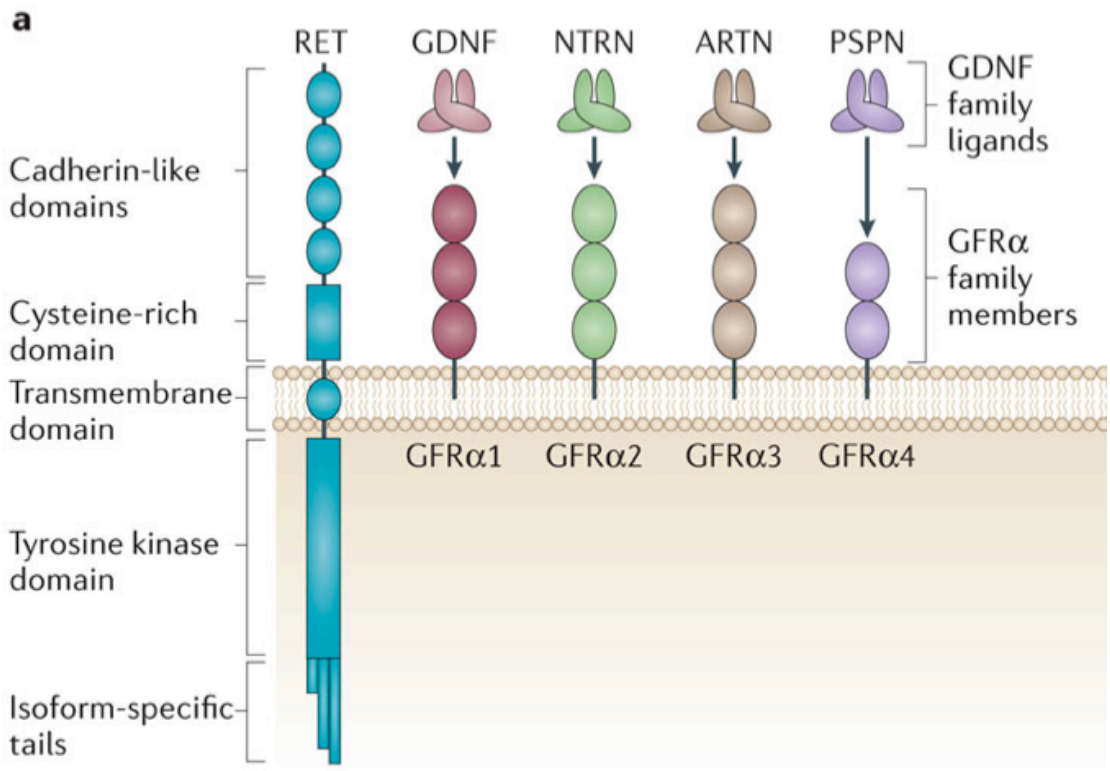

Figure 2. Ret is a transmembrane tyrosine kinase protein. It is the receptor for a family of soluble neurotrophic factors, GDNF. Its ligand specificity is determined by its co-receptors: they are 4 and belong to the GDNF family receptor α (Gfrα) family members. Part of a picture taken from Mulligan, 2014 ((Mulligan, 2014)

1.4.2. Ret in sensory neuron subsets

To date, Ret expression has been detected and characterized in 3 different sensory neuron subpopulations.

Of these, the most abundant and widely studied is the non-peptidergic nociceptor subtype. These neurons are born around embryonic day (E) 11,5 or later and at this stage they express TrkA, the receptor for the nerve growth factor (NGF). NGF controls Ret, Gfrα1 and Gfrα2 expression and these neurons begin to express Ret around E15,5 and, from P0 onward, Gfrα1 and Gfrα2. Ret signalling then autoregulates the expression of its co-receptors and determines the gradual extinction of TrkA expression, completely abolishing it around postnatal (P) day 14 (Luo et al, 2007). Moreover, it regulates the expression of several ion channels that are

known to be enriched in the non-peptidergic nociceptor subset, namely TRPA1, MrgprA1, MrgprA3, MrgprB4. Ret signalling is not necessary for neuronal survival, but it is crucial for normal cell size and for peripheral target innervation (Luo et al, 2007).

Among the Gfrα co-receptors, non-peptidergic nociceptors mainly express Gfrα2 (Luo et al, 2007).

There is a distinct, smaller population of Ret-positive neurons that are born prior to E11, 5 and express Ret from the beginning. These neurons do not express TrkA in any of their developmental stages and have large soma size. This subset, which is also known as the Early-Ret subpopulation, was hypothesised to be constituted by low-threshold mechanoreceptors and thus involved in the perception of discriminative touch stimuli. This was also supported by the observations regarding their peripheral and central afferents that were found around the hair follicle and in the mechanosensitive area of the dorsal horn of the spinal cord, respectively (Molliver et al, 1997).

The Early-Ret subpopulation was characterized more extensively in 2009 (Bourane et al, 2009; Luo et al, 2009) when it was shown that these neurons are Rapidly Adapting Low-threshold mechanoreceptors and therefore responsible for detecting vibration and pressure.

The Ginty group (Luo et al, 2009) used a mouse line in which the early-Ret population was genetically labelled to examine in detail this subset. They demonstrated that early Ret positive neurons have a large soma size and that they project centrally to lamina III-V of the dorsal horn of the spinal cord and peripherally to Meissner corpuscles, longitudinal lanceolate endings and Pacinian corpuscles. They also showed that at P14 all the early-Ret neurons coexpress Gfrα2. Moreover, they used a conditional Ret KO mouse to demonstrate that Ret signalling is not necessary for the survival of RA mechanoreceptors but is crucial for the development of Pacinian corpuscles and for proper central innervation.

Almost identical conclusions were made by Carroll group (Bourane et al, 2009).

By using immunohistochemistry and a RETcKO mouse, they also identified early-Ret neurons as RA mechanoreceptors and showed the importance of Ret in proper development and innervation of this subset. They further found an additional marker for this subset which is expressed during the embryonic stage, the transcription factor MafA.

A third population of Ret-positive sensory neurons has been recently identified (Li et al, 2011): they are small-diameter DRG neurons which do not express any other marker for non-peptidergic nor for peptidergic nociceptors. These neurons are characterized by the expression of TH (tyrosine hydroxylase).

By using an ex vivo skin nerve preparation, the authors observed that this subset was composed of fibers that displayed the classical features of C-fiber Low-Threshold mechanoreceptors. Moreover, more than 80% of TH-positive neurons expressed vGlut3 mRNA, which is found in C-LTMRs (Seal et al, 2009).

The authors genetically labelled TH-positive neurons and observed that their peripheral projections form longitudinal lanceolate endings that associate with 2 types of hair follicles(Driskell et al, 2009): the zig-zag (80% of C-LTMR) and awl/auchene (20% of C-LTMR) hair.

C-LTMRs have dual functions: under normal condition they detect the affective or emotional component of touch, but in pathological conditions they can cause pain and contribute to touch hypersensitivity after injury. Recently, it has been observed that the threshold of activation of these neurons is modulated by TAFA4, a chemokine-like secreted protein (Delfini et al, 2013).

Thus Ret marks several sensory neuron subpopulations and has an important physiological role in the peripheral sensory system. Many recent studies have contributed to elucidate its expression pattern and function, but some of these also pose new questions, especially regarding its distribution within sensory neurons: it is likely, in fact, that Ret marks also other neuronal subsets than the ones described above.

2. Aim of the project

The peripheral nervous system is formed by sensory neurons that differ appreciably in terms of function, morphology and electrophysiological properties. Identification and characterization of defined functional subsets is one of the main challenges of the field, and yet a lot about that is still unknown.

Peripheral sensory neuron cell bodies cluster together in the Dorsal Root Ganglia without any defined arrangement, or at least any known to date, so functional subsets cannot be identified according to their position. Moreover, although several markers have been described, they often display an overlapping pattern and are rarely confined exclusively to only one subpopulation.

In addition, many sensory neurons are polymodal, that is, they can detect different type of stimuli or, for example, change their threshold of activation in presence of injury thus modifying their response.

Identification of discrete subpopulation of DRG neurons is therefore complicated and the tools that are commonly used to study them, such as immunohistochemistry, do not usually allow detection of very small subsets whose presence might be overwhelmed by the multitude of markers and their relative expression.

We therefore sought to develop new tools for the analysis of sensory neuron subpopulations that allows not only identification and isolation of new subsets according to criteria such as expression of membrane markers and independently of their size, but a tool that also offers a pool of data for a complete characterization and proper investigation about systems biology of neuronal subsets.

For that purpose we used fluorescence-activated cell sorting (FACS) techniques. FACS has never been used in the context of peripheral sensory neurons and we sought to develop a protocol for a successful isolation of specific subsets of such a delicate type of cells in order to be able to perform gene expression profiling on them.

Information about expression of genes in a defined subset of sensory neurons has been obtained so far only by isolating neurons, for example, from mice lacking a specific gene and comparing the profile with the one obtained from total DRG of a wild-type mouse. In this way researchers have demonstrated how the expression is altered in absence of a particular gene and therefore its function, but the gene expression profile of a defined subset of sensory neuron has never been tested.

We focused on a group of neurons that specifically express eGFP protein using Ret gene as a driver. As mentioned above, Ret is present in at least 3 different neuronal subsets within the peripheral nervous system. Is it however likely that more Ret-positive populations exist as some studies have detected Ret expression in very small subsets of cells expressing markers different that the widely described IB4, NF200 or TH. For example, costaining with TrkA has been detected (Golden et al, 2010), as well as with TRPV1 and Gfrα3 (Elitt et al, 2006).

The major aim of this proposal was therefore to further characterize and quantify by FACS the Ret-positive subsets, isolate them and perform microarray analysis in order to have a pool of potential markers, transducing molecules, transcription factors, etc that can widen our analysis and allow for a complete characterization of neuronal subsets.

Based upon this strategy we identified at least five different subsets of Ret-positive neurons. We concentrated on the two of them who displayed the most homogeneous distribution and obtained their gene expression profiles.

Our focus therefore became to understand the function of these populations. As a starting point we considered the type of transcripts that were specifically enriched in those subsets and we subsequently performed functional characterization.

3. Main results

3.1. Immunofluorescent screening of Avil-Cre::Ret$^{eGFP/+}$ mouse sensory neurons

In order to visualize all Ret-positive neurons in Dorsal Root Ganglia, we took a genetic approach. We crossed an Avil$^{cre/+}$ mouse line, in which Cre is expressed exclusively in all peripheral sensory neurons (Zurborg et al, 2011), with a Ret$^{eGFP/+}$ line, where a "floxed" RET-eGFP allele has been targeted into allele 1 of the mouse Ret locus and can be conditionally deleted in those cells that express Cre. eGFP expression occurs only when the allele is deleted (Jain et al, 2006).

We obtained Avil-Cre::Ret$^{eGFP/+}$ mice which were viable, fertile and with no obvious abnormalities (> 1 year). With this strategy we specifically targeted Ret-positive DRG neurons avoiding any contamination from surrounding tissues, as Ret is widely expressed in nervous system.

We first characterized Avil-Cre::Ret$^{eGFP/+}$ neurons by immunohistochemistry. We observed very strong endogenous eGFP fluorescence that was present in 55±5 percent of DRG neurons (n=3 mice, total of 4129 cells). Strikingly, we noticed a broad range of intensities among the Ret-positive cells.

We costained DRG sections with markers that are known to mark specific ret-expressing sensory neuron subpopulations. We first used Isolectin B4 (IB4), which is a marker for non-peptidergic nociceptors, and we found an overlap with eGFP-positive cells (Figure 3 A-H): more precisely, 51,5±3,8 percent of Ret-eGFP neurons bound to IB4, confirming that most of the Ret-positive neurons are indeed non-peptidergic nociceptors. Moreover, most of those neurons displayed small or medium size cell bodies and, as mentioned above (1.2.), this is a feature of nociceptive neurons.

We also tested coexpression of Ret and NF200 in order to identify the early-Ret DRG neurons that constitute the RA mechanoreceptor group (Bourane et al, 2009; Luo et al, 2009), responsible for the detection of innocuous touch stimuli such as shape and texture. A small proportion of Ret-eGFP cells (13±1 percent) coexpressed NF200 (Figure 3 E-D, I-L). They also exhibited a large soma size, as typical for mechanoreceptors.

The third Ret-positive subset, known to coexpress TH (tyrosine hydroxylase) (Franck et al, 2011; Li et al, 2011), was also detected in our samples: 22±2,3 percent of Ret-eGFP neurons was positive for TH (Figure 3 E-L).

Importantly, we observed that there were Ret-expressing neurons that did not stain to any of the above-mentioned markers, and their proportion was consistent and worth further investigation.

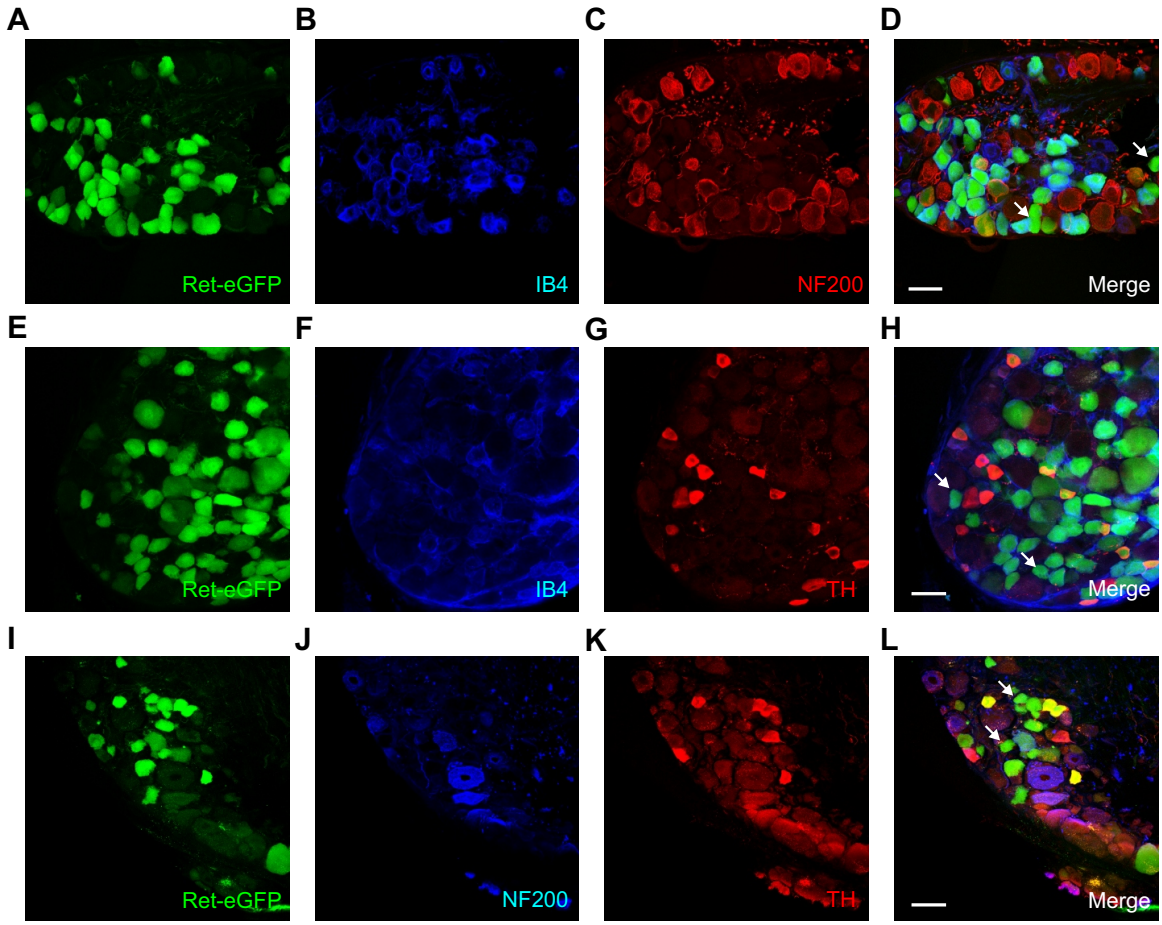

Figure 3. In DRG sections Ret-eGFP expression overlaps with IB4, a marker for non-peptidergic nociceptors (A-H), with the mechanoreceptive marker NF200 (A-D, I-L) and with TH, which marks C-fiber low threshold mechanoreceptors. Arrows in D, H and L indicate neurons that express only Ret-eGFP. Scale bars, 50 μm.

Expression of eGFP was detectable also at the central and peripheral endings, which allowed us to further describe Ret-positive subsets. In the spinal cord Ret-eGFP intensity was detectable from lamina I through lamina V, even though in different proportions (Figure 4). This is consistent with previous observations (Luo et al, 2009). The densest plexus of expression was found in correspondence with IB4 labelling (Figure 4 A-H) in outer lamina II (II_o), delineating the innervation of the nonpeptidergic nociceptors and consistent with the fact that these neurons represent the largest population among the Ret-positive subsets. From lamina III to V Ret-eGFP expression was sparser and overlapped with the NF200-positive mechanoreceptive area (Figure 4 I-L), corresponding to the early-Ret group of neurons. EGFP fluorescence was also present in the internal lamina II (II_i), overlapping with PKCγ-positive interneurons present in that area (Figure 4 E-L). It has been shown that C-LTMR fibers terminate in lamina IIi (Li et al, 2011), so it is likely that those neurons correspond to the TH-positive subset of Ret-positive neurons. We also noticed a small overlap of eGFP-positive fibers with CGRP in lamina I (Figure 4 A-D).

Regarding the peripheral afferents of Ret-eGFP neurons, their wide distribution again confirms the heterogeneity of Ret-positive subsets: in fact the fluorescence was broadly distributed in both dermis and epidermis as free nerve endings and hair-encircling fibers (Figure 5).

During the last years much information about Ret-positive neuronal subtypes has been emerging. We confirmed that there are 3 different groups of Ret expressing cell (IB4-positive nonpeptidergic nociceptors, NF200-positive RA mechanoreceptors and TH-positive C-fiber low-threshold mechanoreceptor) by immuohistochemistry, but our analysis also pointed out to the existence of at least one additional population that did not co-stain with any of those markers.

Even though the utility of immunohistochemical tools is unquestionable, there are only a small number of antibodies that can be used together within the same experiment and it is difficult to investigate multiple subsets at the same time. Moreover, many markers are unknown, as are the majority of the molecules with a functional role in the systems biology of sensory neuron: their discovery would ease the progress of the field.

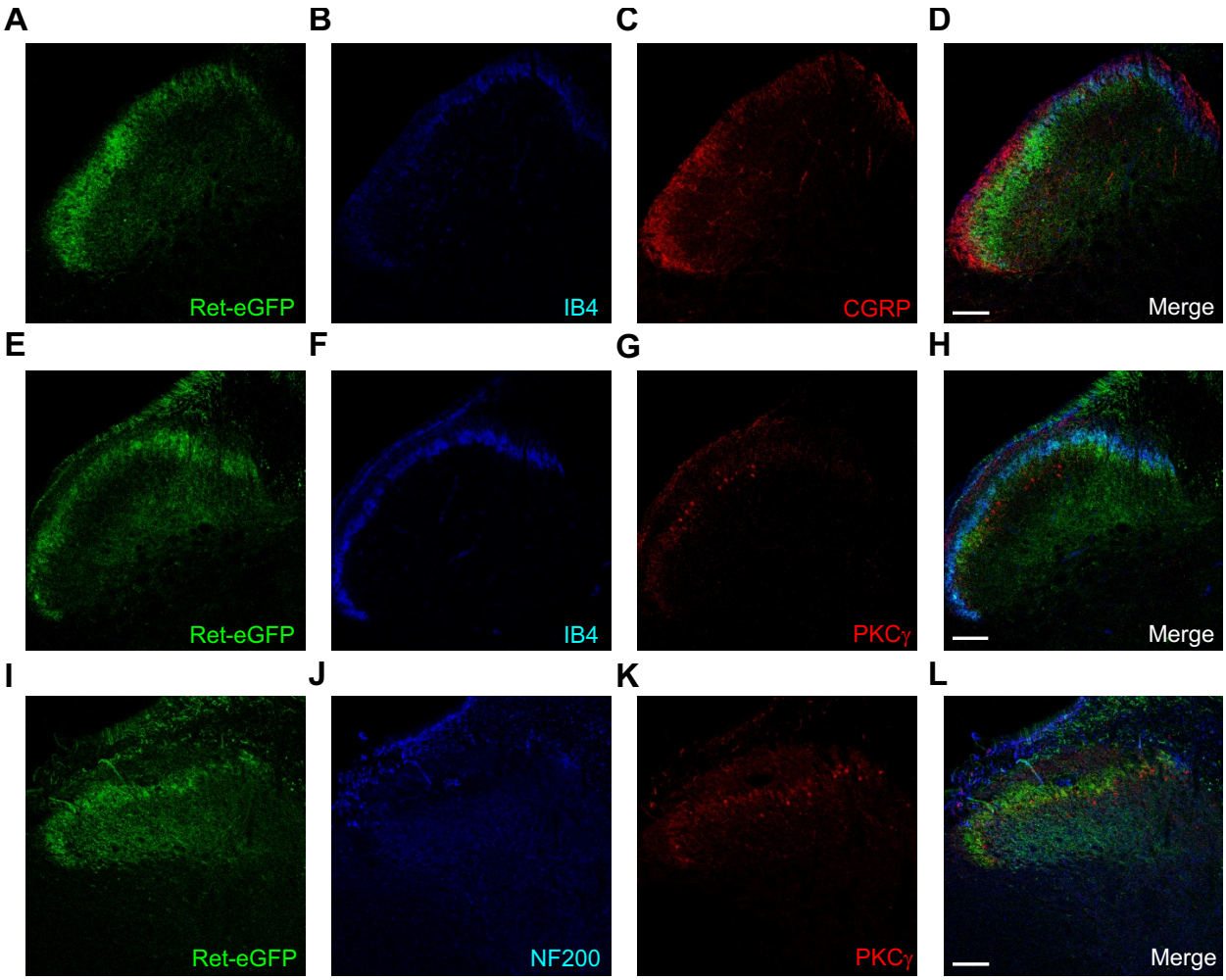

Figure 4. The central terminals of Ret-eGFP expressing neurons are broadly distributed within the dorsal horn of the spinal cord, from lamina I through lamina V. Ret-eGFP fibers are particularly dense in lamina II_o, where they overlap with IB4-binding terminals: this group constitutes the non-peptidergic nociceptor subset. Ret-eGFP expression was also detected in lamina II_i overlapping with PKCγ-positive interneurons, thus representing TH-positive subtype. Ret-eGFP-positive afferents were also present from lamina III to V, the mechanoreceptive area, where they overlapped with NF200. Scale bars, 100 μm

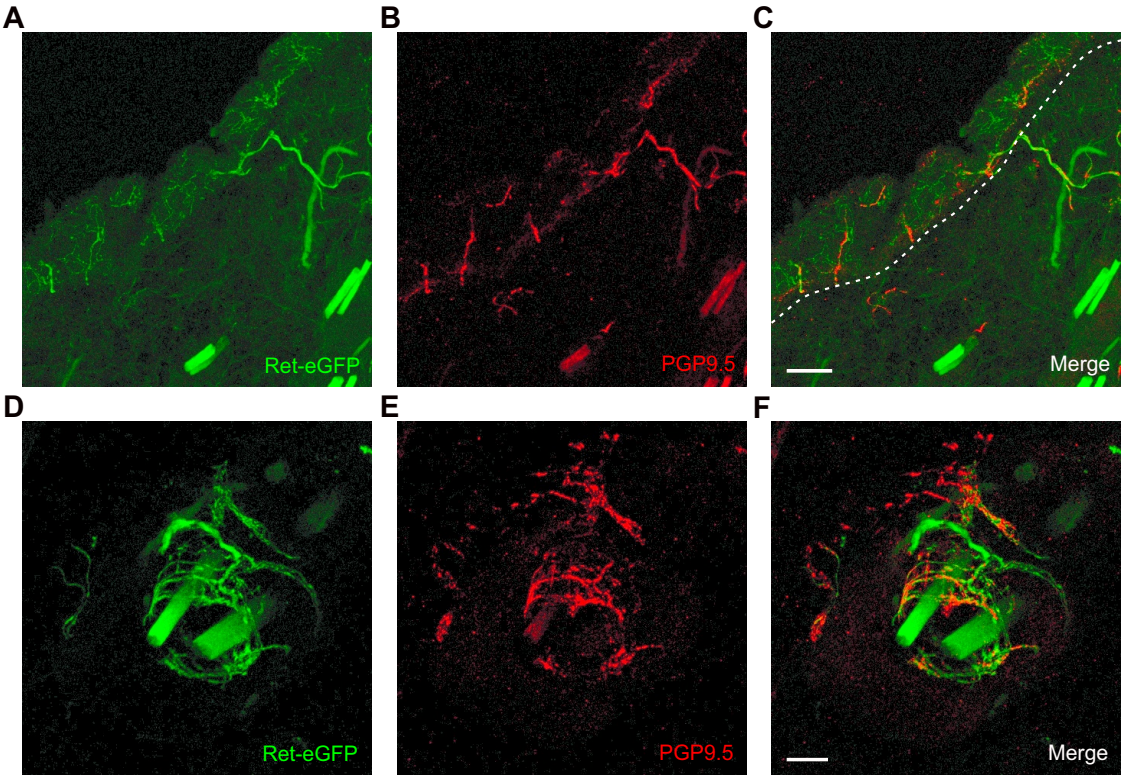

Figure 5. Double immunostaining of Ret-eGFP and the pan-neuronal marker PGP9.5 on Avil-Cre::Ret$^{eGFP/+}$ mouse hairy skin. Ret-eGFP peripheral afferents are widely distributed in the dermis and epidermis, as free nerve endings (A-C) and hair encircling fibers (D-F). The dashed line in C indicates the epidermis/dermis junction. Scale bars, 25 μm

3.2. Fluorescence-activated cell sorting (FACS) as a tool to isolate sensory neuron subsets.

When performing immunohistochemical analyses we noticed that the endogenous eGFP intensity was quite variable within all the classes of Ret-positive cells. We reasoned that such a variability might reflect a further functional diversity among the different Ret-eGFP subsets. An optimal method to investigate the distribution of the different intensities of eGFP is Fluorescence-Activated Cell Sorting (FACS), which can detect diverse fluorescent signals with much more sensitivity than microscopes commonly used to image immunofluorescent assays. Importantly, it can also quantify, describe in terms of size and shape complexity and physically isolate the cells of interest, keeping them alive.

FACS is not a technique commonly used in neuroscience: neurons in general, and adult sensory neurons in particular, are quite delicate and it is very difficult for them to undergo all the processes of isolation, staining and analysis that are necessary for a FACS experiment without compromising their integrity. Moreover, the amount of sensory neurons that is possible to collect from an animal is very low making FACS analysis of DRG neurons very challenging.

We succeeded in applying the flow cytometric analysis to dissociated neurons isolated from Avil-Cre::$Ret^{+/eGFP}$ mice and stained with Isolectin B4. We used IB4 because, among the various markers used for Ret-positive neuron characterization, is the only extracellular marker and can then be used in live imaging.

Cells were additionally stained with a marker for hematopoietic cells, cd45, in order to identify the immune system cells and eliminate them from the analysis. Furthermore, they were stained with a living dye that marks the dead cells that were thus excluded from any further quantification and sorting (Figure E1 in part II).

All the live sensory neurons detected were analysed in terms of eGFP fluorescence intensity and IB4 binding. Among the Ret-positive neurons we observed both IB4-positive and IB4-negative cells, as expected, and also cells with different levels of eGFP fluorescence which were clearly grouping to form well-defined, separate subsets. Combining the information regarding eGFP intensity level and IB4 binding we could identify at least 5 different subpopulations, which we termed Ret-$eGFP^{Lo}$:$IB4^{Neg}$ (A), Ret-$eGFP^{Hi}$:$IB4^{Neg}$ (B), Ret-$eGFP^{Lo}$:$IB4^{Lo}$ (C), Ret-$eGFP^{Hi}$:$IB4^{Lo}$ (D), and Ret-$eGFP^{Hi}$:$IB4^{Hi}$ (E) (Figure 6 A).

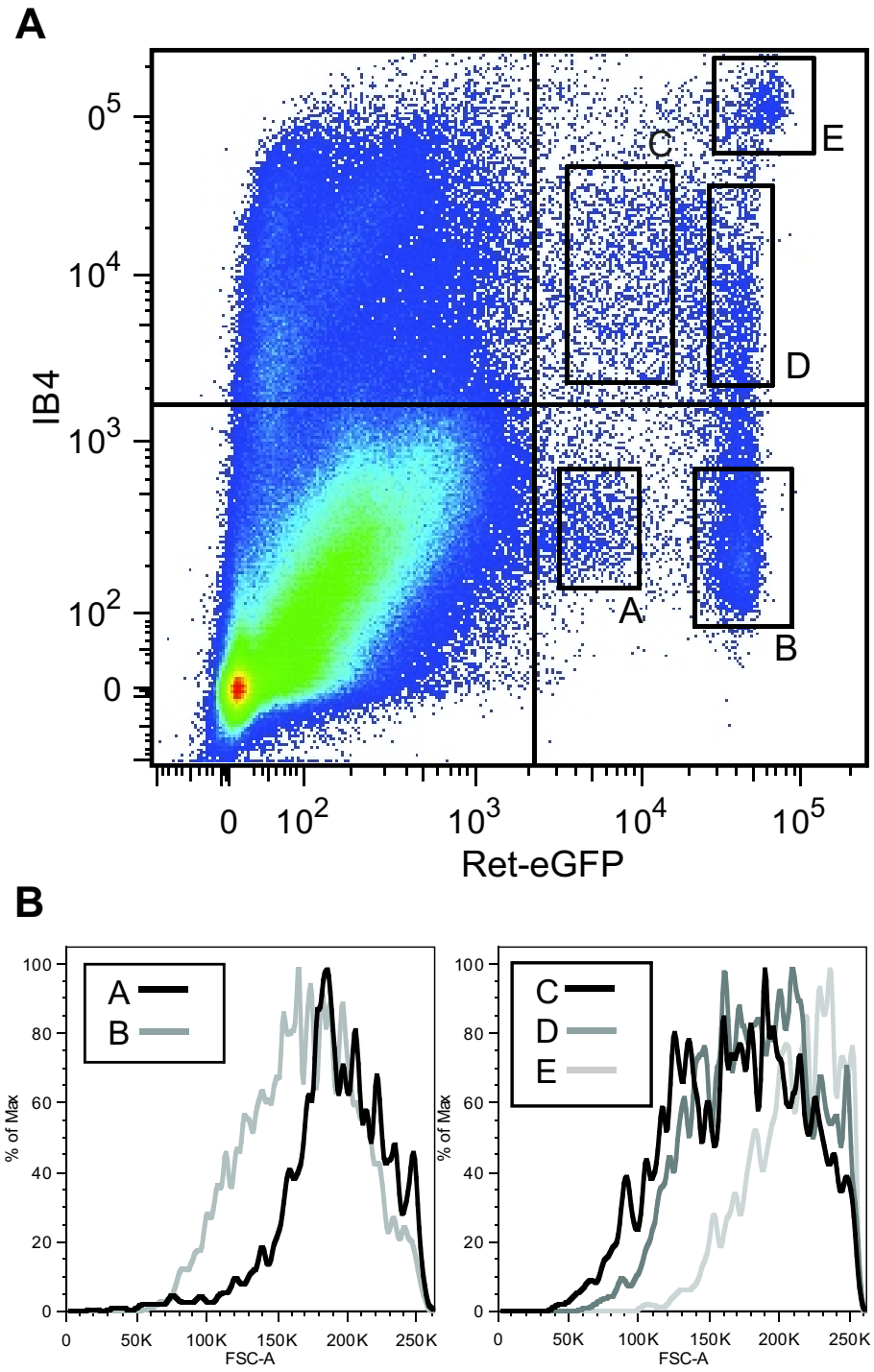

Figure 6. A- Flow cytometric analysis of dissociated Avil-Cre::Ret$^{+/eGFP}$ sensory neurons plotted according to the level of their eGFP intensity and IB4 binding. We observed 5 well-defined populations of Ret-eGFP expressing cells. A and B did not bind to IB4 but expressed Low and High level of eGFP, respectively. C, D and E did bind to IB4 and displayed a range of eGFP intensities. B- eGFP intensity level and IB4 binding were not correlated to the median cell size of the different subsets. The graph shows the forward scatter values plotted against the normalized number of cells, displayed as the percent of Max.

We took advantage of the sorting-function of the FACS to isolate the 2 Ret-positive subsets that did not bind to IB4, namely Ret-eGFP^{Lo}:IB4^{Neg} (A), Ret-eGFP^{Hi}:IB4^{Neg} (B) and perform expression profiling. We choose these 2 populations because they were the best defined and apparently the most homogeneous among the ones observed: in fact, despite the low amount of neurons within these 2 subsets, their distribution fell always down in the same range of size, GFP intensity and IB4 binding.
It is worth noting that the differences that the 2 sorted subpopulations displayed in eGFP expression level was not due to a variance in their size, as the range of their size distribution was the same (Figure 6 B).

3.2.1. Expression profiling of the sorted subsets

Sorting of the Ret-eGFP^{Lo}:IB4^{Neg} and Ret-eGFP^{Hi}:IB4^{Neg} subpopulations was performed in replicates. We then isolated RNA from the sorted cells and performed differential microarray screening.
The results from the 2 screenings were compared in order to analyse the genes that were differentially expressed between the two samples (Figure 7 A).
What we first observed, was that Ret expression was 10 fold greater in the Ret-eGFP^{Hi}:IB4^{Neg} subset compared to Ret-eGFP^{Lo}:IB4^{Neg}, consistent with the level of eGFP expression detected by FACS. Moreover, we found differential expression of the Gfrα co-receptors: Gfrα3 was enriched in Ret-eGFP^{Lo}:IB4^{Neg} while Gfrα2 was upregulated in Ret-eGFP^{Hi}:IB4^{Neg}. Importantly, we found significantly higher level of the mechanosensitive channel Piezo2 (Fam38b) in the Ret-eGFP^{Hi}:IB4^{Neg} subset.
Interestingly, none of the samples were enriched for TH.
Thus Ret-eGFP^{Hi}:IB4^{Neg} subset displays an up-regulation of Ret, Gfrα2 and Piezo 2: Gfrα2 has been shown to be the preferred Ret co-receptor in RA mechanoreceptors (Bourane, Garces et al. 2009; Luo, Enomoto et al. 2009) and only mechanosensitive neurons should express Piezo2. We thus speculate that the Ret-eGFP^{Hi}:IB4^{Neg} population might correspond indeed to the RA mechanosensitive Ret-positive neurons.
Regarding Ret-eGFP^{Lo}:IB4^{Neg} neurons, they express many other interesting molecules, including several with a potential functional role, but almost none of those has been previously associated with Ret-positive neurons.

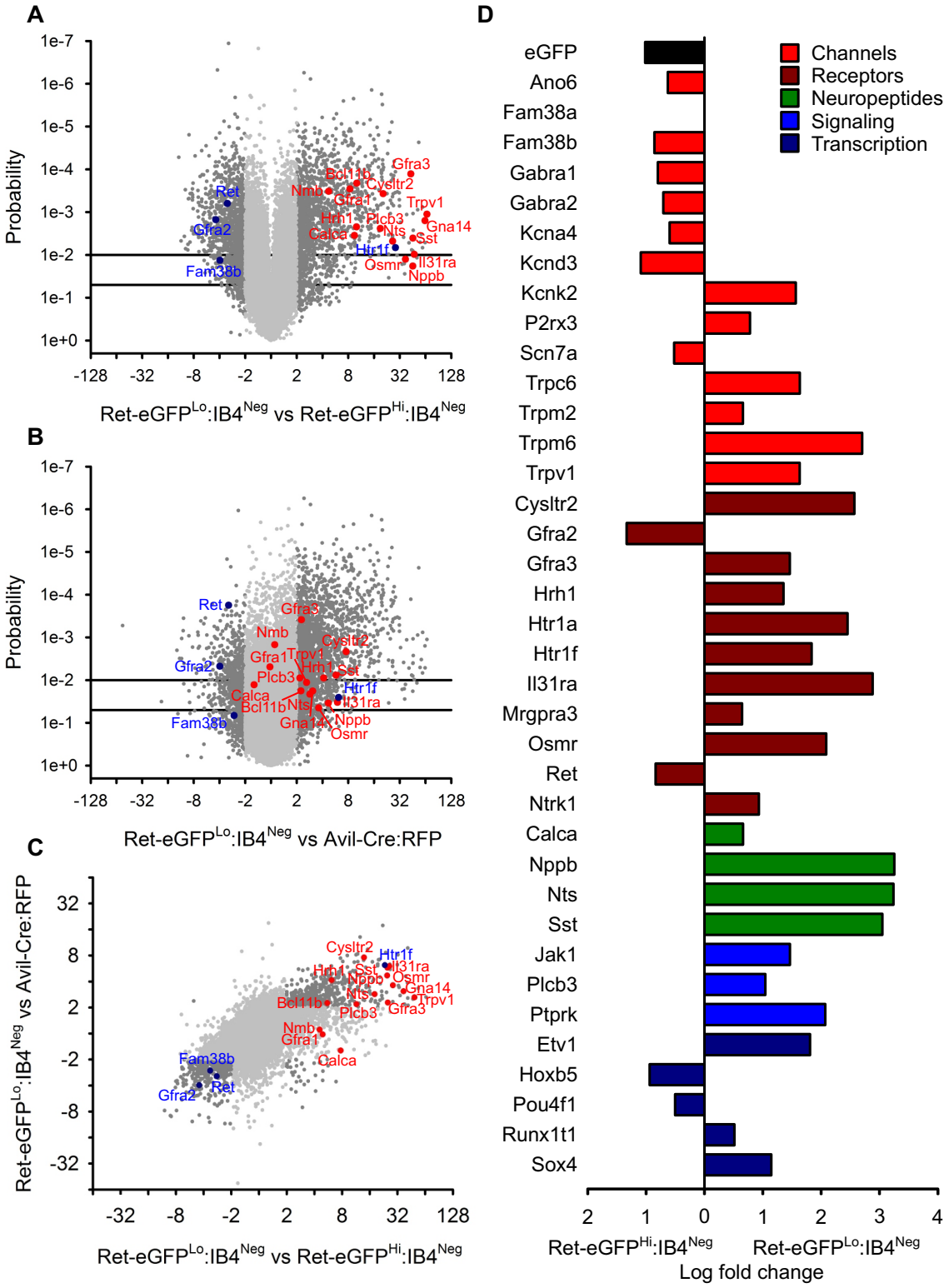

Figure 7. Volcano plots of fold change expression in Ret-eGFP^{Lo}:IB4^{Neg} versus Ret-eGFP^{Hi}:IB4^{Neg} (A) and versus all DRG neurons (B) against probability. A triple comparison between Ret-eGFP^{Lo}:IB4^{Neg}, Ret-eGFP^{Hi}:IB4^{Neg} and Avil-Cre::R26^{tdRFP} datasets is also shown (D). Itch-associated transcripts (marked in red) were enriched in Ret-eGFP^{Lo}:IB4^{Neg} subset, as further confirmed by quantitative RT-PCR (D).

The only exception is TrkA: expression of this receptor, which we found up-regulated in Ret-eGFP^{Lo}:IB4^{Neg}, has been described in a small subset of adult Ret-positive DRG neurons (Golden et al, 2010; Molliver et al, 1997). However the function of this subset is unknown as the scarsity of Ret-positive/TrkA-positive neurons have made difficult its further characterization.

Our Ret-eGFP^{Lo}:IB4^{Neg} subset displays also other features that have been previously annotated, but never fully investigated, in scarce subsets of Ret-positive sensory neurons: for example a different level of Ret expression in DRG has already been noticed, and more precisely a weak expression of Ret has been associated to Gfrα1 and Gfrα3 co-expression (Luo et al, 2007).

Notably, in Ret-eGFP^{Lo}:IB4^{Neg} cells, Gfrα3 is 29 fold up-regulated compared to Ret-eGFP^{Hi}:IB4^{Neg} neurons.

It is also worth noting that this subset does not bind to IB4, so it is not a nonpeptidergic nociceptive population, but on the other hand it does express molecules such as TrkA, CGRP, TRPV1 that are known to be absent from Rapidly Adapting or C-fiber Low Threshold mechanoreceptors. Consistent with our findings, co-expression of TrkA, CGRP and TRPV1 has been observed in Ret and Gfrα3-positive sensory neurons and, in addition, it has been shown that the majority of Gfrα3-positive cells do not bind to IB4 (Orozco et al, 2001).

We thus conclude that the Ret-eGFP^{Lo}:IB4^{Neg} subset that we isolated corresponds to a new, uncharacterized, Ret-positive population, possibly matching the TrkA-positive subset mentioned above. Since it is a very small population, this probably explains the lack of previous characterization. However, the expression profile of this subset described here provides a large amount of information with which to trace the functional identity of this population.

3.2.2. Ret-eGFP^{Lo}:IB4^{Neg} neurons are putative itch receptors

In order to investigate the function of the new Ret-positive subset, we screened all the genes that appeared to be at least two times more expressed in Ret-eGFP^{Lo}:IB4^{Neg} compared to Ret-eGFP^{Hi}:IB4^{Neg}, focusing on the ones that displayed the biggest fold change between the two samples and on those that could have a functional role in neuronal response, such as ion channels, transcription factors, signalling molecules.

Surprisingly, we observed that our subset was highly enriched in molecules that have been previously implicated in itch perception. For example, we found up-regulation of HRH1 (histamine receptor 1) that, together with phospholipase-C-beta-3 (Plcb3) and TRPV1 (also up-regulated in Ret-eGFP^{Lo}:IB4^{Neg} neurons) constitutes part of the pathway responsible for histamine-evoked itch (Han et al, 2006).

Moreover, high expression of the neurotransmitters Nppb and neuromedin B was observed. An important role for Natriuretic Polypeptide B (nppb) in spinal itch transmission has been recently described, and it has been shown to drive itch sensation arousal from several pruritogens (Mishra & Hoon, 2013). Additionally, Nppb is expressed in a subset of small unmyelinated, TRPV1-, MrgprA3- and PLCβ-positive sensory neurons, and its receptor Npra is found in Lamina I spinal neurons. This is again consistent with our findings as the above-mentioned markers are all up-regulated in our sample.

A role for Neuromedin B (Nmb) in itch perception has also been hypothesised as activation of its receptor evokes strong pruritus (Sukhtankar & Ko, 2013).

It is also worth focusing on the enrichment of MrgprA3 in Ret-eGFP^{Lo}:IB4^{Neg}. MrgprA3 is the receptor for Chloroquine, the antimalarial drug that can provoke itch as side effect. It is known that Chloroquine-induced itch follows a non-histaminergic pathway. However, MrgprA3 neurons do respond to histamine (Han et al, 2013), which means that they express histamine receptors, and coexpression of MrgprA3 and HRH1 is what we observed in Ret-eGFP^{Lo}:IB4^{Neg} subset. However, MrgprA3-positive neurons have been shown to coexpress IB4 and CGRP (Han et al, 2013), while in our subset IB4 binding has been excluded. On the other hand, CGRP is expressed.

Importantly, we observed high expression of the co-receptors for Interleukin31, Interleukin 31 receptor α (IL31RA) and Oncostatin receptor M β (OSMR), which indicates a potential clinical relevance for this subset. Indeed high levels of Interleukin 31 in the skin have been linked to pathogenesis of Atopic Dermatitis in both human and mice (Dillon et al, 2004; Sonkoly et al, 2006). IL-31 is mainly produced by TH2 cells but it directly activates sensory neurons, in particular a small subset that coexpress IL31RA, TRPV1 and TRPA1 (Cevikbas et al, 2013).

Considering the identity of the molecules that we found enriched in the Ret-eGFP^{Lo}:IB4^{Neg} population and their relative combination, we believe we have isolated a small, specific subset of Ret-positive cells that has never been characterized before and that this subset is involved in itch sensation.

3.2.3. Gene expression profiling results are specific for Ret-eGFP^{Lo}:IB4^{Neg} subset

In our gene expression analysis we considered the genes that were differentially expressed in 2 populations: Ret-eGFP^{Lo}:IB4^{Neg} and Ret-eGFP^{Hi}:IB4^{Neg}. Up-regulation within one of these subsets is thus not necessarily linked to specificity of gene expression as some of the molecules that I listed above might be expressed as well in populations different from the ones that we analysed.

To overcome this bias, we performed a sorting and expression profiling of total DRG neurons. To do so we used Avil-Cre::R26^{tdRFP} mice in which the majority of peripheral sensory neurons are marked by RFP fluorescence.

DRG neurons were isolated from Avil-Cre::R26^{tdRFP} mice and stained in solution with cd45, the marker for hematopoietic cells, and a living dye, in order to exclude from the analysis all the immune system and all the dead cells, respectively (Figure E3 in part II).

Only the neurons displaying RFP fluorescence were sorted. The sorting was done on 3 biological replicates and for each of them we performed expression profiling, namely microarray, thus obtaining a dataset of levels of gene expression in total DRG neurons.

We then compared Ret-eGFP^{Lo}:IB4^{Neg} to Ret-eGFP^{Hi}:IB4^{Neg} and to this new microarray screen (Figure 7 B and C). In general, fold changes appeared lower than for comparisons with Ret-eGFP^{Hi}:IB4^{Neg} alone, probably reflecting the heterogeneity of total DRG neurons. Moreover, some of the genes that in the previous analysis appeared up-regulated in Ret-eGFP^{Lo}:IB4^{Neg}, such as Calca (that encodes for CGRP), are no longer enriched in this subset. That is however expected as many genes are expressed in more than one neuronal subset and in particular, CGRP is known to be highly expressed in peptidergic nociceptors that form a large Ret-negative population: this population is included within the total DRG sample thus largely contributing to its level of Calca transcript.

However, itch-related molecules such as IL31RA, OSMR and nppb are still enriched in Ret-eGFP^{Lo}:IB4^{Neg} subset and this strengthens our hypothesis that these neurons are indeed involved in itch sensation.

Interestingly, we also detected high and specific expression of the two neuropeptides somatostatin (sst) and neurotensin (nts). These have been studied mostly in rats where they exert an analgesic effect at the spinal cord level (Sarret et al, 2005; Todd et al, 1998).

This new screening provided us with a list of genes that are specifically expressed in Ret-eGFP^{Lo}:IB4^{Neg} neurons and that can be used as markers for this population.

3.2.4. Validation of the gene expression profiling

To validate our microarray analyses, we performed quantitative RT-PCR using a microfluidic platform (Fluidigm).

The cells were collected following the same procedure used for microarray: DRG neurons were isolated from Avil-Cre::Ret$^{+/eGFP}$ mice, stained with IB4, cd45 and the living dye Sytox Blue and analysed by FACS. Ret-eGFP^{Lo}:IB4^{Neg} and Ret-eGFP^{Hi}:IB4^{Neg} subsets were sorted and processed for qRT-PCR.

For the experiment we selected 38 transcripts that we found to be up-regulated in Ret-eGFP^{Lo}:IB4^{Neg} or Ret-eGFP^{Hi}:IB4^{Neg} subsets. We chose the genes that represented itch associated transcripts, but also ion channels, signalling molecules and transcription factors with a demonstrated role in the peripheral nervous system.

Expression of all of these transcripts was tested in both the samples by using a 48.48 Fluidigm Dynamic Array and with technical and biological replicates. Relative expression was normalized to GAPDH.

The results totally confirmed the data obtained by microarray (Figure 7 D and E4 in part II). In particular, Ret expression was 10-fold higher in Ret-eGFP^{Hi}:IB4^{Neg} subset, as well as eGFP expression, thus fully validating the flow cytometry analysis. Moreover, Gfrα2 and Piezo 2 were up-regulated in this sample, supporting the assumption that it represents the RA mechanoreceptors.

As regards to the Ret-eGFP^{Lo}:IB4^{Neg} sample, we found that the highest differential expression was for itch-related transcripts, such as the membrane receptors IL31RA, OSMR, HRH1, and the neuropeptide nppb, again supporting our previous hypothesis that this population of sensory neurons has a role in itch perception.

3.3. Functional characterization of Ret-eGFP^{Lo}:IB4^{Neg} population

3.3.1. Three functional targets: HRH1, IL31RA and 5-HT$_{1f}$

Based upon our microarray screening, we hypothesized that Ret-eGFP^{Lo}:IB4^{Neg} population reflects a new itch-sensitive subset of sensory neurons. To test this assumption we undertook a functional characterization of this population concentrating on 3 membrane receptors: HRH1, IL31RA and 5-HT$_{1f}$.

HRH1 is the histamine receptor preferentially expressed in pruriceptors. Histamine is the most widely used pruritogen in the experimental induction of itch. However it is naturally produced by many cell types and in particular by mast cells in case of tissue inflammation or allergic stimulation.

We selected HRH1 for our functional analysis because, even though it has been widely studied, it has never been observed in Ret-positive cells. Moreover, functional assays for histamine are well assessed and we used it as a positive control for our other investigations.

IL31RA is the receptor for Interleukin31, which has a demonstrated role in the pathogenesis of atopic dermatitis. This is one of the most common itch associated diseases, with a prevalence of 2-5%. It is an inflammation of the dermis and the epidermis, chronically relapsing, causing an intense itch and thus affecting enormously the quality of life of the affected people. Antihistamines are used for relief, but their efficacy is limited and to date a fully efficient long-term therapy is unfortunately lacking.

Sensory neurons expressing Interleukin 31 receptor are therefore potential clinical targets. We have a dataset of transcripts that are enriched in a subset of sensory neurons that might be involved in atopic itch perception: if we demonstrate this involvement we could immediately start searching for a pharmacological target.

When screening the expression profile data we also noticed that a serotonin receptor, namely 5-HT$_{1f}$ (also known as Htr1f), was among the most highly expressed transcripts in Ret-eGFP^{Lo}:IB4^{Neg} population compared to both Ret-eGFP^{Lo}:IB4^{Neg} and total DRG neurons, expression that was also validated by Fluidigm.

Serotonin is pruritogenic and injections in the skin of mice provoke scratching (Akiyama et al, 2009). Several serotonin receptors exist, but a role in itch has been assessed so far only for 5-HT$_2$ (Yamaguchi et al, 1999). 5-HT$_{1f}$ is a receptor that couples to the inhibition of adenylate

cyclase (Adham et al, 1993), so we reasoned that its activation could lead to inhibition of Ret-eGFP^{Lo}:IB4^{Neg} neuronal activity.

Importantly, a selective agonist for 5-HT$_{1f}$ is available, LY344864, which can be used for functional characterization of these neurons

3.3.2. Functional characterization strategy

To assess the function of Ret-eGFP^{Lo}:IB4^{Neg} neurons, we decided to perform first calcium imaging experiments on cultured neurons isolated from Avil-Cre::Ret$^{+/eGFP}$ mice. Calcium is an essential intracellular messenger in mammalian neurons and its concentration rises during their electrical activity. Through calcium imaging techniques it is possible to monitor the intracellular calcium increase in cultured cells and thus detect activated neurons. For our experiments we used the calcium indicator Fura-2AM, a membrane-permeable ratiometric fluorescent dye. It is excitable by ultraviolet light at 340 nm and 380 nm and the ratio of the emissions at those wavelengths is directly correlated to the amount of intracellular calcium, and thus neuronal activation.

We cultured neurons from Avil-Cre::Ret$^{+/eGFP}$ mice and stained them with Isolectin B4. Unfortunately the sensitivity of the microscope was not high enough to distinguish in an objective way different levels of Ret expression, so we concentrated on 3 neuronal subsets: Ret-eGFP/IB4-negative, Ret-eGFP/IB4-positive and Ret-eGFP-negative cells

We then loaded the cells with Fura-2AM and applied histamine, IL-31 or LY344864 via perfusion. The cells that were activated by each compound had an increase of intracellular calcium that provoked a switch in the emission peak of Fura-2AM and an increase in the 380/340 ratio. We could then determine the proportion and the identity, in terms of eGFP expression and IB4 binding, of the cells responsive to each of the above-mentioned compounds.

We sought to explore the function of Ret-eGFP^{Lo}:IB4^{Neg} neurons also in vivo. To do so we injected into the nape of Avil-Cre::Ret$^{+/eGFP}$ mice histamine, IL-31 or LY344864 and monitored the scratching behaviour that each of the substances evoked. Each animal was recorded for 30 minutes after the injection and bouts of scratching were counted.

In addition, we assessed the induction of c-fos expression in spinal cord slices. Upregulation of C-fos in spinal cord interneurons indicates recent cell activity, so it represents an indirect

marker of neuronal activation and indicates where the central afferents of our subset terminate within the dorsal horn of the spinal cord.

3.3.3. Histamine and HRH1

We first investigated the activation of Ret-eGFP/IB4-negative cells by histamine. After culturing DRG neurons isolated from Avil-Cre::Ret$^{+/eGFP}$ mice, staining them with IB4 and loading them with Fura-2AM ,we performed calcium imaging.

When we perfused the cells with histamine, we saw that 9,7% of all neurons were activated (542 total cells). Among these, we assessed the proportion of Ret-eGFP/IB4-negative, Ret-eGFP/IB4-positive and Ret-eGFP-negative cells and we found that 54% of the responding cells belonged to the Ret-eGFP/IB4-negative subset, confirming that some cells that express Ret but do not bind to IB4 are actually activated by histamine (Figure 8A). The fact that we found responding cells also within the other 2 subsets is not surprising as histamine can act on several neuronal populations.

We then performed an in vivo analysis by injecting subcutaneously 8 wild-type or Avil-Cre::Ret$^{+/eGFP}$ mice with 1μM histamine. The mice injected with histamine scratched much more than controls injected with saline, as expected (Figure 8A in part II). We then collected the cervical part of the spinal cord of the Avil-Cre::Ret$^{+/eGFP}$ mice treated with histamine and performed c-fos staining. We observed a widespread activation of spinal cord interneurons mainly located in superficial lamina I of the dorsal horn and in lamina III and few of them were overlapping with eGFP (Figure E6 A-D in part II). Therefore, we can conclude that some of the Ret-eGFP/IB4 negative neurons do respond to histamine.

There is a long-standing debate about the coexistence within the same cells of histaminergic and non-histaminergic pathways. In our subset we found high expression of both HRH1 and MrgprA3: the latter is the receptor that mediates chloroquine response, which occurs through a non-histaminergic pathway (Wilson et al, 2011). The histamine and chloroquine receptors function through activation of TRPV1 and TRPA1 channels respectively. We thus performed calcium imaging on Avil-Cre::Ret$^{+/eGFP}$ neurons with chloroquine, capsaicin (the TRPV1 agonist) and allyl isothiocyanate (AITC, the TRPA1 agonist). The three compounds activated a broad range of DRG neurons, including Ret-eGFP/IB4 negative cells (Figure 8 B-D). In particular, 72% of the neurons that responded to chloroquine (475 total cells) belonged to that population. This data support the idea that histamine-dependent and histamine-independent

pathways might coexist within the same neurons. A convergence of these different mechanisms has been shown in a subset in trigeminal ganglia (Roberson et al, 2013) which could be the correlate of Ret-eGFP/IB4-negative in the DRG.

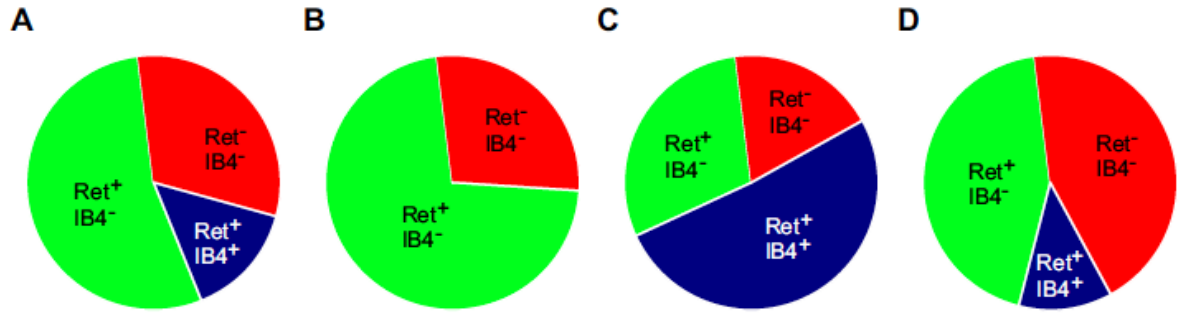

Figure 8. Calcium imaging was performed on dissociated Avil-Cre::Ret$^{+/eGFP}$ neurons. Here we show the proportions of responding cells that were Ret-eGFP/IB4 negative, Ret-eGFP/IB4 positive and Ret-eGFP negative. Histamine and Chloroquine (A and B, respectively) activate mostly Ret-eGFP/IB4- negative neurons. AITC (C) activates predominantly IB4-positive neurons, while capsaicin (D) mainly IB4-negative cells.

3.3.4. Interleukin 31 and IL31RA

As mentioned above, Interleukin 31 is a clinically important molecule because of its involvement in the pathogenesis of atopic dermatitis. We first examined the expression of its receptor IL31RA in DRG neurons. There are no dedicated antibodies that work for immunofluorescence, so we used an active, fluorescently labelled IL-31 molecule to mark IL31RA positive cells. This consisted of a mono-biotinylated IL-31 conjugated with streptavidin alexa-fluor 546 that was applied directly to cultured Avil-Cre::Ret$^{+/eGFP}$ neurons. A small subset of cells was marked: the majority was represented by neurons that weakly expressed Ret and did not bind to IB4, thus confirming our microarray data (Figure 9 A-D).
As a further proof we performed calcium imaging on Avil-Cre::Ret$^{+/eGFP}$ neurons, using 2nM of IL-31. We demonstrated that a small subset of neurons (5,6% of all neurons, 502 total

cells) was robustly activated by IL-31 and the majority of those (83,9%) were Ret-eGFP/IB4 negative cells (Figure 9 E-H).

This result indicates, as recently shown (Cevikbas et al, 2013) that IL-31 may function by directly activating sensory neurons: we further believe that it activates in particular a subset that expresses Ret but does not bind to IB4. This activation could then be the first step in transducing an inflammatory cascade into a behavioural response, which in this case would be scratching.

A similar finding has been recently made for another cytokine, thymic stromal lymphopoietin (TSLP) that acts directly on sensory neurons to produce robust scratching (Wilson et al, 2013). TSLP has also been suggested to trigger initiation and maintenance of Atopic Dermatitis, but it activates a subset of sensory neurons that was not sensitive to either histamine or chloroquine. Moreover TSLP receptor transcripts (Il7r and crlf2) were not enriched in our Ret-eGFP^{Lo}:IB4^{Neg} subset, thus excluding that the 2 populations might coincide.

We also sought to examine the role of IL-31 in vivo. We injected 8 wild type or Avil-Cre::Ret$^{+/eGFP}$ mice with 55pM IL-31 into the nape of the neck and counted the bouts of scratching in the 30 minutes following the injection. A single low dose of the cytokine was enough to provoke robust scratching (Figure 10)

Cervical spinal cord from Avil-Cre::Ret$^{+/eGFP}$ mice injected with IL-31 was collected after the behavioural test and c-fos staining was performed. C-fos expression was sparse and involved few cells, the majority of which were in Lamina I (Figure 8B in part II).

3.3.5. LY344864 and 5-HT$_{1f}$

Serotonin acts through a variety of membrane-bound receptors that are found in both central and peripheral nervous system, as well as non-neuronal tissues such as gut, cardio-vascular system and blood (Hoyer et al, 2002).

In mouse DRG neurons only 5-HT$_2$ has been previously implicated in itch sensation (Yamaguchi et al, 1999). We found a very high expression of another serotonin receptor transcript, 5-HT$_{1f}$, in Ret-eGFP^{Lo}:IB4^{Neg} subset.

We performed calcium imaging on cultured sensory neurons from Avil-Cre::Ret$^{+/eGFP}$ mice, stained with IB4, by applying 30 nM LY344864. Surprisingly, we observed that a population of neurons was activated.

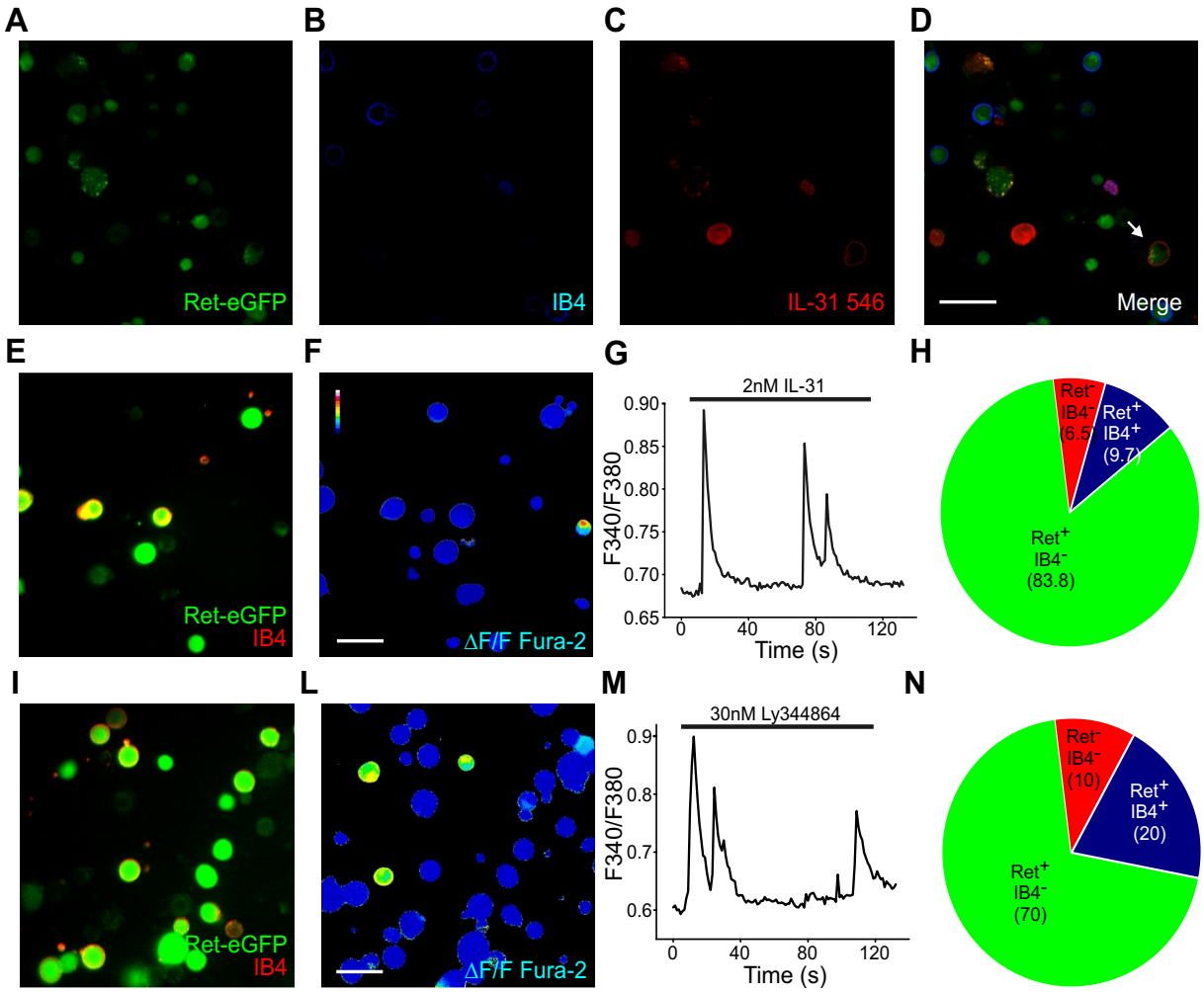

Figure 9. Biotinylated IL-31 binds to cells that weakly express Ret-eGFP and do not bind to IB4 (A-D). It also elicits neuronal response when used in calcium imaging (E-G), especially in Ret-eGFP/IB4 negative sensory neurons (H). LY344864 can drive neuronal activation as well (I-M) and the majority of activated DRG neurons were Ret-eGFP/IB4 negative (N).

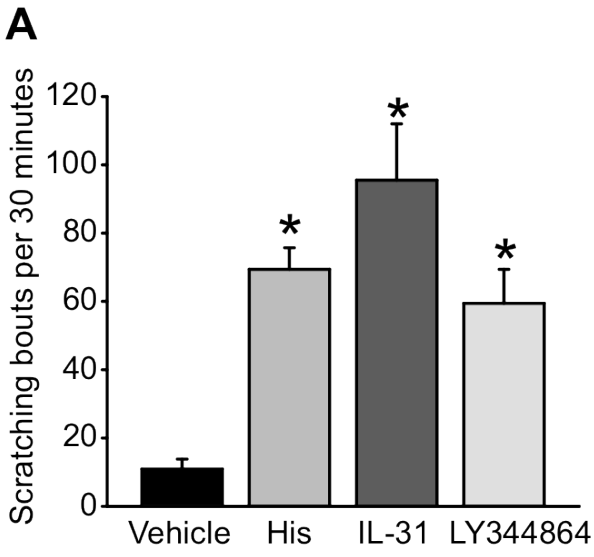

Figure 10. Histamine, IL-31 and LY344864 cause scratching in vivo after single injection. Animals were observed for 30' aftert the injections and the number of bouts of scratching was always significantly bigger compared to what observed in animals treated only with vehicle.

The main reason we chose this receptor as a target is because it is coupled to G_i signalling which should inhibit neuronal activation, so we did not expect to observe calcium influx in any neuronal subset. Instead we observed neuronal firing in 3,5% of total neurons (292 total cells) and 70% of these were Ret-eGFP/IB4-negative (Figure 9 I-N). Intriguingly, it has been reported that many G_i coupled receptors can interact with a G_{14} subunit that has the capacity to link G_i coupled receptors to activation of PLCβ (Ho et al, 2001). Notably, the G_{14} subunit is encoded by Gna14 gene and we found its transcript to be among the most differentially expressed in Ret-eGFP^{Lo}:IB4^{Neg} population. Its presence could therefore expand the signalling repertoire of this subset and explain why neuronal activation through 5-HT_{1f} occurs.

We investigated further the effect of LY344864 *in vivo* by injecting it in 7 wild-type or Avil-Cre::Ret$^{+/eGFP}$ mice at a 1mM concentration. We detected a number of bouts of scratching in the 30 minutes following the injection that was significantly higher than what we observed in control mice injected with saline (Figure 10). We therefore believe that activation of 5-HT_{1f} receptor in DRG neurons triggers itch and that this happens in a subset of Ret-eGFP/IB4 negative population.

We collected the cervical part of the spinal cord of Avil-Cre::Ret$^{+/eGFP}$ mice injected with LY344864 and checked for c-fos immunoreactivity: we detected a few interneurons positive for anti-c-fos antibody located mainly in Lamina I and III (Figure 8B in part II).

It is worth mentioning that this serotonin receptor has been proposed as clinical target in the treatment of migraine (Goadsby, 2005): such a 5-HT_{1f} based drug, however, could activate Ret-eGFP^{Lo}:IB4^{Neg} population causing itch as a side effect.

3.3.6. Role of Ret-eGFP^{Lo}:IB4^{Neg} neurons in itch sensation

Our calcium imaging and scratching behaviour analyses point towards the fact that subsets of Ret-eGFP/IB4-negative neurons are activated by pruritogens. Histamine is a well-known itching substance but it has never been observed to act on neurons that express Ret but do not bind to IB4. Here we show that such activation occurs. The interaction of interleukin 31 with its receptor IL31RA on DRG neurons has been recently described (Cevikbas et al, 2013) but it is the first time that Ret-positive and IB4-negative neurons are being involved in a pruritic stimulus detection that is linked to atopic dermatitis. As regards the serotonin agonist LY344864, we demonstrated that it acts as an itch promoting compound, thus discovering both a new receptor, 5-HT$_{1f}$, and a new sensory neuron subset involved in pruritus.
Our microarray data indicate that it is the Ret-eGFP^{Lo}:IB4^{Neg} subset of sensory neurons that mediates response to Histamine, IL-31 and LY344864. We were however not able to directly address this as we could not objectively target Ret-eGFP^{Lo}:IB4^{Neg} cells but a bigger Ret-eGFP/IB4-negative population which also includes Ret-eGFP^{Hi}:IB4^{Neg} neurons. Therefore, we sought to specifically target Ret-eGFP^{Lo}:IB4^{Neg} neurons by using a genetic approach: among the transcripts specifically enriched in this subset, according to the microarray data, we found that somatostatin (sst) was highly expressed in this population. Given its function as neuropeptide and especially the availability of a Sst-Cre driver line, we choose it as a target gene for further analyses.

3.4. First characterization of Sst-Cre::Ret^{eGFP} mice

Somatostatin is a peptide produced by neurons and other cell types. It has been originally discovered as the hypothalamic growth hormone release-inhibiting hormone (Siler et al, 1973) but soon expression in tissues other than central nervous system was reported (Hokfelt et al, 1975). Somatostatin is also expressed in dorsal root ganglia and it has been shown that in rats, it exerts an inhibitory action on dorsal horn neurons (Chapman & Dickenson, 1992) and, in particular, an anti-nociceptive and anti-inflammatory effect. Moreover, it relieves pain in humans (Shi et al, 2014). Because we found that somatostatin was enriched in our Ret-eGFP^{Lo}:IB4^{Neg} population, we reasoned that having a mouse line where somatostatin drives Cre expression could allow us to exclusively target this subset.

We obtained an Sst-*ires*-Cre driver line (Taniguchi et al, 2011) and crossed it with the $Ret^{eGFP/+}$ line. In this way we should observe eGFP fluorescence in cells that express both Sst and Ret and therefore specifically mark Ret-$eGFP^{Lo}$:$IB4^{Neg}$ neurons. We were expecting to see only few fluorescent neurons, given the low amount of cells belonging to this subset, and expressing eGFP at low level.

Indeed, we observed when we performed immunohistochemistry on DRG sections from Sst-Cre::Ret^{eGFP} mice that eGFP-positive cells constituted 1,3 ± 0,28 percent of the total number of neurons (3 adult mice, 8820 tot cells) and eGFP endogenous expression appeared quite faint, comparable with the palest fluorescence detected in DRG from Adv-Cre::Ret^{eGFP}.

We performed stainings with NF200, IB4 and TH in order to identify mechanoreceptors, non-peptidergic nociceptors and C-fiber low threshold mechanoreceptors, respectively, and exclude that the eGFP-positive cells belong to any of these populations (Figure 11) .

The vast majority of eGFP-positive cells (87,5 ± 8,9 percent) did not coexpress any of the above-mentioned markers, confirming our hypothesis that these neurons belong to a new uncharacterized subset of Sst-positive and Ret-positive cells.

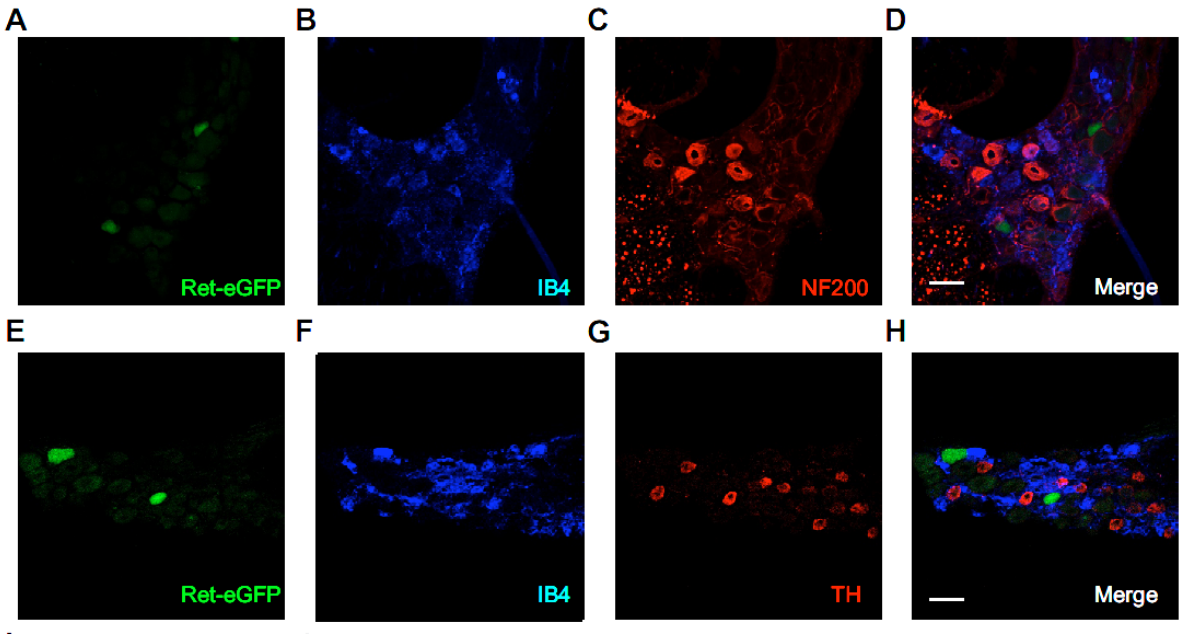

Figure 11. A small proportion of DRG neurons from Sst-Cre::Ret^{eGFP} mice displays eGFP fluorescence (1,6 ± 0,28 percent of total cells) which in 87,5 ± 8,9 percent of the the neurons does not overlap with IB4, NF200 or TH. Scale bars: 50 µm.

We could not detect the precise central patterns of innervation of the Sst-Cre::Ret^{eGFP} DRG neurons due to the fact that the paucity of the fluorescent afferent fibers was overwhelmed by Sst-expressing interneurons: in fact somatostatin expression has been detected also in spinal cord interneurons (Proudlock et al, 1993). As regards the peripheral projections of Sst/Ret-eGFP positive neurons, we detected faint fluorescence at the dermal/epidermal border, forming free nerve endings (Figure 6 I-L).

To strengthen our analysis and see whether the Sst-*ires*-Cre line could be used to unequivocally mark the Ret-eGFP^{Lo}:IB4^{Neg} population, we investigated whether somatostatin expression in DRG is actually confined to a subset of Ret-positive cells. The fact that we found somatostatin transcript to be up-regulated in Ret-eGFP^{Lo}:IB4^{Neg} subset compared to total DRG neurons, in fact, does not automatically exclude a potential low-level expression in other cell subtypes. Therefore we crossed the Sst-*ires*-Cre line with a Rosa-tdRFP strain to obtain Sst-Cre::R26^{tdRFP} mice that have all the sst-expressing neurons marked with RFP fluorescence.

We quantified the number of RFP-positive sensory neurons and found the similar percentages observed for eGFP-positive cells from Sst-Cre::Ret^{eGFP} DRG. Namely, the cells expressing RFP were 1,86 ± 0,74 percent of total sensory neurons (3 adult mice, 3300 tot. cells), which was not statistically different from the proportion of Sst/Ret-eGFP-positive cells (student's t-test, p-value=0,19) (Figure 12). We further stained DRG with NF200, IB4 and TH and again we found similar distribution of overlaps detected for Sst/Ret-eGFP-positive cells. We thus believe to have now the tools to specifically target the Ret-eGFP^{Lo}:IB4^{Neg} subset by using the Sst-*ires*-Cre: this will simplify any further analysis and characterization of this population.

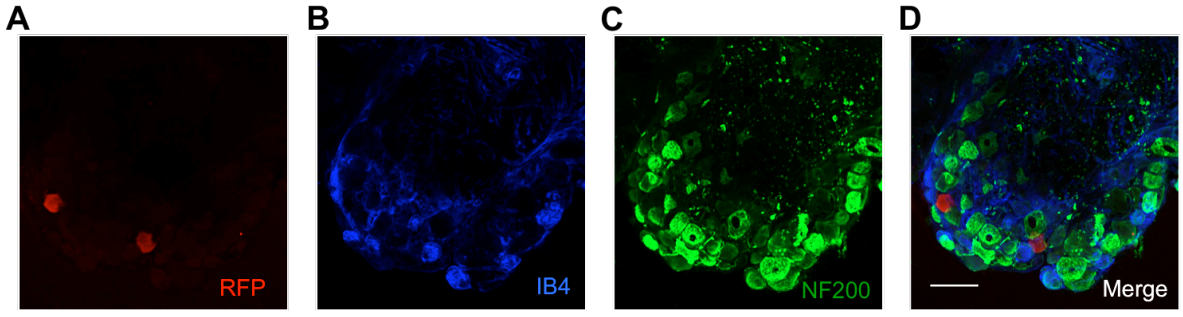

Figure 12. DRG section from Sst-Cre::R26^{tdRFP} mouse stained with RFP, IB4 and NF200. Sst/RFP-positive cells were 1,86 ± 0,74 percent of total cells, and 84,4 ± 4,13 percent of them did not stain with IB4 or NF200. We could not perform TH staining because of the incompatibility of the antibodies. Scale bar: 50 µm.

4. Conclusions and future prospects

During my PhD work I developed methods for applying flow cytometric techniques to Dorsal Root Ganglia neurons, thus proposing a new way of isolating and characterizing discrete sensory neuron subpopulations. Flow cytometry has not been used so far to analyse sensory neurons due to the fragility of these cells and their low number within the organism. However I demonstrated that this technique is not only feasibly appliable on DRG neurons, but it can provide data that are not acquirable with the common techniques used in the somatosensory field. Indeed, by using FACS, it is possible to isolate discrete subsets of cells, even those that are numerically very small and thus almost undetectable with immunohistochemistry, and to obtain their gene expression profiles. The knowledge of the type of transcripts that are enriched in a given population opens a lot of possibilities regarding its functional characterization thus easing the understanding of the systems biology of sensory neurons.

I applied these techniques to Avil-Cre::Ret^{eGFP} DRG neurons bringing new insights into Ret-expressing neuron function and distribution. I isolated 2 Ret-positive subsets that displayed different levels of eGFP fluorescence and did not bind to IB4. Their expression profiles indicated that one of them, namely Ret-$eGFP^{Hi}$:$IB4^{Neg}$ subset, probably represents the RA mechanoreceptive subset of Ret-positive cells, while the second one, Ret-$eGFP^{Lo}$:$IB4^{Neg}$ subset, is likely to be a new population involved in itch perception. This assumption was made based on the type of transcripts that we found up-regulated in the 2 subsets. In fact, many of the genes enriched in Ret-$eGFP^{Lo}$:$IB4^{Neg}$ neurons have been linked, in previous researches, to itch sensation.

We functionally characterized this population by focusing on 3 molecules whose receptors were specifically expressed in the putative itch subset: histamine, IL-31 and the agonist of the serotonin receptor 5-HT_{if}, LY344864. By performing calcium imaging, we demonstrated that all the three substances could activate a subset of Ret-eGFP/IB4-negative cells. Notably, for none of the compounds a role in Ret-positive subset activation has been observed before. It is also interesting that 5-HT_{1f}, despite being a G_i coupled receptor, could provoke a neuronal response.

We further analysed the function of the three compounds *in-vivo*, by injecting them in the neck of the mice and assessing scratching behaviour. While for histamine and IL-31 a direct role in pruritus processing has already been assessed, this was not the case for LY344864. My

data indicates that it actually does provoke itch, therefore discovering a new serotonin receptor involved in itchy sensation.

By genetically marking with Sst-Cre::Ret^{eGFP} mice exclusively the Ret-eGFP^{Lo}:IB4^{Neg} subset, we further demonstrated that there is a sensory neuron population that expresses Ret and does not bind to any of the markers known to be coexpressed in Ret-positive neurons.

In addition to the scientific implications of these findings, we also believe that the new Ret-positive subset could be of clinical importance. For example, IL-31-driven activation underlines the potential role of these neurons in the pathogenesis of atopic itch. Moreover while the fact that 5-HT$_{if}$ receptor also drives pruritic response must be considered by the researchers that have proposed that receptor as a target for migraine treatments as they could provoke side effects.

It is also worth noting the coexistence within this new subset of the histamine receptor with molecules that have been described as initiators of histamine-independent itch pathways, such as MrgprA3. It is still under debate whether these mechanisms act in different subpopulations of sensory neurons or they can also be simultaneously present in the same cells. Our findings argue against a complete separation of the two pathways within DRG neurons.

A further important observation stems from the small amount of neurons that belong to the population we discovered: despite their scarcity, they are able to elicit scratching behaviour, which suggests that many other small but functionally important DRG subsets might exist that have likely never been detected by the commonly used techniques. They could be identified, however, by flow cytometry.

In further work exploring this population, we have demonstrated that we now have a good tool to examine deeply the new Ret-positive subpopulation: the Sst-*ires*-Cre mouse line. First of all, we are planning to perform FACS on these mice in order to sort the eGFP+ cells and check specific gene expression by quantitative RT-PCR (Fluidigm) with the aim of comparing it to the results obtained from Ret-eGFP^{Lo}:IB4^{Neg} subset and see whether they coincide. Preliminary results with FACS showed that Sst/Ret-eGFP-positive neurons follow a distribution, in terms of numbers, size and eGFP intensity, which is very similar to Ret-eGFP^{Lo}:IB4^{Neg} subset. Sst-Cre::Ret^{eGFP} mice could be further characterized with calcium imaging experiments to prove that Sst/Ret-eGFP–positive cells do respond to histamine, IL-31 and LY344864. The experiment that would ultimately confirm the role of our Ret-positive subset in itch sensation is ablation of these neurons. Our study suggests that somatostatin is specifically expressed in the new subpopulation, therefore the Sst-*ires*-Cre line could be used to specifically drive its ablation, for example, by crossing it with a line that expresses the

diptheria toxin receptor after Cre recombination and specifically in DRG neurons. Functional analyses would then assess whether sensory neurons still respond to IL-31 and LY344864 and wheter scratching behaviour following injections of those substance is reduced, thus confirming, or not, our hypothesis.

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PART II

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A subpopulation of itch receptors marked by Ret expression

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ABSTRACT

Itch, the unpleasant sensation that elicits a desire to scratch is mediated by specific subtypes of cutaneous sensory neuron. Here, we identify a subpopulation of itch sensing neuron that express the receptor tyrosine kinase Ret. We applied flow cytometry to isolate Ret positive neurons from dorsal root ganglia (DRG) and detected a distinct population marked by low levels of Ret expression and absence of isolectin B4 (IB4) binding. We determined the transcriptional profile of these neurons and demonstrate that they express neuropeptides, the NGF receptor TrkA, and multiple transcripts associated with itch. Neurons in this population were responsive to both histamine and histamine-independent itch stimuli. Moreover, interleukin 31 (IL-31), a cytokine with a prominent role in atopic dermatitis, and agonists at the $5HT_{1F}$ receptor, directly activated these cells and evoked robust scratching behavior. Our data provide a molecular signature for a subpopulation of neurons activated by multiple pruritogens.

INTRODUCTION

The perception of physical and chemical stimuli through the skin is initiated by peripheral sensory neurons that have their cell body in the DRG. The complexity of somatosensation is reflected by the fact that a myriad of sensations including touch, pain, itch and temperature are recognized by the peripheral nervous system. It has long been debated whether this functional complexity arises from activation of specific subtypes of sensory neuron for each stimulus modality, or from encoding and summation of neuronal activity generated by neurons that can detect a broad range of stimuli (Ma, 2010). Recently, it has been proposed that the sensation of itch is a discrete sensory modality that utilizes a dedicated neuronal pathway tuned to perceive only this sensation (Han et al, 2013; Mishra & Hoon, 2013; Sun et al, 2009).

Itch, the sensation that elicits a desire to scratch, serves a protective function against potentially harmful environmental irritants (Ikoma et al, 2006). Although unpleasant, itch is inherently different from pain, both in its sensory quality and its behavioral outcome (scratching compared to withdrawal). The neuronal pathways that mediate itch versus pain also appear to be distinct. In the spinal cord, ablation of neurons expressing receptors for the neuropeptides gastrin-releasing peptide (Grp) (Sun et al, 2009) or Natriuretic polypeptide b (Nppb) (Mishra & Hoon, 2013) reduces itch responses to multiple pruritogens but does not affect nociceptive behavior. Similarly, subpopulations of C-fiber primary afferents are activated by pruritogens (Davidson et al, 2007; Schmelz et al, 1997; Schmelz et al, 2003) and ablation or selective activation of DRG neurons positive for the Mas-related G protein coupled receptor A3 (MrgprA3) impacts upon scratching behavior but not pain (Han et al, 2013).

Itch is further categorized by its dependence upon histaminergic or non-histaminergic mechanisms. Histamine-dependent itch is elicited through activation of the H1 receptor

(HRH1) and signaling through phospholipase-β3 (PLCβ3) and the ion channel TRPV1 (Han et al, 2006; Imamachi et al, 2009). The existence of further histamine-independent pathways is supported by observations that many chronic pruritic syndromes such as atopic dermatitis are resistant to antihistamine therapy (Yosipovitch & Papoiu, 2008). Mechanistically, histamine-independent itch is likely to be mediated by activation of the ion channel TRPA1. For example, injection of the antimalarial agent chloroquine induces itch (Ajayi et al, 1989) via MrgprA3 receptors (Liu et al, 2009) functionally coupled to TRPA1 (Wilson et al, 2011), and TRPA1 is also required for itch produced by oxidative stress and leukocyte accumulation (Fernandes et al, 2013; Liu & Ji, 2012). Other antihistamine resistant itch responses include those elicited by cytokines such as interleukin 31 (IL-31) released from T-cells during allergic itch (Akiyama & Carstens, 2013; Cevikbas et al, 2013). IL31 induces severe pruritus and may be a key mediator in atopic dermatitis (Dillon et al, 2004), although the mechanism by which it activates distinct neuronal itch pathways is unknown.

While histamine-dependent and independent itch utilize distinct molecular mechanisms to activate neurons, it is unclear whether this occurs in discrete populations of sensory neuron. All MrgprA3 expressing neurons respond to histamine (Liu et al, 2009) and ablation of these cells reduces histamine-evoked scratching (Han et al, 2013). However, it has also been reported that histamine and non-histamine evoked itch occurs in separate peripheral pathways (Davidson et al, 2012), and indeed silencing of TRPV1 or TRPA1 positive neurons selectively inhibits histamine or chloroquine evoked itch respectively (Roberson et al, 2013). Further information on the molecular profile of subpopulations of sensory neurons and identification of molecular markers for different subtypes of itch neuron would resolve these conflicting observations.

To distinguish different populations of sensory neuron and ultimately define their function we examined the expression pattern of the glial-derived neurotrophic factor (GDNF) receptor Ret

in mouse DRG. Almost every DRG neuron expresses at least one neurotrophic factor (Marmigere & Ernfors, 2007), and approximately 60 percent of cells are marked by Ret (Molliver et al, 1997). The Ret tyrosine kinase is the signaling receptor for GDNF family ligands GDNF, neurturin, artemin and persephin which bind via GPI-anchored co-receptors termed GFRα1-4 to initiate signaling through Ret (Airaksinen & Saarma, 2002). Two distinct waves of Ret expression arise during development with the first occurring prior to E11.5 and the second emerging subsequently (Luo et al, 2007; Molliver et al, 1997). Early Ret positive neurons develop into rapidly adapting mechanoreceptors and express the co-receptor GFRα2 and high levels of Ret (Bourane et al, 2009; Luo et al, 2009). Late Ret positive neurons form a large heterogeneous group of non-peptidergic nociceptors that can be distinguished by their binding of the plant lectin IB4 (Luo et al, 2007; Molliver et al, 1997). A further population of Ret positive neuron co-expresses the enzyme tyrosine hydroxylase (TH) (Franck et al, 2011; Li et al, 2011) and forms C-fiber low threshold mechanoreceptors which have been implicated in the affective component of touch (Olausson et al, 2010). Ret signaling is required for the differentiation, maturation and anatomical projections of these populations indicating that it has powerful regulatory actions on primary sensory neurons (Bourane et al, 2009; Franck et al, 2011; Golden et al, 2010; Honma et al, 2010; Luo et al, 2009; Luo et al, 2007; Molliver et al, 1997).

We sought to define functionally distinct populations of Ret-positive neuron by first examining Ret co-expression with conventional markers in the DRG and then determining the molecular profile of identified populations to establish their function. To this end we took a genetic approach and generated mice where eGFP expression was driven from the Ret locus exclusively in peripheral sensory neurons (Jain et al, 2006; Zurborg et al, 2011). We identified multiple subpopulations of Ret-positive neurons in DRG which were quantified using flow cytometry. Microarray analysis of Ret expressing neurons that were negative for

IB4 uncovered a rare population of cells enriched for pruritogen receptor transcripts. Characterization of these neurons revealed a novel subtype of Ret-positive neuron selectively activated by multiple classes of pruritogen.

RESULTS

Ret-eGFP expression in primary sensory neurons

To examine Ret expression in the adult peripheral nervous system, $Ret^{eGFP/+}$ mice (Jain et al, 2006) were crossed with $Avil^{cre/+}$ mice to obtain heterozygote Avil-Cre::$Ret^{eGFP/+}$ mice. This approach allowed us to target all DRG neurons and avoid extraneous GFP expression in surrounding tissues (Zurborg et al, 2011). Heterozygous mice were viable, exhibited no overt behavioural phenotype, and displayed robust eGFP fluorescence in peripheral sensory ganglia.

We investigated Ret-eGFP distribution in DRG by co-staining sections from Avil-Cre::$Ret^{eGFP/+}$ mice with a selection of markers for different subtypes of sensory neuron. Ret-eGFP was present in 55±3 percent of neurons (n=12 DRG cultures) and displayed a broad range of fluorescence intensities across different cells. We examined expression with IB4 and NF200, markers of non-peptidergic nociceptors and myelinated neurons respectively, and observed overlap with the majority of Ret-eGFP positive neurons (Figures 1A-1D), reflecting the early and late Ret neurons described previously (Luo et al, 2007; Molliver et al, 1997). We further investigated Ret-eGFP co-expression with TH, a marker of C-fiber low threshold mechanoreceptors (Franck et al, 2011; Li et al, 2011). eGFP fluorescence was evident in many TH-positive neurons and these cells were not co-labeled with NF200 or IB4 (Figures 1E-1L). Our analysis also indicated that a small proportion of Ret-eGFP positive neurons were not marked by either IB4, NF200 or TH suggesting the existence of a novel subtype of Ret expressing neuron. To characterize this population, we co-stained sections with CGRP, a

marker of peptidergic nociceptors that has not been previously associated with Ret-positive sensory neurons. Intriguingly, Ret-eGFP fluorescence coincided with a small proportion of CGRP expressing cells (Figures 1M-P) that may reflect a functionally uncharacterized subset of primary afferent neuron.

We utilized Avil-Cre driven Ret-eGFP expression to examine the peripheral and central projections of Ret-positive sensory neurons. In the skin Ret-eGFP fluorescence was broadly distributed and present in free nerve endings terminating in the dermis and epidermis (Figures 2A-2C), and in lanceolate endings encircling hairs (Figures 2D-2F). Similarly in spinal cord sections, Ret-eGFP was widely expressed across the dorsal horn. This was evident as a dense plexus of expression in lamina II_o corresponding to IB4 positive non-peptidergic nociceptors (Figures 3A-D and I-L) and more diffusely through laminae III to V overlapping with NF200 labeled mechanoreceptor inputs (Figures 3E-H). Notably, we also detected Ret-eGFP expression immediately ventral to IB4 positive terminals that coincided with PKCγ, a marker for lamina II_i/III interneurons (Figures 3A-H), and in lamina I co-expressed with CGRP. Thus RET-eGFP expression distinguishes multiple populations of peripheral sensory neuron that are likely to be functionally distinct.

To obtain quantitative data on the distribution of Ret positive neuronal populations in DRG we applied flow cytometric analysis to acutely dissociated neurons. We focused on levels of IB4 binding and native Ret-eGFP fluorescence as this would allow for quantitative measurements in live cells. In line with histological data, we observed both IB4-positive and IB4-negative populations of Ret-eGFP neuron (Figures 4A and E1). Importantly however, flow cytometric analysis revealed multiple well-defined subpopulations delineated by their levels of eGFP fluorescence and IB4 binding (termed Ret-eGFP^{Lo}:IB4^{Neg} (A), Ret-eGFP^{Hi}:IB4^{Neg} (B), Ret-eGFP^{Lo}:IB4^{Lo} (C), Ret-eGFP^{Hi}:IB4^{Lo} (D), and Ret-eGFP^{Hi}:IB4^{Hi} (E)). There was a broad distribution in cell size across all populations as determined by forward

scatter values, and no correlation between size and levels of IB4 binding or fluorescence (Figure 4B). We further validated flow cytometric analysis using fluorescent microscopy and observed that in both sensory neuron cultures (Figure 4C) and sections of DRG (Figure 4D) from Avil-Cre::$Ret^{+/eGFP}$ mice, native eGFP fluorescence varied by an order of magnitude across IB4 positive and negative cells.

Transcription profiling of Ret-eGFP, IB4 negative neurons

We reasoned that by defining the molecular composition of Ret-eGFP populations we may be able to gain clues as to their function. We focused on IB4-negative neurons because flow cytometric data indicated that these cells formed two well defined and potentially homogeneous populations. Moreover, while one of these populations presumably corresponds to RA mechanoreceptors (Bourane et al, 2009; Luo et al, 2009), the other may reflect an as yet uncharacterized population of Ret-positive neurons. We performed differential microarray screening on sorted Ret-$eGFP^{Hi}$:$IB4^{Neg}$ and Ret-$eGFP^{Lo}$:$IB4^{Neg}$ cells and determined that these two populations do indeed cluster into distinct, homogeneous subsets (Figure E2). We further identified several functional markers in each population that gave a first indication as to their identity. For example, Ret-$eGFP^{Lo}$:$IB4^{Neg}$ cells were enriched in transcripts for TRPV1, CGRP (Calca), and the Ret co-receptors GFRα1 and GFRα3, (Figure 5A), while Ret-$eGFP^{Hi}$:$IB4^{Neg}$ neurons expressed significantly higher levels of Ret, GFRα2, and the mechanosensitive ion channel Piezo 2 (Fam38b) (Figure 5A). Intriguingly, neither population was enriched for TH. We thus speculate that Ret-$eGFP^{Hi}$:$IB4^{Neg}$ neurons may correspond to RA mechanoreceptors, while Ret-$eGFP^{Lo}$:$IB4^{Neg}$ cells represent a novel, functionally distinct subtype of nociceptor.

We sought to define the function of the novel Ret-$eGFP^{Lo}$:$IB4^{Neg}$ population and observed that this subset was highly enriched in molecules previously implicated in itch (Figure 5A, red

dots). For example, transcripts for histamine-dependent itch mediators HRH1, PLCβ3 and TRPV1 were all differentially expressed in Ret-eGFP^{Lo}:IB4^{Neg} neurons, as were, itch-associated neurotransmitters such as Nppb (Mishra & Hoon, 2013) and neuromedin B (Nmb) (Jensen et al, 2008). Importantly, Il31ra and Osmr, co-receptors for IL-31 were also highly expressed in Ret-eGFP^{Lo}:IB4^{Neg} neurons suggesting that this population could contribute to IL-31 mediated pruritus in atopic dermatitis.

To further investigate whether itch-associated receptor transcripts were indeed specifically expressed in Ret-eGFP^{Lo}:IB4^{Neg} neuron, we performed a second microarray screen where we assessed differential expression in this population with respect to all DRG neurons. We sorted DRG neurons from Avil-Cre::R26^{tdRFP} mice in which the majority of peripheral sensory are marked by RFP fluorescence (Figure E3) and subjected them to microarray analysis. Similar to differential screening between Ret-eGFP^{Lo}:IB4^{Neg} and Ret-eGFP^{Hi}:IB4^{Neg} populations, this dataset was also enriched in itch-associated molecules (Figure 5B). Fold change levels were lower than for comparisons with Ret-eGFP^{Hi}:IB4^{Neg} neurons (Figure 5C), presumably reflecting the mixed molecular profile of all DRG neurons, and the fact that some transcripts such as Calca (CGRP) mark large populations of Ret-negative neurons.

To validate the microarray analysis we performed parallel quantitative RT-PCR analysis of 38 transcripts in Ret-eGFP^{Lo}:IB4^{Neg} and Ret-eGFP^{Hi}:IB4^{Neg} populations using a microfluidic platform (Fluidigm). We selected genes that represented not only itch associated transcripts but also ion channels, signaling molecules and transcription factors with a demonstrated role in the peripheral nervous system. In agreement with microarray data, the highest differential expression between Ret-eGFP^{Lo}:IB4^{Neg} and Ret-eGFP^{Hi}:IB4^{Neg} neurons was for itch related genes such as the neuropeptides Nppb, Sst (somatostatin) and Nts (neurotensin), the membrane receptors Il31ra, Osmr, Cysltr2, MrgprA3 and Hrh1, and the signaling molecule Plcb3. We also observed higher expression in this population for Ntrk1 (TrkA), Calca

(CGRP) and Trpv1 transcripts, marking it as a novel population of Ret-positive neurons, as well as the ion channels Trpm6, Trpc6, Trpm2 and P2rx3 and the serotonin receptors Htr1f and Htr1a (Figures 5D and E4). In the Ret-eGFP^{Hi}:IB4^{Neg} population, we detected almost 10-fold higher expression of Ret and eGFP, validating the flow cytometry analysis. In addition Gfra2 and the mechanosensitive ion channel Piezo 2 (Fam38b) (but not Piezo 1 (Fam38a)) were upregulated, supporting the assumption that these neurons function as RA mechanoreceptors. Intriguingly, we also observed that this population was enriched in transcripts involved in chloride transport including the GABA channel subunits α1 and γ2 (Gabra1, Gabrg2) and the putative chloride channel anoctamin 6 (Ano6) (Figures 5D and E4).

Functional characterization of Ret-eGFP^{Lo}:IB4^{Neg} neurons

We reasoned that further analysis of upregulated transcripts in the Ret-eGFP^{Lo}:IB4^{Neg} population would allow us to determine the function of these neurons and establish whether they do indeed form a novel population of Ret-positive cells. We selected three highly enriched transcripts in Ret-eGFP^{Lo}:IB4^{Neg} cells, Sst, Il31ra and Htr1f, and applied genetic and pharmacological approaches to determine their function. We first assessed the anatomical properties of putative Ret-eGFP^{Lo}:IB4^{Neg} neurons by crossing an Sst^{Cre} driver line (Taniguchi et al, 2011) with Ret^{eGFP} mice to generate heterozygote Sst-Cre::Ret$^{eGFP/+}$ mice. In DRG sections from these animals we detected weak eGFP fluorescence in a rare population of neurons corresponding to approximately two positive cells per section (Figure 6). We explored this expression pattern in more detail by co-staining sections with IB4, NF200 and TH. Importantly, Sst^{Cre} driven Ret-eGFP expression did not overlap with any of these markers (Figures 6A-H) suggesting that Sst does indeed mark the Ret-eGFP^{Lo}:IB4^{Neg} population and that this population is unique. We further investigated the peripheral projections of Sst/Ret-eGFP positive sensory neurons by examining skin sections from Sst-Cre::Ret$^{eGFP/+}$ mice. We

detected faint eGFP immunofluorescence at the dermal/epidermal border that overlapped with expression of the pan-neuronal marker PGP9.5 and formed free nerve endings (Figures 6 I-L). Importantly, this staining pattern was absent from sections taken from control mice not expressing Sst-Cre.

To examine the function of Ret-eGFP^{Lo}:IB4^{Neg} neurons we performed ratiometric calcium imaging on acutely dissociated DRG from Avil-Cre::Ret$^{eGFP/+}$ mice. Using microscopy we were not able to unequivocally assign Ret positive neurons as being either Ret high or low; we thus assessed the proportion of Ret-eGFP-negative, Ret-eGFP/IB4-positive and Ret-eGFP/IB4-negative neurons activated by various stimuli. Based upon the microarray analysis we assumed that these neurons may form a population of itch receptors and thus focused on their responses to pruritogens. We first investigated their activation by histamine and observed that histamine application induced calcium flux across a broad range of cells, of which Ret-eGFP/IB4-negative neurons formed a large population (9.7% of all neurons responded to histamine, 54% of these were Ret-positive/IB4 negative, Figure E5A). Similarly, immunostaining of DRG sections with an antibody against HRH1indicated that many of the neurons positive for Ret and negative for IB4 co-expressed HRH1 (Figures E5E-H). We next examined neuronal activation by a histamine-independent stimulus by applying low concentrations of chloroquine to cells. Again we found that the majority of responses to chloroquine were clustered in Ret-eGFP/IB4-negative neurons (5.6% of all neurons responded to chloroquine, of which 72% were Ret-eGFP/IB4-negative, Figure E5B). Since the ion channels TRPV1 and TRPA1 may act as receptor operated channels for histamine-dependent and independent itch respectively, we also investigated response properties to agonists of these channels. We observed that the TRPV1 agonist capsaicin and the TRPA1 agonist allyl isothiocyanate evoked calcium influx across all three populations. However capsaicin responses were confined predominantly to IB4-negative neurons while allyl isothiocyanate

activated mainly IB4 positive neurons (Figures E5C and D). Thus itch different inducing stimuli activate a broad range of DRG neurons but have a preferential distribution towards the Ret-eGFP/IB4-negative population.

In light of the potential clinical importance of IL-31 to atopic dermatitis and pruritus (Sonkoly et al, 2006), we next examined the actions of IL-31 on Ret-eGFP^{Lo}:IB4^{Neg} cells. We first investigated the expression of IL-31RA in DRG neurons. We were unable to obtain selective IL-31RA antibodies that worked for immunofluorescence, and thus developed a method for identifying IL-31RA positive neurons based upon fluorescent labeling of recombinant IL-31 ligand. Mono-biotinylated IL-31 was conjugated with streptavidin Alexa Fluor 546 and applied directly to acutely dissociated DRG neurons from Avil-Cre::Ret$^{eGFP/+}$ mice. In agreement with microarray analysis we observed that a small population of DRG neurons bound IL-31 Alexa Fluor 546 conjugates and that this coincided with weakly fluorescent Ret-eGFP cells that were mainly negative for IB4 labeling (Figures 7A-D). We next assayed the response properties of these cells to IL-31 application using calcium imaging. Strikingly, we observed that IL-31 robustly activated a subset of sensory neurons and this effect was almost exclusively confined to the Ret-eGFP/IB4-negative population (5.6% of all neurons responded to IL-31, of which 83.9% were Ret-eGFP/IB4-negative, Figures 7E-H). Thus IL-31 may exert some of its pruritogenic effects by directly activating Ret-eGFP/IB4-negative sensory neurons.

Finally we wished to examine a receptor/signaling pathway that has not been explored in the context of itch. We selected $5HT_{1F}$ (Htr1f) because this receptor couples to the inhibition of adenylate cyclase (Adham et al, 1993) and we reasoned that its stimulation may therefore inhibit neuronal activity. We first immunostained DRG sections with an antibody against $5HT_{1F}$ and observed immunoreactivity in a rare subpopulation of neuronal soma that were positive for Ret-eGFP and mostly negative for IB4 binding (Figures 7I-L). We explored the

function of $5HT_{1F}$ receptors in this population further by applying the $5HT_{1F}$ agonist LY344864 to neurons from Avil-Cre::$Ret^{+/eGFP}$ mice. Unexpectedly, we found that application of low concentrations of LY344864 evoked calcium flux in a subpopulation of neurons that was qualitatively similar to that evoked by IL-31, histamine and chloroquine (Figures 7M-O). Moreover, this activity was concentrated mainly in the Ret-eGFP/IB4-negative population (3.5% of all neurons responded to LY344864, of which 70% were Ret-eGFP/IB4 negative, Figure 7P) suggesting that in these cells, $5HT_{1F}$ signaling can directly evoke calcium flux.

To explore the role of Ret-eGFP/IB4 negative neurons in generating itch at the in vivo level, we reasoned that application of agonists that activate this population should evoke scratching behavior and activate second order spinal cord neurons when applied in vivo. We therefore injected low concentrations of IL-31 and LY344864 subcutaneously (as well as histamine as a positive control) and monitored scratching responses and the induction of C-fos expression in spinal cord slices. Strikingly we observed that a single subcutaneous injection of either Il-31 or LY344864 rapidly evoked scratching behavior to the same extent as histamine (Figure 8A). Moreover, application of Il-31 or LY344864 also induced sparse C-fos expression in lamina I and lamina II_i/III, that overlapped with Ret-eGFP expression but was largely separate from IB4 labeling (Figures 8B-D and E6). Collectively our data indicate that a small subpopulation of Ret-positive cells may mediate itch sensation, and that remarkably, very few cells may be required to elicit a behavioral scratching response.

DISCUSSION

Here, we show that a previously uncharacterized population of sensory neurons that express the receptor tyrosine kinase Ret may function as itch receptors. Using flow cytometry of dissociated DRG neurons we isolated this population based upon its weak expression of Ret and absence of IB4 binding. We demonstrate that these neurons express markers common to

peptidergic nociceptors such as TrkA and CGRP, and importantly, are highly enriched for transcripts associated with itch sensation. Anatomical and functional analysis of these cells indicates that they form a rare subtype of DRG neuron that project to the dermal/epidermal border of the skin and function to detect itch inducing stimuli.

Three populations of Ret positive sensory neuron have been previously identified and characterized. Early Ret neurons develop into RA mechanoreceptors (Bourane et al, 2009; Luo et al, 2009) while late Ret neurons become C-fiber low threshold mechanoreceptors and non-peptidergic nociceptors (Li et al, 2011; Molliver et al, 1997). Intriguingly, a fourth population of Ret positive neurons has also been described that expresses TrkA in adult mice but has unknown function ((Golden et al, 2010; Molliver et al, 1997). This population is scarce, corresponding to around 10% of all Ret positive cells (Golden et al, 2010; Molliver et al, 1997), and presumably this rarity has made further analysis difficult. By taking advantage of the sensitivity and analytic power of flow cytometry, we have isolated these cells and performed gene expression profiling to determine their function.

We initially used histochemistry to identify populations of Ret-eGFP positive neuron expressing markers of mechanoreceptors (NF200), non-peptidergic nociceptors (IB4) and peptidergic nociceptors (CGRP). However, limited by the number of markers that can be applied simultaneously to the same section, we turned to flow cytometry to analyze Ret-expression in more detail. We based our analysis on the observation that Ret-expression levels differ considerably across the DRG, and that low Ret levels correlate with GFRα1/3 expression (Luo et al, 2007), while high Ret expressing neurons express GFRα2 and form RA mechanoreceptors (Bourane et al, 2009; Luo et al, 2009). By co-labeling cells with IB4 we were able to distinguish multiple subtypes of Ret positive neurons of which a Ret-low, IB4 negative population was clearly evident. That this population corresponds to the fourth, uncharacterized type of Ret neuron is supported by several lines of evidence. Firstly it is IB4

negative, indicating that it is not part of the non-peptidergic nociceptor population. Secondly, it expresses almost 10 fold less Ret transcript compared to Ret-eGFP^{Hi}:IB4^{Neg} neurons. Thirdly, Gfra3 is up-regulated 29 fold in these cells while Gfra2 has 21 fold higher expression in the Ret-eGFP^{Hi}:IB4^{Neg} population. Finally, we detected many differentially expressed genes such as TrkA, CGRP and TRPV1, in the Ret-eGFP^{Lo}:IB4^{Neg} population which are known to be absent from C-LTMRs or RA mechanoreceptors (Li et al, 2011). While we did not perform a similar analysis on IB4 positive neurons, flow cytometry data indicates that a further three populations can also be differentiated here which may also form distinct functional populations.

Given the powerful regulatory actions of neurotrophic factors in the peripheral nervous system, a major emphasis of previous research has been to investigate how deletion of these factors impacts upon the survival, differentiation and maintenance of distinct populations. Here, we have not considered the effects of Ret ablation on Ret-eGFP^{Lo}:IB4^{Neg} negative neurons, concentrating instead upon the initial functional characterization of this population. Of note, the number of Ret positive/ TrkA positive neurons is not reduced in Ret nociceptor specific conditional knockout mice (Golden et al, 2010), suggesting that this population does not require Ret for its survival. It is however possible that the termination pattern of these neurons in the skin and spinal cord, and the expression of ion channels and signaling molecules is regulated by Ret signaling as has been shown for other populations (Bourane et al, 2009; Franck et al, 2011; Golden et al, 2010; Honma et al, 2010; Li et al, 2011; Luo et al, 2009; Luo et al, 2007; Molliver et al, 1997). The identification of markers, and molecular tools with which to target Ret-eGFP^{Lo}:IB4^{Neg} negative cells described here would now allow for further investigation of these phenotypes.

Amongst the differentially expressed genes present in Ret-eGFP^{Lo}:IB4^{Neg} cells we observed a strong enrichment of the neuropeptides CGRP, Nppb, neurotensin (Nts) and somatostatin

(Sst). Of these, we focused our attention on Sst because of the availability of an Sst-Cre driver line (Taniguchi et al, 2011) which we reasoned could be used to target the Ret-eGFP^{Lo}:IB4^{Neg} population. Indeed, recombination of the Ret locus by Sst-Cre occurred in very few neurons of the DRG, resulting in low eGFP fluorescence in IB4, NF200 and CGRP negative cells, analogous to the Ret-eGFP^{Lo}:IB4^{Neg} population. In agreement, these neurons formed sparse terminations in the skin consistent with a potential role for this population in detecting itch. Using this genetic strategy we were unable to assay the central projections of Sst-Cre::Ret^{eGFP} positive sensory neurons because of native expression of Sst in spinal cord interneurons (Proudlock et al, 1993). However, previous studies have shown that the Sst receptor Sst2a (as well as the Nts receptor Nts2) are present in laminae I-IV of the spinal cord (Sarret et al, 2005; Todd et al, 1998), suggesting the possibility that Ret-eGFP^{Lo}:IB4^{Neg} may synapse with these neurons. Future experiments, perhaps using intersectional genetics to limit Sst-Cre expression to peripheral sensory neurons would be able to address these questions.

We observed that many molecules that have previously been implicated in itch sensation showed the highest enrichment in Ret-eGFP^{Lo}:IB4^{Neg}. These included transcripts for the histamine and chloroquine receptors Hrh1 and Mrgpra3, the signaling molecule Plcb3 and the putative neurotransmitter Nppb. Moreover enrichment of itch associated transcripts was also evident when assessing expression levels relative to all DRG neurons suggesting that although rare, this population may play a role in itch sensation. The expression profile of Ret-eGFP^{Lo}:IB4^{Neg} neurons therefore represents a molecular inventory for these neurons, and many more molecules with an as yet unexplored role in itch may be contained within this list.

To validate the gene expression analysis we performed calcium imaging on acutely dissociated DRG neurons from Avil-Cre::Ret^{eGFP} mice. In agreement with previous studies we observed that histamine activated a broad range of dissociated neurons (Imamachi et al, 2009), while chloroquine was more specific in its activation profile, evoking calcium influx in

approximately 5% of cells (Liu et al, 2009). Of note, our expression analysis and functional data indicate that both histamine and chloroquine can activate Ret positive/IB4 negative cells, supporting the observation chloroquine sensitive neurons form a subset of histamine responders ((Liu et al, 2009). It has been reported that these two pruritogens activate separate neuronal pathways in vivo, and distinct neurons in in vitro calcium imaging assays (Roberson et al, 2013). Our data argue against a complete separation of histamine dependent and independent pathways, and suggest that in Ret positive/IB4 negative cells these different mechanisms converge. Indeed Robertson et al (2013) observed a small population of trigeminal neurons that respond to both histamine and chloroquine and it is conceivable that these neurons may be the trigeminal correlate of Ret positive/IB4 negative neurons in the DRG.

Strikingly, we observed that IL-31 directly activated Ret positive/IB4 negative cells, suggesting that this could be the first step in transducing an immune cell derived inflammatory cascade into a behavioral scratching response. A similar finding has also been recently reported for Thymic Stromal Lymphopoietin (TSLP), an epithelial cell derived cytokine that plays a critical role in the development of atopic dermatitis (Wilson et al, 2013). TSLP receptor transcripts (Il7r and Crlf2) were not expressed in Ret-eGFP^{Lo}:IB4^{Neg} cells and accordingly, TSLP directly activated a novel subpopulation of sensory neurons that were not sensitive to either histamine of chloroquine (Wilson et al, 2013). Investigation of the relative roles of these populations of neuron and their interaction with immune and epithelial cells may have important clinical consequences for understanding the progression of allergic diseases.

We were intrigued by the high expression levels of the serotonin receptors Htr1f and Htr1a in Ret-eGFP^{Lo}:IB4^{Neg} neurons. Serotonin has a well-established pruritogenic action when applied subcutaneously and this effect is mediated by 5-HT$_2$ receptors (Nojima & Carstens,

2003; Yamaguchi et al, 1999). Transcripts for 5-HT_2 receptors were not upregulated in Ret-eGFP^{Lo}:IB4^{Neg} cells and we did not investigate further whether serotonin directly activates these cells. Instead we focused on the $5HT_{1F}$ receptor because it is coupled to G_i signaling and should therefore inhibit neuronal activation. Unexpectedly we observed an apparent activation of Ret-eGFP/IB4-negative sensory neurons by the $5HT_{1F}$ agonist LY344864, and robust scratching behavior when LY344864 was injected subcutaneously. A potential basis for this effect may come from the observation that many G_i coupled receptors are able to interact with G_{14} subunits promoting the stimulation of phospholipase C (Ho et al, 2001). Indeed the transcript for G_{14} (Gna14) was amongst the most differentially expressed genes in Ret-eGFP^{Lo}:IB4^{Neg} neurons (Figures 5A-C), and thus its presence could expand the signaling repertoire of these cells allowing for G_i coupled receptors such as $5HT_{1F}$ to generate neuronal activity. Of note, $5HT_{1F}$ receptor agonists have also been proposed as clinical targets for the treatment of migraine (Goadsby, 2005). Our data indicates that these compounds could have unwanted side-effects via activation of Ret-eGFP^{Lo}:IB4^{Neg} neurons and generation of itch.

An important question that arises from our expression data is whether the presence of multiple itch associated transcripts in Ret-eGFP^{Lo}:IB4^{Neg} neurons is sufficient to define them as itch receptors. In support of this notion, we observed that agonists which selectively activate these neurons at the cellular level (IL-31, LY344864) evoked scratching behavior when injected in vivo. Similarly, the chloroquine receptor Mrgpra3 was enriched in Ret-eGFP^{Lo}:IB4^{Neg} cells, and chloroquine application induced calcium flux predominantly in the Ret-eGFP/IB4-negative population. Since ablation of Mrgpra3 positive neurons impacts upon scratching behavior but not pain (Han et al, 2013), this would suggest that Ret-eGFP^{Lo}:IB4^{Neg} neurons are indeed itch receptors and that multiple itch inducing pathways coexist within these neurons. Thus therapeutic strategies for treating itch should target common signaling molecules or disrupt neuronal function, rather than inhibit specific receptors. The

transcriptional profiling approach described here is a first step towards identifying these molecules.

MATERIALS AND METHODS

Transgenic Mouse lines

To study Ret expression in adult primary sensory neurons we crossed $RET^{eGFP/+}$ mice (Jain et al, 2006) with the DRG neuron specific Cre line $Avil^{cre/+}$ (Zurborg et al, 2011) to obtain Avil-Cre::$RET^{eGFP/+}$ mice. We also used the $Avil^{cre/+}$ line to mark sensory neurons within the DRG by crossing it with a Rosa26-tdRFP strain (Luche et al, 2007) to generate Avil-Cre::$R26^{tdRFP}$ mice. To specifically mark Ret-$eGFP^{Lo}$:$IB4^{Neg}$ neurons we bred Sst-Cre (Taniguchi et al, 2011) and $RET^{eGFP/+}$ mice, thus obtaining Sst-Cre::Ret $^{eGFP/+}$ animals. Heterozygous mice were all viable and exhibited no overt behavioural phenotype.

Mice were bred and maintained at the EMBL Mouse Biology Unit, Monterotondo, in accordance with Italian legislation (Art. 9, 27. Jan 1992, no 116) under licence from the Italian Ministry of Health.

Immunofluorescence

DRG and spinal cord from adult mice (6 weeks or older) were postfixed in 4%PFA for 30 minutes (DRG) or two hours (SC), embedded in 12% bovine gelatin and sectioned using a vibratome at 50-100 μm. Sections were incubated for 30 minutes in 50% ethanol and then overnight in 0,3% TritonX , and 5% goat or donkey serum in PBS at 4C containing one or more antibodies diluted as shown below. Secondary antibodies and streptavidin-647 were diluted (1:1000 and 1:600 respectively) in 0,3%TritonX , 5% goat or donkey serum in PBS and left for 1-2h at 4C. Slides were mounted with Prolong gold antifade (Invitrogen, P36930).

For immunofluorescence analysis of the skin, the hair was removed and skin collected and post-fixed in 4% PFA for 8-12h, incubated in 30% sucrose overnight and frozen in OCT. 35 μm sections were cut with a cryostat. Antibody staining was performed as described above.

We used the following primary antibody dilutions: Rabbit anti-NF200 (Sigma Aldrich, N0142) 1:500, goat anti-CGRP (Santa Cruz, SC8856), rabbit anti-PKCγ (Santa Cruz, SC-211) 1:100, rabbit anti-Htr1f (Novus, NBP1-19625) 1:100, Rabbit anti-H1 histamine receptor (Alamone labs AHR-001) 1:200, rabbit anti-PGP9.5 (Dako, Z5116) 1:200, Isolectin GS-B4-biotin XX conjugate (Invitrogen I21414) 1:100, Rabbit anti-c-Fos (EMD Millipore, PC38), 1:20000, anti-RFP (Rockland, 600-401-379), 1:200, Rabbit anti-TH, (Millipore, AB152), dilution 1:1000. All images were visualized with a Leica SP5 Confocal microscope and analyzed with ImageJ.

Flow Cytometry and Microarray Analysis

To quantitavely analyze the distribution of Ret^{+} sensory neurons in DRG we used flow cytometry on Avil-Cre::$RET^{eGFP/+}$ mice: DRG were collected from 10 adult Avil-Cre::$RET^{eGFP/+}$ mice (6 weeks or older) and pooled. Cells were dissociated with 1mg/ml collagenase IV and 0.05% Trypsin (Gibco, 25300-054) for 25 min each at 37C and subsequently kept on ice in 1%FBS in PBS. Cells were labeled in suspension with 1:50 anti-mouse CD16/32 (eBioscience 14-0161-85) for 5 minutes and Isolectin GS-B4-biotin XX conjugate (1:100) for 10 minutes. After washing, cells were incubated immediately with a cocktail containing Streptavidin-Alexa 647 and anti-CD45-PeCy7 for 5 min, washed, and resuspended in 1%FBS in PBS.

To isolate the whole sensory neuron population, we applied flow cytometric analysis and subsequent sorting on Avil-Cre::$RFP^{tg/+}$ mice: DRG were collected from 3 adult Avil-Cre::$RET^{eGFP/+}$ mice (6 weeks or older) and pooled. Cell dissociation was performed as described above. Cells were then labeled in suspension with 1:50 anti-mouse CD16/32 (eBioscience 14-0161-85) for 5 minutes and after washing cells were incubated in anti-CD45-PeCy7 (1:200) for 5 min, washed, and resuspended in 1%FBS in PBS.

Flow cytometric online and offline analyses were performed in a FACS Aria III SORP (BD Bioscience) using FACS Diva software (BD Bioscience) and FlowJo (Tree Star, Inc.), respectively. For gene expression analysis, DRG cells were sorted (see Supp. Fig. 1a) using an 85 µm nozzle (40 PSI) directly into RLT buffer (RNeasy kit, Qiagen) and RNA was purified according to manufacturer instructions. Two rounds of RNA amplification, labeling and hybridization to Affymetrix® MOE430 2.0 GeneChip® expression arrays were performed by the EMBL Gene Core Facility. Microarray data was analyzed as detailed in Expanded View Information.

Fluidigm

To validate the microarray data we used the microfluidic platform Fluidigm to perform quantitative RT-PCR. DRG from Avil-Cre::RET$^{eGFP/+}$ mice were collected and processed as described above. Cells were sorted directly into 5µl of CellsDirect (Invitrogen, PN 11753-100 and 11753-500) 2x Buffer, and combined with 0.2µl of RT / Taq mix, 1µl of Assay pool (formed by 38 individual assay pairs that were previously pooled and diluted to a final concentration of 500nM) and 2.8µl of water. The samples went through target specific RT and pre-amplification (16 cycles) following manufacturer instructions. Pre-amplified cDNA was then diluted 1:10 and combined together with 2.5µl 2x SsoFast Evagreen Supermix (Bio-Rad, PN 172-5211) and 0.25µl 20x Sample Loading Reagent (Fluidigm, PN 100-0388) and loaded onto the sample inlets of a 48.48 Fluidigm Dynamic Array. All 38 individual assays pairs (100uM) were diluted by combining 0.25µl of each assay with 2.25µl of TE Buffer (TEKnova, PN T0224) and 2.5µl of Assay Loading Reagent (Fluidigm, PN 85000736). The 48.48 chip was then loaded on the IFC Controller MX and 30 cycles of qPCR was performed on the Fluidigm BioMark HD system. Data was analyzed using Fluidigm Real-Time Analysis Software.

Mono-biotinylated IL-31 labeling

Monobiotinylated IL-31 was produced and biotinylated as described in Expanded View Experimental Procedures. Equimolar biotinylated IL-31 was conjugated with streptavidin

Alexa Fluor 546 for 30 minutes at 4C. Dissociated DRG neurons from Avil-Cre::Ret^{eGFP} mice were plated on glass coverslips and washed for one hour in CIB with 1% BSA (140mM NaCL, 4mM KCl, 2mM CaCl2, 1mM MgCl2, 4,55 mM NaOH, 5 mM Glucose, 10 mM HEPES, pH 7,4). IL-31 Alexa Fluor 546 conjugate was then applied at 1nM concentration in CIB plus 1% BSA for 30 minutes at 4C along with IB4-647 at 1:800 dilution. Following labeling, cells were washed 3 times with CIB plus 1% BSA and then fixed for 5 minutes in 4% PFA before mounting. As a negative control, 1nM streptavidin Alexa Fluor 546 was applied to cells using an identical procedure and no membrane staining was evident

Calcium Imaging

DRG from adult Avil-Cre::RET$^{eGFP/+}$ mice were collected and incubated in 1mg/ml collagenase IV (Sigma Aldrich, C5138) and 0,05% Trypsin (Gibco, 25300-054) for 25 minutes each at 37C. They were then suspended in DRG medium (DMEM -Gibco, 41966-029- with 10% heat inactivated Fetal Bovine Serum –PAA, A15101-, 0,8% Glucose and 100U of Penicillin/Streptomycin –Gibco, 15140-122-). Cells were plated in a droplet of medium on glass coverslips treated with Poly-L-lysine and left to attach for 3 hours at 37C.

IB4 staining (Isolectin GS-B4-biotin XX conjugate, Invitrogen, I21414) was performed at 1:100 dilution (in 1%FBS/PBS) followed by 5 minute incubation in 1:600 Streptavidin 546 (Invitrogen, S11225). Cells were then incubated in 3 μM Fura2-AM (Invitrogen, F1221) in CIB (140mM NaCL, 4mM KCl, 2mM CaCl2, 1mM MgCl2, 4,55 mM NaOH, 5 mM Glucose, 10 mM HEPES, pH 7,4) and calcium imaging was performed as described (Caspani et al, 2009). Concentrations of compounds used were: Histamine 1 μM (Sigma, H7250), 30 nM LY344864 30 nM (Abcam, ab120592), IL31 (Peprotech, 210-31) 2 nM, Choloroquine (Sigma, C 6628) 500 μM, allyl isothiocyanate (Sigma) 100 μM

Behavior

6-8 week old C57BL/6J or Avil-cre::RET$^{eGFP/+}$ mice were acclimated for one day. The following day they were injected subcutaneously with 100 µl solution in the nape of the neck with one of the following reagents: 10mM Histamine (Sigma, H7250) in saline, 1mM LY344864 (Abcam, ab120592) in saline, IL31 (Peprotech, 210-31) in 0,1% BSA/PBS. Control injections were performed with appropriate vehicle. Animals were observed for 30 minutes and bouts of scratching counted. In some cases, 1 hour after injections, the cervical part of the spinal cord was collected for c-Fos staining

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AUTHOR CONTRIBUTION

The study was designed by PAH and KKS. KKS, LI, RD, MP, CP, GB and DB performed experiments and TP performed bioinformatics analysis. PAH wrote the paper with contributions from other authors.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Figure 1. Ret-eGFP is Expressed in Multiple Sensory Neuron Subsets

(A−L) Ret-positive neurons largely overlap with markers for myelinated neurons (NF200), non-peptidergic nociceptors (IB4) and C-fiber low-threshold mechanoreceptors (TH). However some Ret positive neurons are negative for these markers (indicated by the arrows in D,H and N) suggesting the existence of a further subset of Ret^{+} neurons. (O−R) A small proportion of Ret positive cells coincides with CGRP-expressing neurons (arrowheads in P), reflecting an uncharacterized subpopulation of sensory neurons. Scale bars, 50 μm.

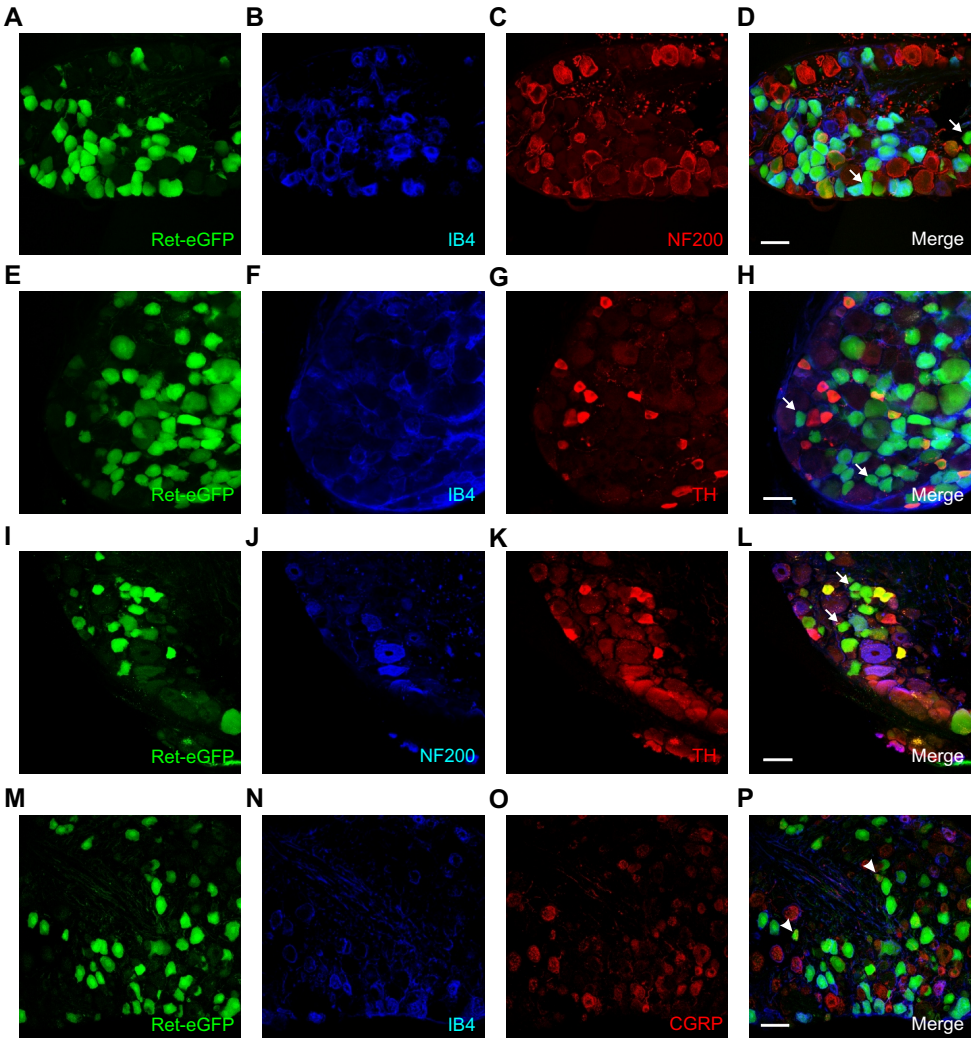


Figure 2. Ret-eGFP Fluorescence Is Widely Distributed in the Skin

(A−C) Ret-eGFP free nerve endings are broadly distributed in the dermis and epidermis of the skin. Double immunofluorescence of Ret-eGFP (A) and the pan-neuronal marker PGP9.5 (B) in adult Avil-Cre::$Ret^{+/eGFP}$ mouse hairy skin. Dashed line indicates the dermis/epidermis junction. Scale bar, 50 μm. (D−F) Ret-eGFP fibers also terminate as lanceolate endings encircling hairs. Double immunostaining of Ret-eGFP (D) and the pan-neuronal marker PGP9.5 (E) in adult Avil-Cre::$Ret^{+/eGFP}$ mouse hairy skin. Scale bar, 25 μm,

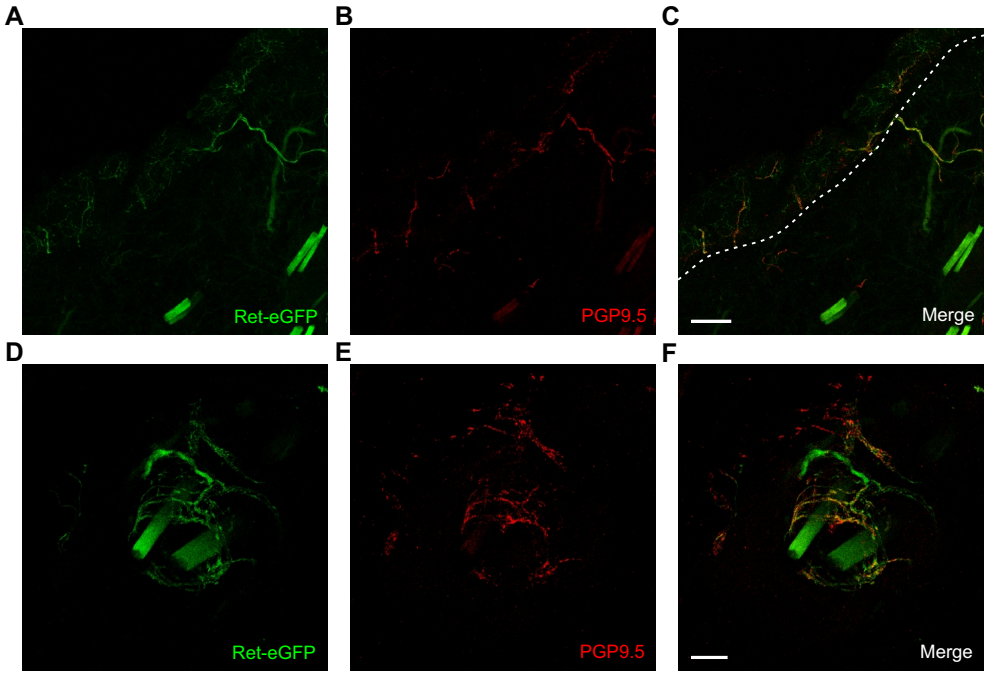


Figure 3. Central Terminals of Ret-eGFP Expressing Primary Sensory Neurons Are Broadly Distributed Within The Dorsal Horn of the Spinal Cord

Spinal cord cross-sections from adult Avil-Cre::Ret$^{+/eGFP}$ mice labeled with the indicated markers. (A−D) Ret-eGFP fluorescence is strongly expressed in lamina II_o and partially overlaps with Lamina I. Triple immunofluorescene of Ret-eGFP (A) with IB4 (B) and CGRP (C). (E−H) Ret-eGFP expression in lamina II_i/III. Triple immunofluorescence of Ret-eGFP (E) with IB4 (F) and PKCγ (G), a marker for lamina II_i/III interneurons. (I−L) Ret-eGFP is expressed diffusely through laminae III to V. Triple immunostaining of Ret-eGFP (I) with NF200 (J) and PKCγ (K). Scale bar, 100 μm.

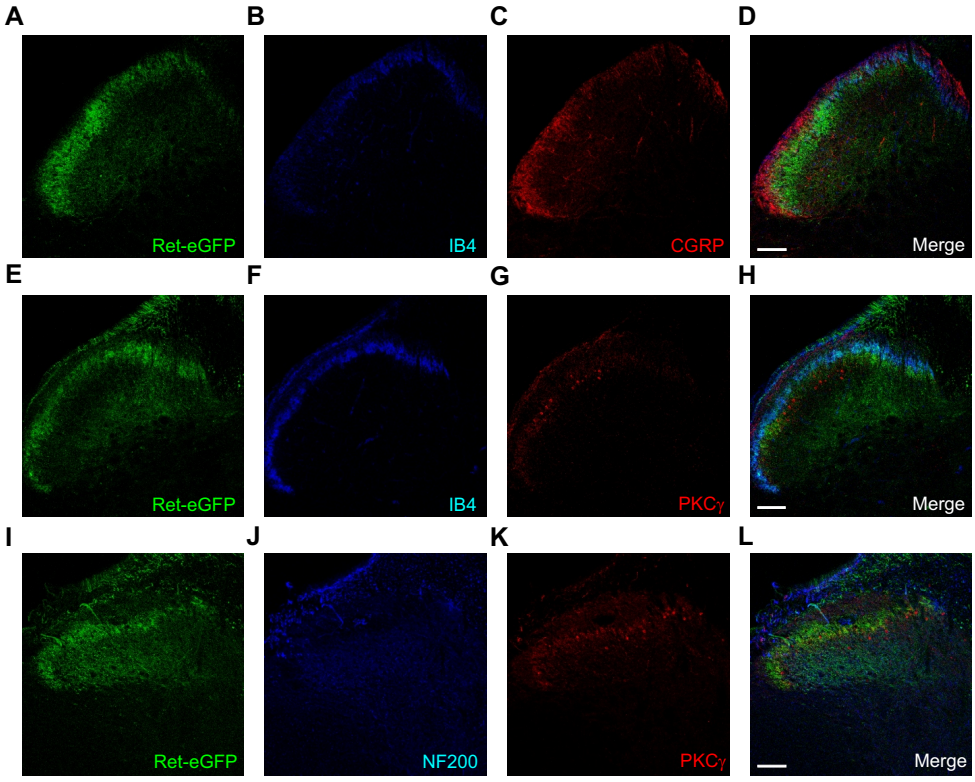


Figure 4. Multiple Ret-positive Subpopulations as Determined by Flow Cytometric Analysis of Dissociated Sensory Neurons from Avil-Cre::Ret$^{+/eGFP}$ Mice

(A) Flow cytometric analysis of dissociated sensory neurons plotted according to their level of endogenous Ret-eGFP expression and IB4 binding. There are 5 well-defined subsets, 2 out of which do not bind to IB4 but display a differential level of eGFP intensity (termed Ret-eGFP^{Lo}:IB4^{Neg}, A, and Ret-eGFP^{Hi}:IB4^{Neg} ,B, respectively). The other 3 subsets bind IB4 and display a range of eGFP intensities, termed Ret-eGFP^{Lo}:IB4^{Lo}, C, Ret-eGFP^{Hi}:IB4^{Lo}, D, and Ret-eGFP^{Hi}:IB4^{Hi}, E. (B) No correlation between the median cell size of different Ret^{+} subsets and eGFP intensity or IB4 binding. The graph shows the forward scatter values plotted against the normalized number of cells, displayed as the percent of Max. (C−D) Variations in endogenous eGFP intensity are clearly visible with fluorescent microscopy. (C) Cultured neurons and (D) DRG section from Avil-Cre::Ret$^{+/eGFP}$ mice displaying native eGFP fluorescence and stained with IB4. Different levels of eGFP intensity (high -indicated by arrows and low -indicated by arrowheads) are detected across IB4^{+} and IB4^{-} cells. Scale bar, 50 μm.

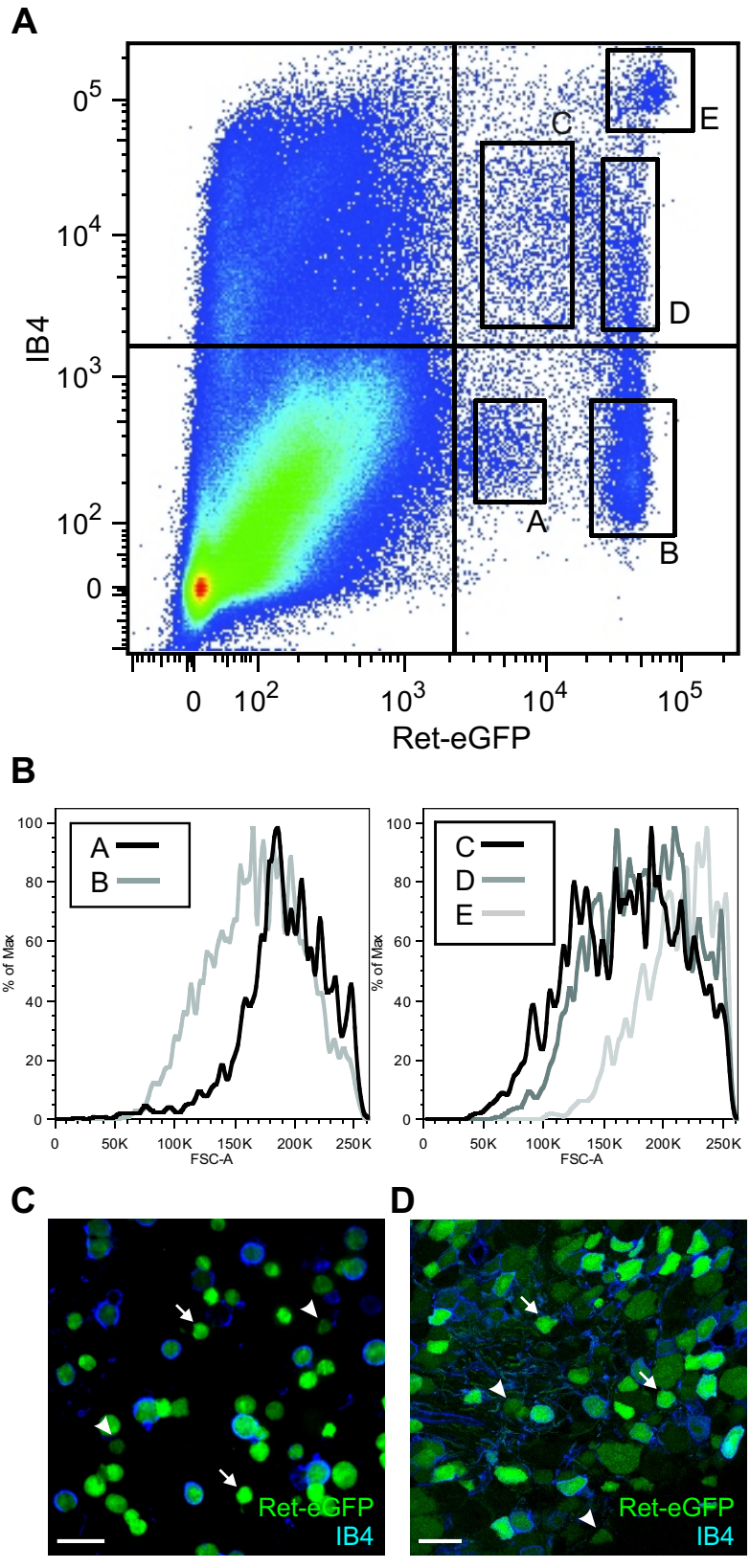

A
IB4
Ret-eGFP
C
E
D
A
B
B
% of Max
FSC-A
A
B
C
D
E
C
D
Ret-eGFP
IB4
Ret-eGFP
IB4

Figure 5. Transcriptional profiling of Ret-eGFP^{Hi}:IB4^{Neg} and Ret-eGFP^{Lo}:IB4^{Neg} Sensory Neurons.

(A) Ret-eGFP^{Hi}:IB4^{Neg} and Ret-eGFP^{Lo}:IB4^{Neg} neurons display distinct expression profiles. A volcano plot of fold change expression in Ret-eGFP^{Lo}:IB4^{Neg} versus Ret-eGFP^{Hi}:IB4^{Neg} against probability. Ret has higher expression in the Ret-eGFP^{Hi}:IB4^{Neg} subset, which also shows an up regulation of the Ret co-receptor Gfra2 and Fam38b, encoding for the mechanosensitive ion channel Piezo2. The Ret-eGFP^{Lo}:IB4^{Neg} subset, displays an array of molecules previously associated with itch (marked in red). (B) Itch-associated transcripts are enriched in the Ret-eGFP^{Lo}:IB4^{Neg} population compared to all DRG neurons. Volcano plot of fold change expression in Ret-eGFP^{Lo}:IB4^{Neg} neurons versus sorted neurons from Avil-Cre::R26^{tdRFP} mice against probability. Molecules linked to itch perception are significantly upregulated in the Ret-eGFP^{Lo}:IB4^{Neg} subset. (C) Gene expression within the Ret-eGFP^{Lo}:IB4^{Neg} population is confirmed by triple comparison between Ret-eGFP^{Lo}:IB4^{Neg}, Ret-eGFP^{Hi}:IB4^{Neg} and Avil-Cre::R26^{tdRFP}datasets. (D) Validation of differential microarray screening by quantitative RT-PCR. Transcripts encoding ion channels, receptors, neuropeptides, signaling molecules and transcription factors were selected for quantitative RT-PCR. Differential expression between Ret-eGFP^{Lo}:IB4^{Neg} and Ret-eGFP^{Hi}:IB4^{Neg} populations correlates with microarray analysis.

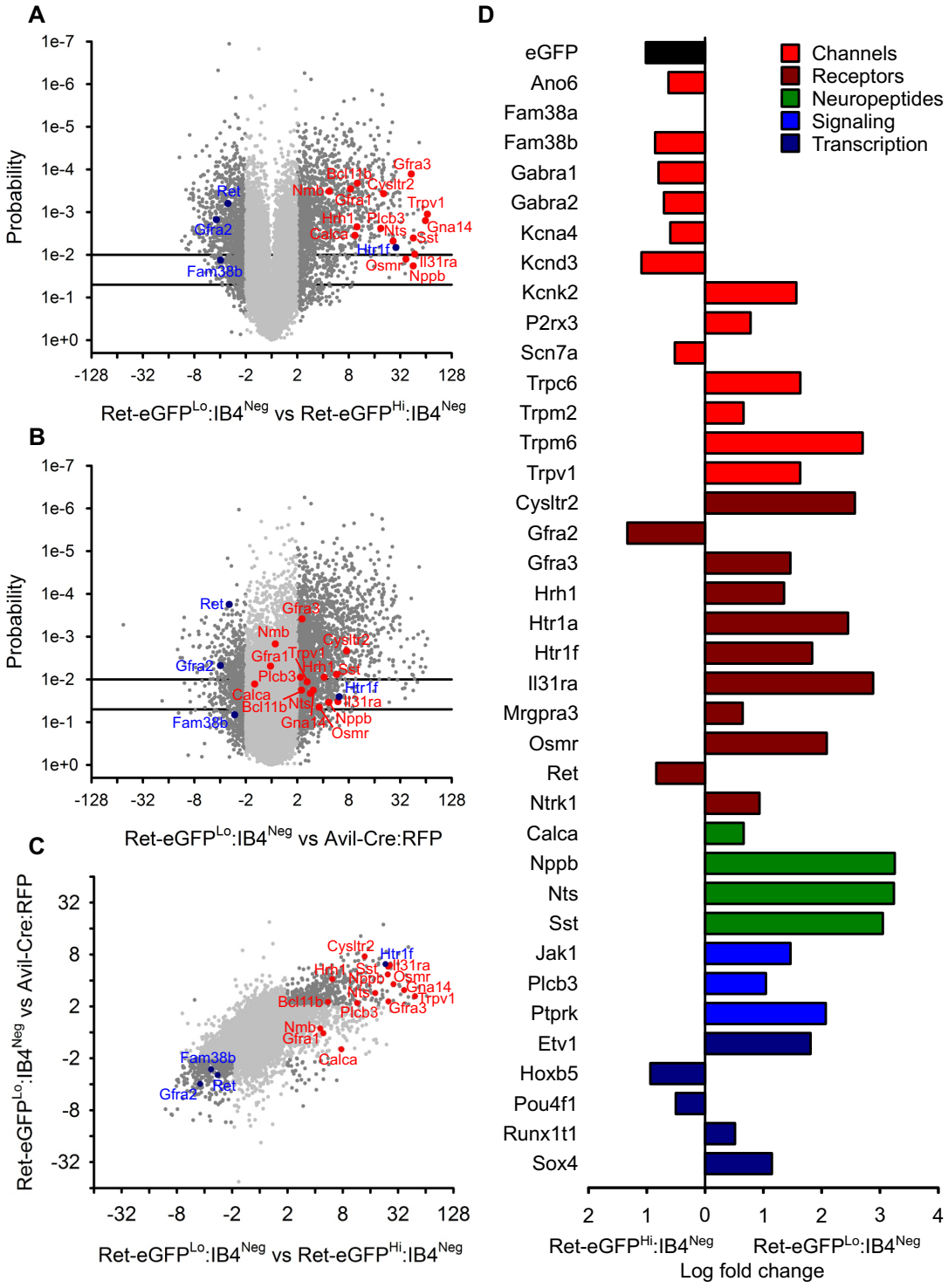

A
Probability
1e-7
1e-6
1e-5
1e-4
1e-3
1e-2
1e-1
1e+0
-128 -32 -8 -2 2 8 32 128
Ret-eGFP^Lo:IB4^Neg vs Ret-eGFP^Hi:IB4^Neg
Ret
Gfra2
Fam38b
Gfra3
Bcl11b
Cysltr2
Nmb
Gfra1
Trpv1
Hrh1
Plcb3
Gna14
Calca
Nts
Sst
Htr1f
Osmr
Il31ra
Nppb
B
Probability
Ret-eGFP^Lo:IB4^Neg vs Avil-Cre:RFP
C
Ret-eGFP^Lo:IB4^Neg vs Avil-Cre:RFP
32
8
2
-2
-8
-32
-32 -8 -2 2 8 32 128
Ret-eGFP^Lo:IB4^Neg vs Ret-eGFP^Hi:IB4^Neg
D
eGFP
Ano6
Fam38a
Fam38b
Gabra1
Gabra2
Kcna4
Kcnd3
Kcnk2
P2rx3
Scn7a
Trpc6
Trpm2
Trpm6
Trpv1
Cysltr2
Gfra2
Gfra3
Hrh1
Htr1a
Htr1f
Il31ra
Mrgpra3
Osmr
Ret
Ntrk1
Calca
Nppb
Nts
Sst
Jak1
Plcb3
Ptprk
Etv1
Hoxb5
Pou4f1
Runx1t1
Sox4
Channels
Receptors
Neuropeptides
Signaling
Transcription
2 1 0 1 2 3 4
Ret-eGFP^Hi:IB4^Neg
Ret-eGFP^Lo:IB4^Neg
Log fold change

Figure 6. Sst^{Cre} Driven Ret-eGFP Expression in DRG and Hairy Skin.

(A−H) Sst^{Cre} mediated recombination of the Ret locus drives eGFP expression in sensory neurons that do not bind to IB4 or co-express NF200 or TH. Triple immunostaining of DRG from Sst-Cre::$Ret^{eGFP/+}$ with RetGFP (A), IB4 (B) and NF200 (C), and RetGFP (E), IB4 (F) and TH (G). (I−L) Peripheral projections of sensory neurons from Sst-Cre::$Ret^{eGFP/+}$ mice terminate in the hairy skin as free nerve endings (arrows). Triple immunostaining of Ret-eGFP (I), DAPI (J) and PGP9.5 (K). Dashed line indicates the dermis/epidermis junction. Scale bars, 50 μm.

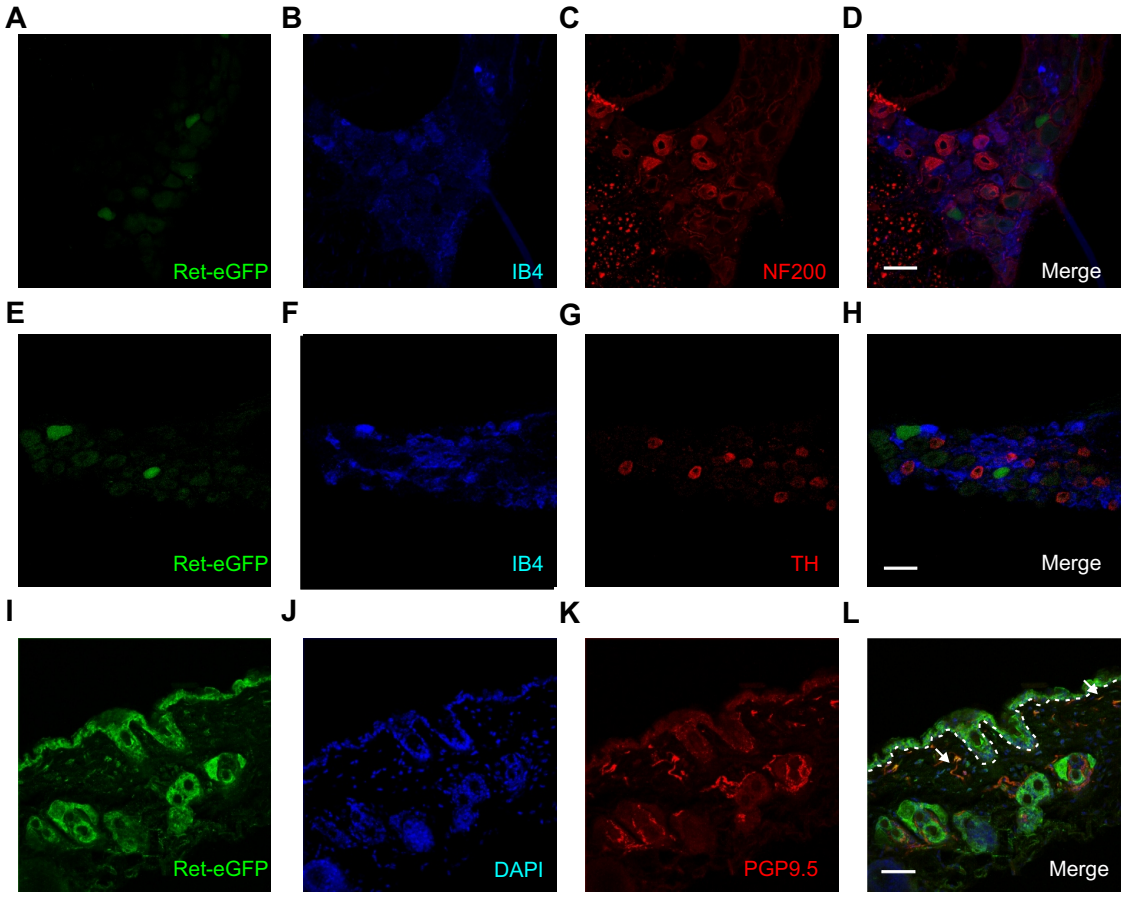


Figure 7. Il-31 and the $5HT_{1F}$ Agonist LY344864 Activate Ret-eGFP/IB4 Negative Neurons

(A−H) IL-31 binds to and activates Ret-eGFP/IB4 negative neurons. (A−D) Biotinylated IL-31 binds to the plasma membrane of a small subset of weakly fluorescent Ret-eGFP^{+} cells which are IB4 negative (arrow). Live imaging of cells isolated from Avil-Cre::Ret$^{+/eGFP}$ mice and co-stained with IB4 (B) and 1nM biotinylated IL31 conjugated to streptavidin Alexa Fluor 546. (C). (E−H) IL-31 evokes calcium flux in Ret-eGFP/IB4 negative neurons. (E) Dissociated sensory neurons isolated from adult Avil-Cre::Ret$^{+/eGFP}$ mice and stained with IB4. (F) Fura-2 fluorescent intensity changes for the same cells upon application of 2nM IL-31, and (G) example of IL-31 response. (H) IL-31 responses are mainly confined to Ret-eGFP/IB4 negative neurons (numbers in parenthesis indicate percentages, n=3 mice). (I−P) The $5HT_{1F}$ receptor is expressed in Ret-eGFP/IB4 negative neurons. (I−L) Triple immunostaining of DRG sections of Avil-Cre::Ret$^{+/eGFP}$ showing Ret-eGFP (I), IB4 (J) and $5HT_{1F}$ (K). Arrowheads (L) indicate $5HT_{1F}$ cells that are IB4 positive and weakly express Ret-eGFP. (M) Dissociated sensory neurons isolated from adult Avil-Cre::Ret$^{+/eGFP}$ mice and stained with IB4. (N) Fura-2 fluorescent intensity changes for the same cells upon application of 30nM LY344864, and (O) an example of their response. (P) LY344864 activates mainly Ret-eGFP/IB4

negative neurons (numbers in parenthesis indicate percentages, n=3 mice). Scale bars, 50 μm

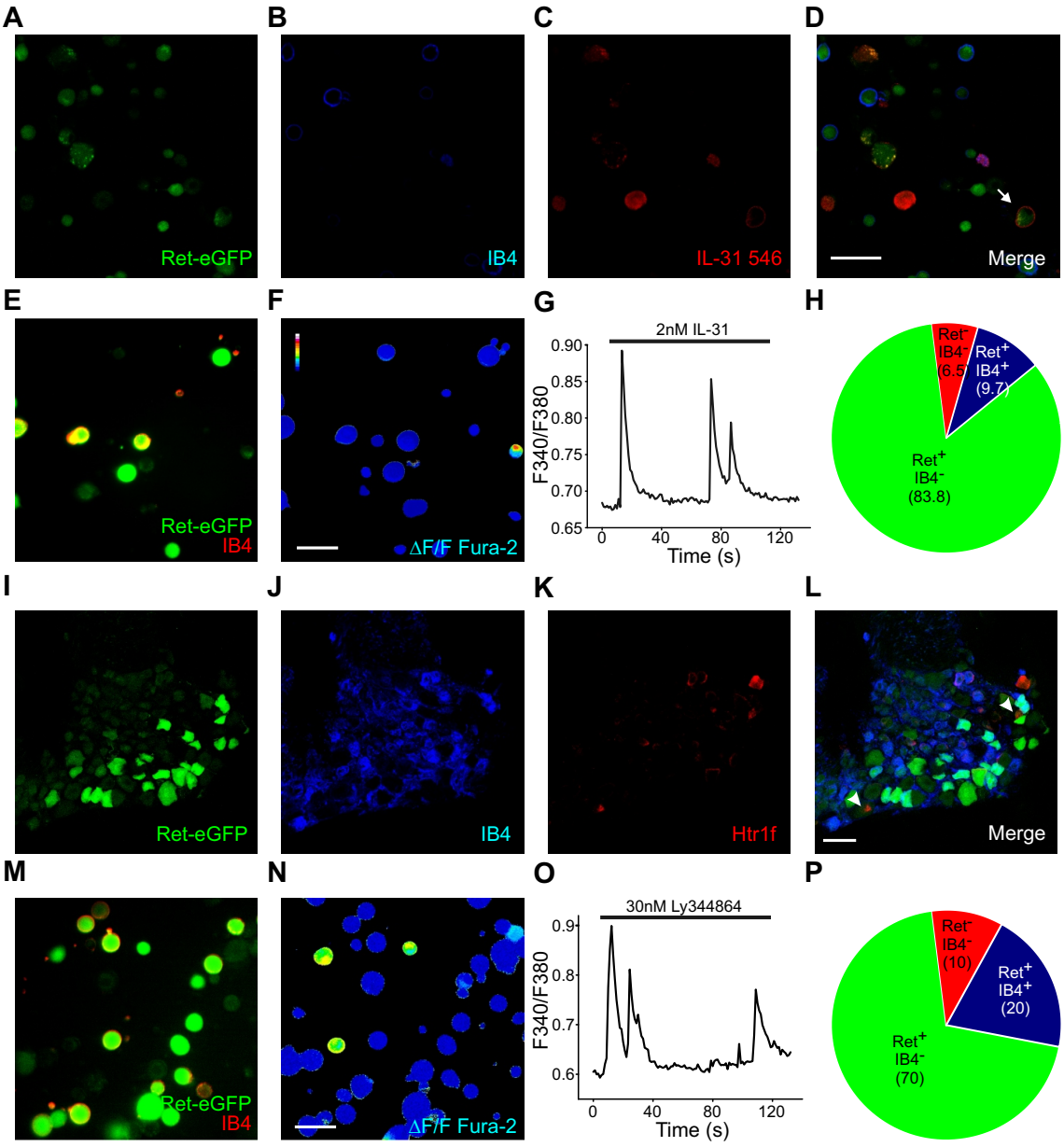
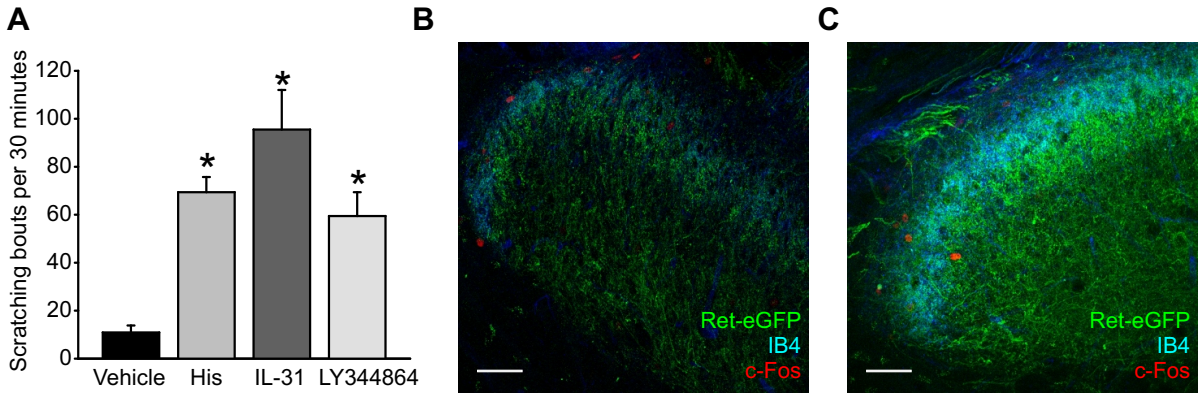

A
B
C
D
Ret-eGFP
IB4
IL-31 546
Merge
E
F
G
H
Ret-eGFP
IB4
ΔF/F Fura-2
2nM IL-31
F340/F380
Time (s)
Ret⁻ IB4⁻ (6.5)
Ret⁺ IB4⁺ (9.7)
Ret⁺ IB4⁻ (83.8)
I
J
K
L
Ret-eGFP
IB4
Htr1f
Merge
M
N
O
P
Ret-eGFP
IB4
ΔF/F Fura-2
30nM Ly344864
F340/F380
Time (s)
Ret⁻ IB4⁻ (10)
Ret⁺ IB4⁺ (20)
Ret⁺ IB4⁻ (70)

Figure 8. IL-31 and LY344864 Elicit Scratching Behavior in Mice and C-fos Activation in the Spinal Cord.

(A) A single subcutaneous injection of histamine (positive control), IL-31 or LY344864 evokes significantly more scratching bouts than injection of vehicle alone. The scratching behavior was observed for 30 minutes after the injection. (B−C) Application of IL-31 or LY344864 induces sparse activation of C-fos expression which overlaps with the Ret-eGFP positive neurons in laminae I and II_i/III. IL-31 (B) and LY344864 (C) induced c-fos activation in Avil-Cre::$Ret^{+/eGFP}$ mouse cervical spinal cord cross-sections. Scale bars, 50 µm.



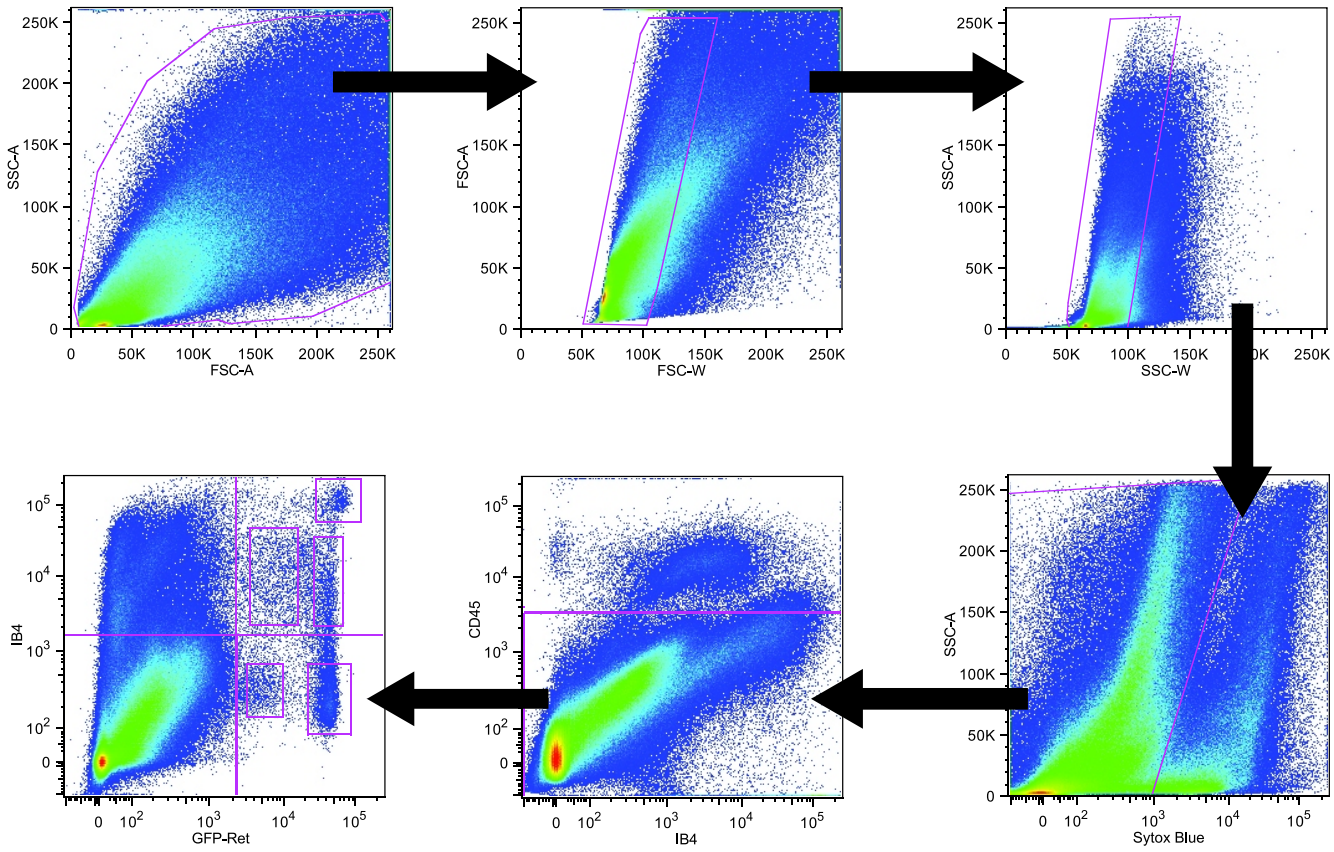
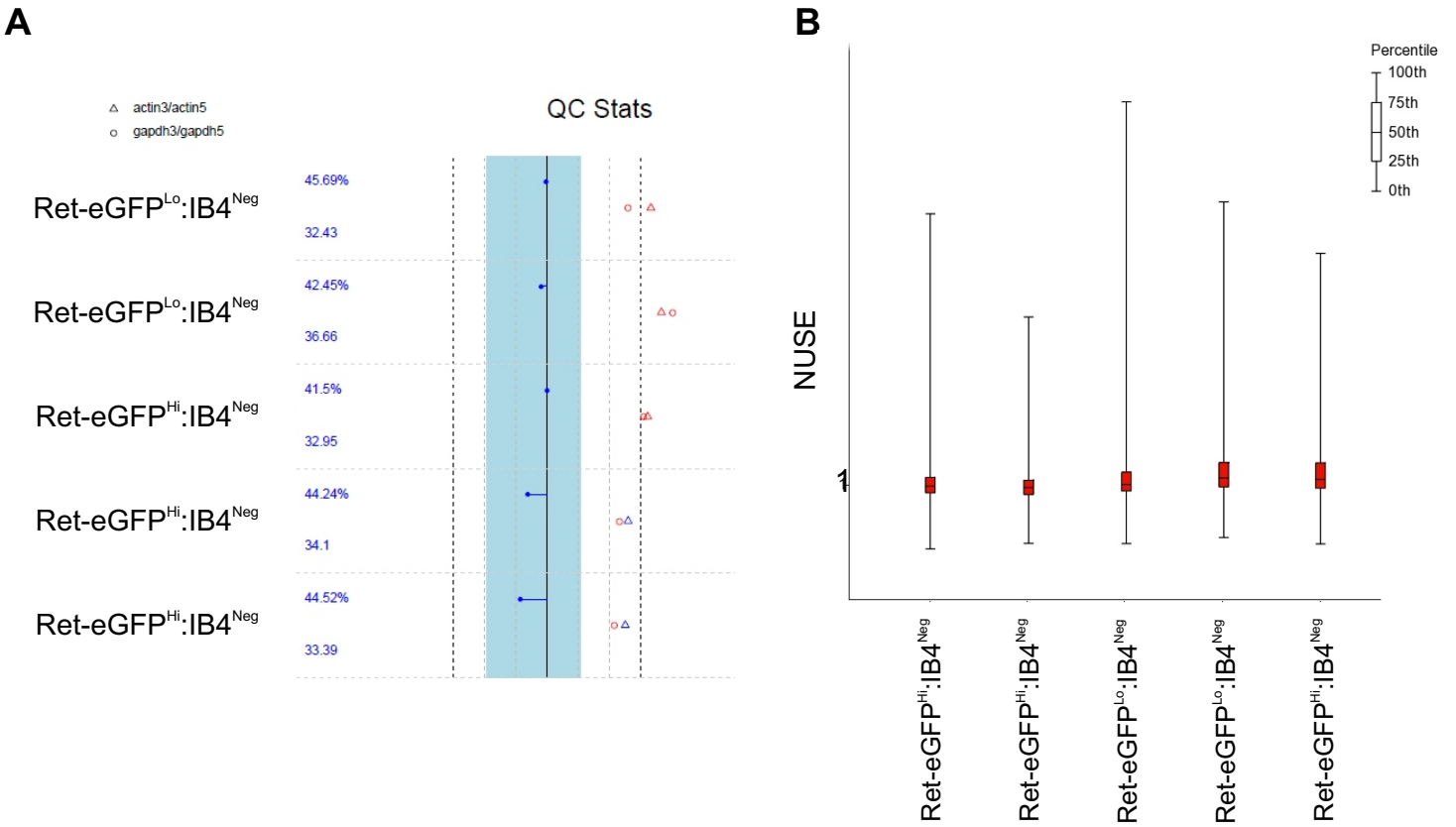


Figure E1. Avil-Cre::Ret$^{+/eGFP}$ DRG Cell Sorting Strategy

Hierarchical gating strategy for the isolation of live Ret^{+} sensory neurons. DRG cells were isolated from Avil-Cre::Ret$^{+/eGFP}$ mice, quantified and selected according to their size and complexity. Dead cells and immune cells were excluded using the living dye Sytox Blue, and an antibody against cd45. Live sensory neurons were defined by their levels of eGFP fluorescence and IB4 binding. Percentages from parent populations are indicated in each graph.


A
actin3/actin5
gapdh3/gapdh5
QC Stats
Ret-eGFP^Lo:IB4^Neg
45.69%
32.43
Ret-eGFP^Lo:IB4^Neg
42.45%
36.66
Ret-eGFP^Hi:IB4^Neg
41.5%
32.95
Ret-eGFP^Hi:IB4^Neg
44.24%
34.1
Ret-eGFP^Hi:IB4^Neg
44.52%
33.39
B
Percentile
100th
75th
50th
25th
0th
NUSE
1
Ret-eGFP^Hi:IB4^Neg
Ret-eGFP^Hi:IB4^Neg
Ret-eGFP^Lo:IB4^Neg
Ret-eGFP^Lo:IB4^Neg
Ret-eGFP^Hi:IB4^Neg

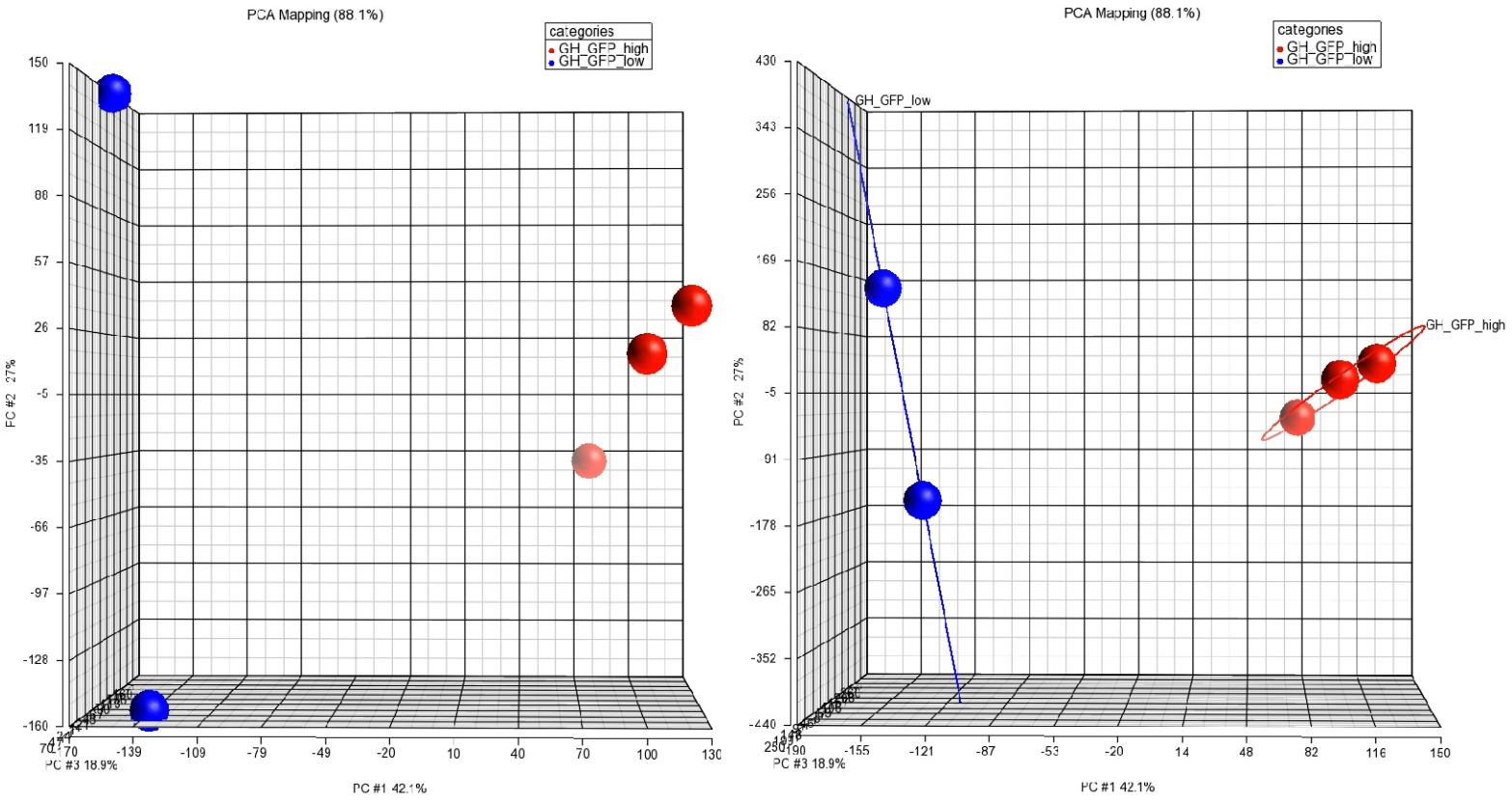
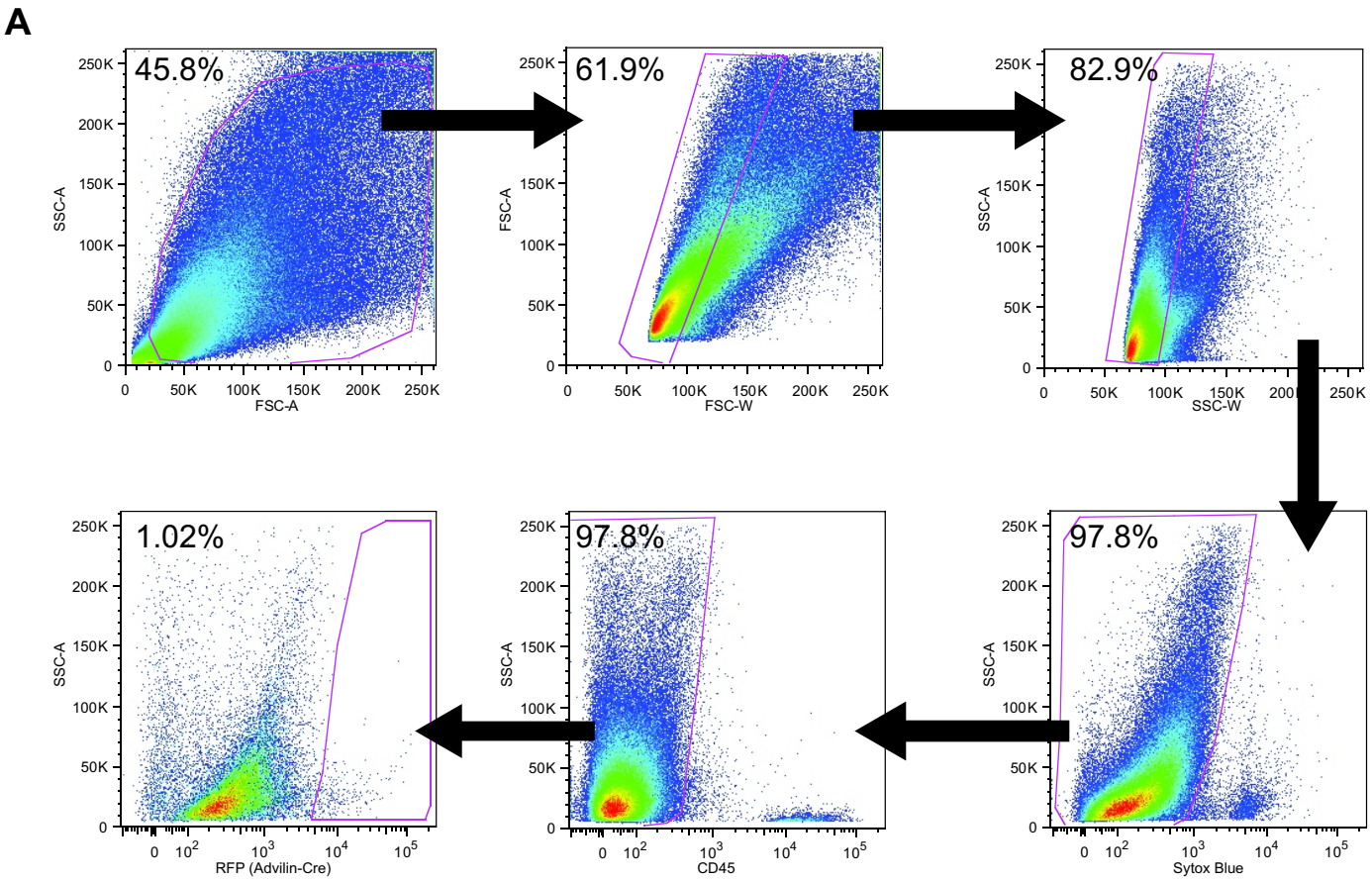

C
PCA Mapping (88.1%)
categories
GH_GFP_high
GH_GFP_low
PC #2 27%
PC #3 18.9%
PC #1 42.1%
PCA Mapping (88.1%)
categories
GH_GFP_high
GH_GFP_low
GH_GFP_low
GH_GFP_high
PC #2 27%
PC #3 18.9%
PC #1 42.1%

Figure E2. Microarray quality control

(A) R ratios between the expression values for 3' and 5' ends of GAPDH and β-actin mRNA transcripts. No suspect 3' to 5' signal intensity ratio was found. Probesets marked as Present range around 41.5% – 47.62%, while the background levels are up to 38.88. Each row shows the %present, average background, scale factors and GAPDH / -actin ratios for an individual chip. GAPDH 3 ': 5' values are plotted as circles. GAPDH values that are considered potential outlier (ratio > 1.25) are coloured red, otherwise they are blue. β-actin, 3': 5' ratios are plotted as triangles. Because this is a longer gene, the recommendation is for the 3': 5' ratios to be below 3; values below 3 are colored blue, those above, red. Blue stripe in the image represents the range where scale factors are within 3-fold of the mean for all chips. Scale factors are plotted as a line from the center line of the image. A line to the left corresponds to a down-scaling, to the right, to an up-scaling. If any scale factors fall outside this 3-fold region, they are colored red, otherwise they are blue. (B) The Normalized Unscaled Standard Errors (NUSE) were plotted to account for differences in variability between genes. The standard error estimates obtained for each probe set on each array were taken and standardized across arrays so that the median standard error for each type of probe set was 1 across all arrays. All boxplots on the graph should be similar and the median should be 1 across all arrays. All arrays were observed to be of similar quality. (C) PCA plot of RMA8 normalized, GC content adjusted array data. Both panels show a different angle of the PCA plot. Low intragroup variability was observed, while group differences are discernible. The first principal component (X- axis) explains 30.6% of data variation, while the second principal component (Y-axis) explains 19.6% of data variation. The third principal component (Z-axis) explains 16.2% of data variation. Group color codes Blue: GH_GFP_low, Red: GH_GFP_high. Illustrations made with Partek® Genomics suite software ver6.3.



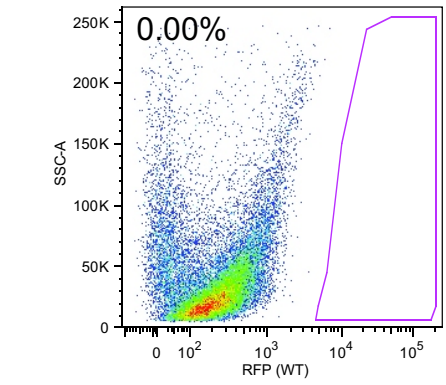


Figure E3. Avil-Cre::R26^{tdRFP} DRG Cell Sorting Strategy

(A) Hierarchical gating strategy for the isolation of RFP^{+}CD45^{-} living cells from single cell suspensions of DRG isolated from Avil-Cre::R26^{tdRFP} mice. Percentages from parent populations are indicated in each graph. (B) Last gate corresponding to control animals not expressing Cre recombinase.

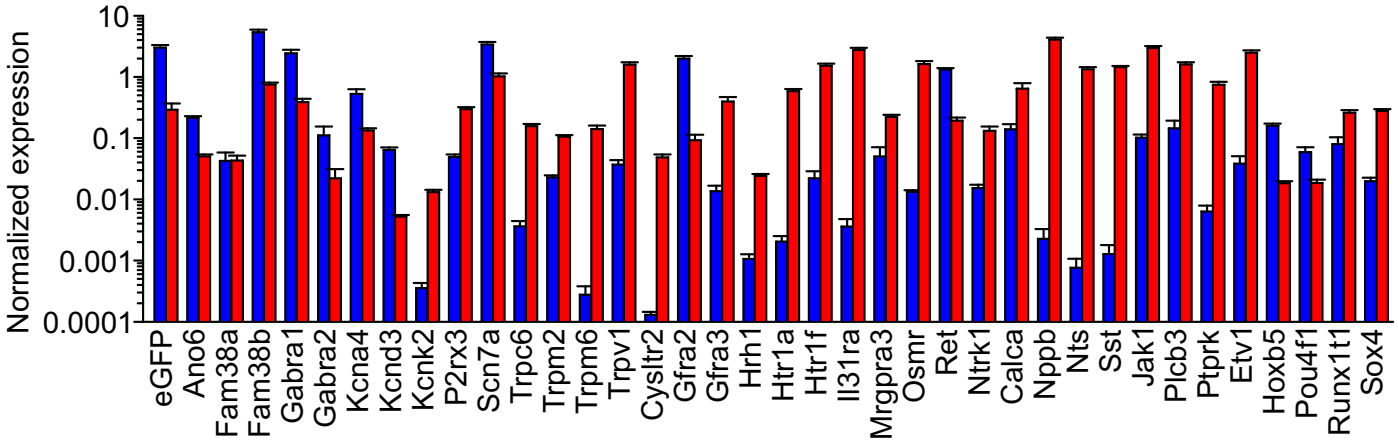


Figure E4. Validation of the Microarray Screens by Quantitative RT-PCR

The expression of 38 genes was validated using the microfluidic platform Fluidigm. Their relative expression, normalized to GAPDH, is shown here (± SEM). Expression in the Ret-eGFP^{Hi}:IB4^{Neg} subset is indicated in blue, and expression in the Ret-eGFP^{Lo}:IB4^{Neg} subset is in red.

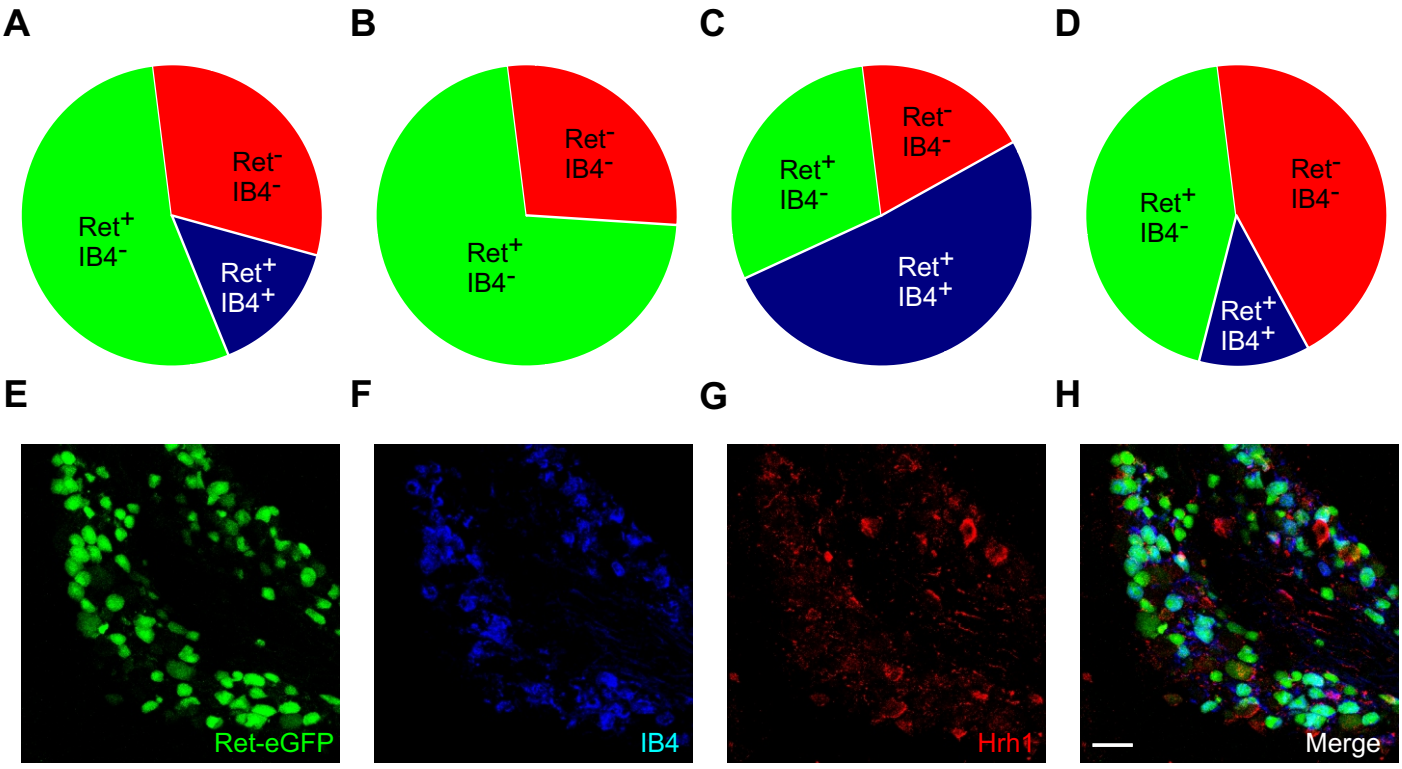


Figure E5. Activation Profile of Ret-eGFP/IB4 Negative Neurons

Ratiometric calcium imaging was performed on sensory neurons dissociated from Avil-Cre::Ret$^{+/eGFP}$ mice, to assess the proportion of (A) Histamine, (B) Chloroquine, (C) Allyl isothiocyanate and (D) Capsaicin responsive cells that were Ret-eGFP-negative, Ret-eGFP/IB4-positive and Ret-eGFP/IB4-negative. The expression of the Histamine receptor 1 in Ret-eGFP/IB4 negative subpopulation is shown by immunostaining. (E- H) Triple immunostaining of RetGFP (E), IB4 (F) and HRH1 (G) on DRG sections from Avil-Cre::Ret$^{+/eGFP}$ mice.

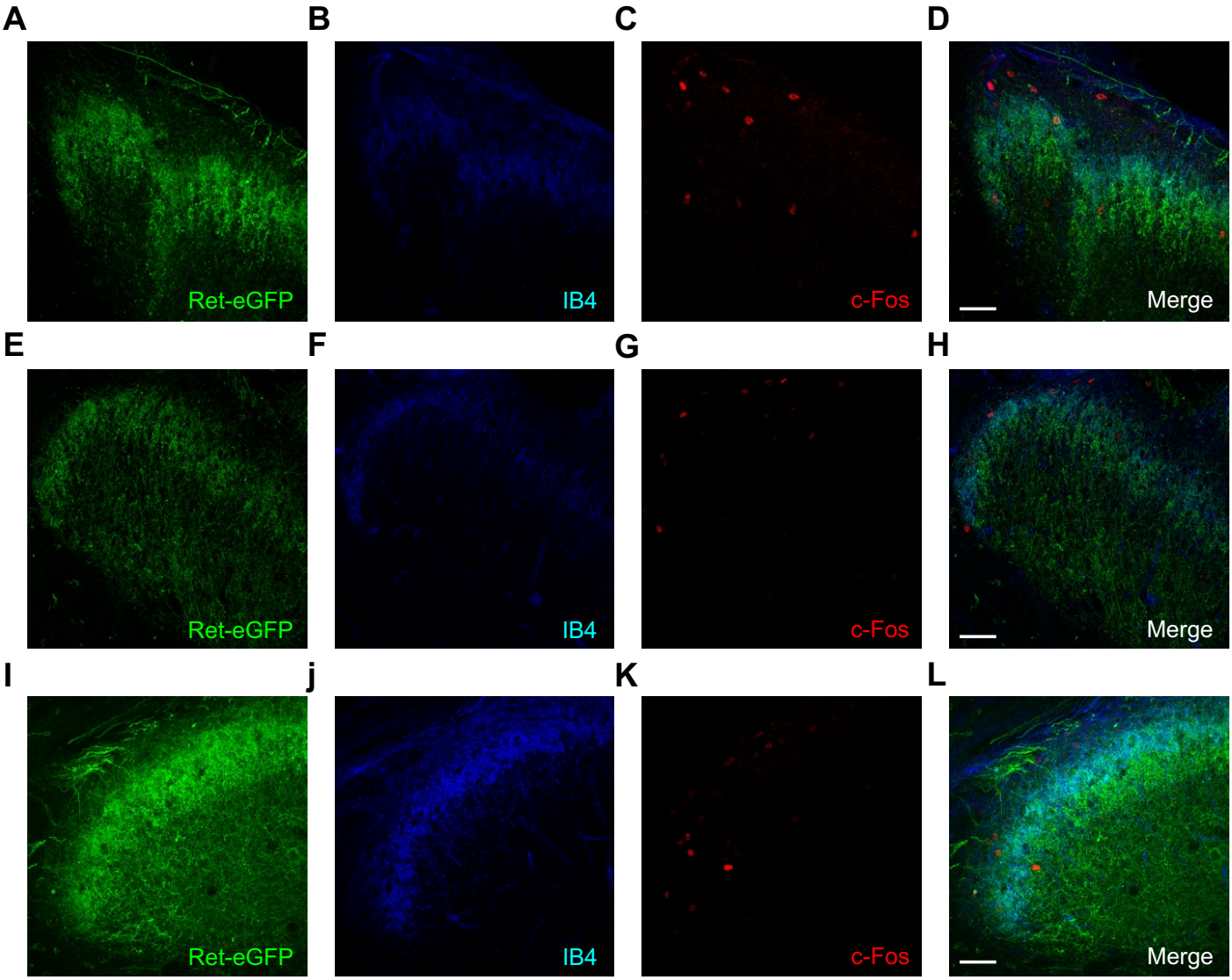


Figure E6. Pruritogens Induce c-Fos Expression in Cervical Spinal Cord After Subcutaneous Injection

C-fos activation in Avil-Cre::Ret$^{+/eGFP}$ mouse spinal cord cross-sections after injection of 1µmol Histamine (A- D), 55pmol IL-31 (E- H), 100nmol LY344864 (I- L). IB4 binding is also shown. Scalebar 50 µm.