Association of $\text{Na}_\text{V}1.8$ with lipid rafts in DRG sensory neurons

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PhD Thesis
Abstract

Voltage gated sodium channels (VGSCs) play a key role in the initiation and propagation of action potentials in neuronal cells. Na\textsubscript{V}1.8 is a Tetrodotoxin resistant VGSC expressed in nociceptors and underlies the majority of sodium currents during action potentials. Many studies have highlighted a key role of Na\textsubscript{V}1.8 in different pain pathways. Lipid rafts are microdomains of the plasma membrane highly enriched in cholesterol and sphingolipids characterised by unique physical features: a liquid ordered phase and the resistance to non-ionic detergent at 4°C. Lipid rafts are thought to act as platforms on the membrane where proteins and lipids can be compartmentalised and functionally clustered. In the present study we investigated Na\textsubscript{V}1.8 sub-cellular localisation and explored the idea that it is associated with lipid rafts in nociceptors. We hypothesised that lipid rafts on primary sensory neurons act as a platform on the membrane where Na\textsubscript{V}1.8 can be trafficked and underlie action potentials generation. We demonstrated that Na\textsubscript{V}1.8 is associated with lipid rafts along the sciatic nerve \textit{ex vivo} and in DRG neurons \textit{in vitro}. We also found that Na\textsubscript{V}1.8 is distributed in clusters along the axons of DRG neurons \textit{in vitro} and \textit{ex vivo}. We investigated the functional meaning of Na\textsubscript{V}1.8-raft association by studying action potential propagation in sensory neurons, in response to mechanical and chemical stimulation, by calcium imaging. Disruption of the association between Na\textsubscript{V}1.8 and lipid rafts in cultured sensory neurons, by methyl-beta-cyclodextrin and 7-ketocholesterol, caused a reduction in the number of cells able to propagate action potentials. In addition, lipid raft depletion caused a remarkable reduction in the conduction velocity upon mechanical stimulation. These findings highlight the importance of the association between Na\textsubscript{V}1.8 and lipid rafts in the conduction of action potentials and could lead to new perspectives in the study of Na\textsubscript{V}1.8 trafficking and nociceptor excitability.
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Bibliography
Declaration of Originality

I hereby declare that this thesis and the work reported herein was composed by and originated entirely from me. Information derived from the published and unpublished work of others has been acknowledged in the text and references are given in the list of sources.

Alessandro Pristerá.
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>7KC</td>
<td>7-ketocholesterol</td>
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<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>CAB</td>
<td>Capsaicin, ATP, Bradykinin</td>
</tr>
<tr>
<td>CTB</td>
<td>Cholera toxin sub-unit B</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene related peptide</td>
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<tr>
<td>DIV</td>
<td>Days in vitro</td>
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<tr>
<td>DRG</td>
<td>Dorsal root ganglia</td>
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<tr>
<td>DRM</td>
<td>Detergent resistant membrane</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>GM1</td>
<td>Ganglioside GM1</td>
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<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>$L_o$</td>
<td>Liquid ordered</td>
</tr>
<tr>
<td>$L_d$</td>
<td>Liquid disordered</td>
</tr>
<tr>
<td>MBCD</td>
<td>Methyl-β-cyclodextrin</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
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<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>TTX-s</td>
<td>Tetrodotoxin sensitive</td>
</tr>
<tr>
<td>TTX-r</td>
<td>Tetrodotoxin resistant</td>
</tr>
<tr>
<td>VGSCs</td>
<td>Voltage gated sodium channels</td>
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<td>WT</td>
<td>Wild-type</td>
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Chapter 1: General Introduction
1 Introduction

1.1 Pain and pain anatomical pathway

1.1.1 Pain: definition and types.

The ability of complex living organisms to detect noxious stimuli and to code the sensation of pain is of fundamental importance. The feeling of pain serves as a protective mechanism against potentially dangerous insults; it prevents or limits bodily harm and preserves physical integrity. The experience of pain is the result of a complex interaction and integration of different parts of the body which include neuronal and non-neuronal cells, the sensory transducers, nerves, the spinal cord, and the brain. Also, at each level of the pain pathway, multiple tuning mechanisms contribute to define the sensation of pain, which ultimately occurs in the brain. Pain also affects the emotional dimension and this, in turn, can affect the feeling of pain itself. Pain is defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Wall and Melzack, 2005). An unequivocal strict classification of different types of pain cannot be made, since there are overlapping mechanisms behind the different conditions. Nevertheless, pain can be broadly classified into different types according to several parameters which include the cause, time course, modality, and region of the body from which it arises. By considering the cause as a determinant, pain can be defined as nociceptive when it results from a direct activation of the cells that are able to transduce the noxious insult. Minor injuries, burns and infections can lead to an inflammatory state, characterised by hypersensitivity towards noxious stimuli. This type of pain is referred to as inflammatory pain. Neuropathic pain results from damage or a malfunction of the nervous system. Examples of conditions that may be associated to neuropathic pain include trauma, diabetes, viral infection and cancer (toxic effect of chemotherapy). From a temporal point of view, pain can be defined as acute, which usually resolves when the noxious stimulus is no longer present, or chronic, which can occur for years. The modality of pain, referring to intensity and description, can also be used in its classification. In fact, there are several degrees of pain intensity (moderate to severe) and it can be described, for example, as burning, stabbing, tingling, numbing, and prickling. Pain becomes of clinical relevance when it loses its protective physiological role.
There are many conditions and diseases where specific symptoms are accompanied by chronic pain. In these conditions pain no longer serves as a defensive mechanism. Usually chronic pain is characterised by hyperalgesia and allodynia. Hyperalgesia is defined as an increased sensation of pain in response to a normally noxious stimulus, while allodynia is the perception of pain in response to a normally non noxious stimulus. Chronic pain heavily affects the quality of life and can aggravate the clinical condition, since chronic pain can be the cause of co-morbidities like stress, anxiety, depression, and lack of sleep. Apart from pure clinical aspects, social issues must be also considered, in terms of both social consequences for the patients (e.g. inability or impairment to perform everyday activities) as well as costs for the health systems. It is estimated that 1% of the UK population suffers from chronic pain. The different types of pain can be managed clinically, albeit with different degrees of effectiveness. Traditionally, acute nociceptive and inflammatory pain can be efficiently treated with painkillers (e.g. paracetamol, NSAIDs). On the other hand, even if drugs (e.g. opioids, tricyclic antidepressant, and pregabalin) can alleviate chronic pain in some patients, effective treatments able to abolish severe ongoing pain are unavailable (O'Connor and Dworkin, 2009). For this reason, a wide-spectrum approach is being carried out, from scientists to clinicians, in an effort to understand the exact mechanisms behind untreatable pain, in order to tackle this condition.

1.1.2 Pain anatomical pathway: nociceptors.

Sensory information from the limb and trunk is conveyed to the spinal cord by sensory neurons, whose cell bodies are clustered in dorsal root ganglia (DRG) within the vertebral column adjacent to the spinal cord. DRG neurons are pseudo-unipolar, with a single axon arising from the cell body which bifurcates in two branches. The peripheral branches, bundled in peripheral nerves, terminate in the target tissues (e.g. skin, viscera, muscle spindles) while the central processes enter the spinal cord, through the spinal roots, and connect in the superficial laminae of the grey matter (dorsal horns) inter-neurons or neurons projecting to the brain (Fig. 1.1) (Kandel et al., 2000). This anatomical organization provides the base for the transmission of stimuli from the peripheral and visceral tissues to the central nervous system. Supra-threshold stimulation of the peripheral endings of these neurons triggers action potentials that are propagated through the axons to the spinal cord.
Fig. 1.1) Schematic organization of the sensory and motor systems. Sensory afferent system is shown in red. Sensory neurons, whose cell bodies are contained in the dorsal root ganglia (DRG), convey information from the periphery to the dorsal horn of the spinal cord. In the spinal cord they connect inter-neurons (not shown) or neurons that project to the brain, which ultimately processes and integrates the incoming information. The descending pathway is shown in green. Efferent system is shown in blue. Neurons that control skeletal muscle contraction have cell bodies in the ventral horns of the spinal cord, where they receive information from the brain. Efferent axons project to the peripheral system through the peripheral nerves.

DRG neurons are a heterogeneous population of cells and can be discriminated according to different properties including morphology, extent of myelination/speed of conduction, and sensory function. Also, DRG neurons can be classified according to the expression of specific molecular markers, like structural protein, receptors, and ion-channels (Hunt and Mantyh, 2001). From a morphological point of view there are three classes of neurons. Related to this parameter, DRG neurons are also divided according to their myelination state which, together with the size of the axons, determines the speed of conduction. By considering the aforementioned features, DRG neurons are classically defined as: Aα/β, Aδ and C fibre neurons.
Aα/β are large, heavily myelinated fibres, with an axonal diameter larger than 10 µm; they conduct action potentials at high speeds (30-100 m/sec). Aδ are medium size, thinly myelinated neurons, with a diameter of 2 to 6 µm, and an intermediate conduction velocity (12-30 m/sec). C fibres are small unmyelinated neurons, with axon diameters between 0.4 and 1.2 µm; these cells conduct action potentials at the lowest speed (0.5-2 m/sec). The different dimensions are also mirrored at the level of the cell bodies, with large neurons having a diameter smaller than 25 µm, medium neurons having a diameter between 25 µm and 32 µm and large neurons with a diameter greater than 32 µm (Lawson et al., 1984). In the skin, even though the proportion can vary, their relative abundance is 20%, 10% and 70%, respectively (Millan, 1999). Action potential propagation in myelinated neurons relies on the effect of myelin and on the clustering of voltage gated sodium channels (VGSCs) in discrete positions along the axons, the Nodes of Ranvier, which lack the myelin envelope (Cusdin et al., 2008). Myelin wrap between the Nodes of Ranvier increases the resistance between the cytoplasm and the extracellular space, preventing current leakage between the Nodes. It also drastically reduces the capacitance of the axons, which contributes to the high speed of conduction in myelinated axons. Clusters of VGSCs at the Node of Ranvier prevent the depolarisation (which passively spreads between the nodes) from dying-out, by boosting action potentials. Action potentials are only generated in the nodes and they propagate by quickly “jumping” from node to node. For this reason, action potentials are said to propagate by saltatory conduction (from the Latin saltare, to jump) (Kandel et al., 2000). On the other hand, unmyelinated fibres conduct depolarisations more slowly than myelinated fibres. In contrast to the myelinated fibres, the action potentials are not propagated by saltatory conduction in these fibres. Once the membrane has been depolarized beyond the VGSCs activation threshold, an action potential is generated. This local depolarisation is spread passively down the axon, causing the adjacent patch of membrane to reach the threshold for generating an action potential. Ultimately, action potentials are propagated by continuous conduction. In contrast to the myelinated fibres, events regulating VGSCs trafficking, their subcellular localisation and the detailed mechanisms of action potential propagation in unmyelinated axons are still unknown.

DRG neurons also exhibit differences from a functional point of view. Aα neurons carry proprioception information from muscle spindles and the joints. Aβ neurons respond to light touch and in normal condition they are not activated by noxious stimuli. DRG neurons that
respond to noxious stimuli are called nociceptors, and they include the Aδ and C neurons. Both neuron types end as free nerve endings in the peripheral tissue. Nociceptors typically respond to stimuli of different nature (e.g. thermal, mechanical, and chemical), hence they are called polymodal. As a very simplified rule, Aδ are responsible for the sharp, acute and spatially localised pain while C fibres mediate the dull, long lasting, more diffused pain (Julius and Basbaum, 2001). There are two types of Aδ neurons, with different sensitivity to thermal and mechanical stimuli. Aδ type-I are present in both glabrous and hairy skin. They respond to chemicals and high threshold mechanical stimuli (e.g. pinching of the skin). These cells are weakly responsive to high intensity heat. Importantly, these cells can be sensitised, and in this condition they will be also activated by thermal stimuli. Aδ type-II neurons are mostly present in the hairy skin. Compared to type-I, they have higher and lower thresholds to mechanical and thermal stimuli, respectively. These cells are thought to mediate the fast component of noxious heat. C fibres are generally polymodal, even though some neurons responding to unique stimuli have been described. C fibres respond to chemical, thermal and mechanical noxious stimuli. There is also a class of C fibres named as silent nociceptors; in normal conditions these neurons are not gated by noxious stimuli, but they become responsive when sensitised. It must be noted that not all C and Aδ neurons are nociceptors; neurons responding to non noxious stimuli have been discovered in these sub-groups and the functional classification between Aβ, Aδ and C fibres must be considered dynamic. Indeed DRG neurons can change their properties in inflammatory and chronic pain conditions (Gold and Gebhart, 2010).

DRG neurons can be grouped according to the expression of specific markers. Myelinated neurons express the structural protein NF200, while unmyelinated C fibres are positive for cytoskeletal protein Peripherin. C fibres can be further subdivided into two major groups, peptidergic and non peptidergic. The first group expresses and releases neurotransmitters Substance P and Calcitonin Gene Related Peptide (CGRP). These cells are trophic for Nerve Growth Factor (NGF) and express its receptor TrkA. The non peptidergic population is trophic for Glial Derived Neurotrophic Factor (GDNF) and expresses its receptor c-Ret and GFRα. Also, it binds the Isolectin IB4 (glycoprotein isolated from Griffonia simplicifolia). Myelinated neurons are trophic for Brain Derived Neurotrophic Factor (BDNF) and NT-3 and express their cognate receptors TrkB and TrkC (Fig. 1.2).
Fig. 1.2) Nociceptor sub-populations. The pie-chart shows a simplified representation of the different sub-population of nociceptors. Marker molecules are also presented for each subgroup.

DRG sub-populations differentially project into the spinal cord. The dorsal horn of the spinal cord is organized into laminae with distinct anatomical and functional properties. C fibres terminate in the superficial laminae. More specifically, peptidergic fibres connect spinal neurons in lamina I and in the most superficial area of lamina II, while non peptidergic fibres terminate more deeply in lamina II. Aδ fibres project to lamina I and deeper into lamina V. Lamina V is also, with lamina IV and III, the area where Aβ fibres terminate to. Spinal neurons in lamina V that receive inputs from nociceptors and non nociceptors cells are called Wide Dynamic Range neurons. Myelinated neurons also terminate into the deeper area of lamina II, enriched with spinal neurons expressing PKC (Basbaum et al., 2009). C fibres act on postsynaptic neurons by releasing glutamate, which binds to post-synaptic α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) and N-methyl D-aspartate (NMDA) receptors and elicits excitatory post-synaptic potentials. If the noxious stimulus intensity is strong enough Substance P will be also released; this neuropeptide binds to NK1 receptors and will contribute to an augmented post-synaptic response (Woolf and Costigan, 1999).

Apart from transducing acute nociception, DRG neurons contribute to the development and/or maintenance of hyperalgesia and allodynia in pain conditions. Indeed, during inflammatory conditions pro-inflammatory compounds released in the site of trauma (e.g. Prostaglandin E2 (PGE2), NGF, Bradykinin, Histamine, Tumor Necrosis Factor alpha (TNFα) trigger intracellular pathways that sensitise the nociceptors, by decreasing the threshold of activation and/or increasing firing upon stimulation (Hucho and Levine, 2007). These effects are collectively named as peripheral sensitization, and are distinguished from events that occur at the level of
the spinal cord, termed central sensitization, which also contribute to hyperalgesia and allodynia. The different mechanisms underlying peripheral and central sensitization are reflected in the ultimate output: peripheral sensitization leads to heat and mechanical primary hyperalgesia, while central sensitization contributes to mechanical secondary hyperalgesia only (Ali et al., 1996; Richard A. Meyer, 2005). In neuropathic pain conditions (e.g. injury of nervous system, diabetic neuropathy, HIV related neuropathy, post herpetic neuralgia) altered nociceptor excitability contributes to the development and/or maintenance of chronic pain, also characterized by hyperalgesia and allodynia (Sah et al., 2003). In fact, even though changes in the central nervous system are important for the development of painful symptoms in peripheral neuropathies, several evidence indicates that changes in the excitability of primary nociceptive afferents are the single most crucial factor in the generation and maintenance of chronic neuropathic pain (Arner et al., 1990; Gracely et al., 1992; Koltzenburg et al., 1994; Ochoa and Yarnitsky, 1993; Rowbotham et al., 1995; Wall and Melzack, 2005).

Damaged nociceptors show aberrant excitability and firing properties which can be caused by several factors such as: 1) lowered threshold of activation of the excitatory transducers, 2) mislocalisation of mechano/heat/chemical transducers at the injured end or at the level of the cell bodies and 3) altered distribution of voltage gated ion channels (Gold and Gebhart, 2010). Because of these factors, nociceptors may fire more vigorously upon activation and may develop the ability to generate ectopic discharges. This translates into an augmented or an ongoing electric activity towards the central nervous system. In abnormal conditions Aδ fibres have the tendency to fire at regular intervals at high frequency while C fibres fire in an irregular fashion; usually C fibres develop abnormal firing patterns later than Aδ fibres. DRG neurons, because of cellular insults (e.g. injury, metabolic imbalance, viral infection) and consequential cell death, may release chemicals that, either directly or indirectly, affect neighbouring uninjured fibres. It has been reported that these intact fibres can become sensitised, fire irregularly and contribute to pain states (Ali et al., 1999). Nociceptors represent an interesting subject for studies aiming at the identification of therapeutic targets, because they gate the noxious stimuli and play a key role in the development and maintenance of chronic pain states.
1.1.3 Pain anatomical pathway: Spinal cord and brain.

Spinal cord neurons are heterogeneous and their properties, together with the type of sensory neurons they connect to, define the spinal cord output upon sensory neuron excitation. Spinal cord neurons that receive inputs solely from nociceptors are found in the superficial laminae. Another class of dorsal horn neurons only connect Aβ afferents and fire upon light touch stimulation. Neurons that receive inputs from nociceptors and non nociceptors cells are called Wide Dynamic Range neurons and reside in lamina V. Both excitatory (glutamatergic) and inhibitory (GABAergic) inter-neurons in the dorsal horn add a further level of modulation in spinal processing (D'Mello and Dickenson, 2008). Glial cells are also present in the spinal cord and have been demonstrated to play an important role in pain states (Scholz and Woolf, 2007).

Dorsal horn neurons represent an important site of synaptic plasticity. The changes that occur at the level of the spinal cord during pain states are referred to as central sensitisation. Modulation of the synaptic strength between primary afferents and dorsal horn neurons is at the foundation of hypersensitivity that accompanies pain states. High frequency firing of nociceptors results in the engagement of NMDA receptors in the dorsal horn neurons, which leads to increased intracellular calcium concentration. Calcium, by acting as an intracellular messenger, activates pathways that determine an increased responsiveness of dorsal horn neurons upon stimulation by afferent fibres. These changes are mainly due to post-translational modification of channels, receptors and signaling proteins and/or to their increased expression and trafficking to the membrane. Central sensitisation also accounts for the secondary mechanical hyperalgesia that develops in the area surrounding an injury. In normal conditions Aβ neurons activation would not normally engage pain transmission circuit. Central sensitisation results in the recruitment of previously subliminal low-threshold Aβ fibre inputs which elicit allodynia (Basbaum and Woolf, 1999).

Dorsal horns also represent the site where the major ascending pathways project from: the spinothalamic and spinobulbar projections. Neurons of the spinothalamic tract directly project to the thalamus and originate from different regions of the spinal cord: lamina I, laminae IV-V and laminae VII-VIII. This anatomical pathway is the one most closely associated with pain, temperature and itch sensation. The spinobulbar projection originates from the same regions of the spinothalamic tract but the cells of origin are different. It terminates in the parabrachial nucleus (PB), the periaqueductal grey (PAG) and the brain stem reticular formation (Hunt and Mantyh, 2001). From the thalamus, PB and PAG the nociceptive information is conveyed to
different regions of the brain. The current model proposes that there is not a distinct “pain” area, rather there is a “brain matrix” of different areas which may be differently activated during pain processing. These areas comprise the amygdala, the insula, the primary and secondary somatosensory, insular, anterior cingulate, and prefrontal cortices (Tracey, 2007; Tracey and Mantyh, 2007).

The brain exerts its influence, both facilitatory and inhibitory, on the dorsal horn nociceptive information through the descending pathway. Neurons of this pathway originate in the rostroventral medulla and act on the primary sensory afferents, interneurons and projecting neurons of the spinal cord. The cellular population of the descending pathway is mixed and consists of “on cells”, “off cells” and “neutral cells”. “On cells” discharges just prior to the occurrence of withdrawal from noxious stimuli, “off cells” stop firing just prior to a withdrawal reflex “neutral cells” show no consistent changes in activity when withdrawal reflexes occur (Millan, 2002).
1.2 Voltage gated sodium channels: an overview

Voltage gated sodium channels (VGSCs) are necessary for the generation and propagation of action potentials in the nervous system (Hille, 2001). This class of protein comprises nine traditional isoforms (Na\textsubscript{V}1.1-9) plus an atypical channel (Na\textsubscript{X}) (Goldin et al., 2000). VGSCs are highly homologous proteins with an amino acid identity greater than 50% and are thought to have evolved from one common ancestor (Catterall et al., 2003). VGSCs are composed of a main α sub-unit, which associates to β sub-units. The peptide core of the α sub-unit has an apparent molecular weight of approximately 220 kDa, which can considerably vary according to isoform specific glycosylation states (Diss et al., 2004). The α sub-unit, once folded, forms the ion passage pore and encodes the main electrophysiological properties: voltage-dependent activation, rapid inactivation and selective ion conductance. The crystal structure of eukaryotic VGSCs is not available. Hence, the folding topology of VGSCs has been predicted from the amino acid sequence, homology to other proteins, from the crystal structure of a presumptive bacterial VGSC purified from Bacillus halodurans (Ren et al., 2001) and from the structure (obtained by cryo-electron microscopy) of VGSC purified from Electrophorus electricus electroplax (Sato et al., 2001). This analysis has revealed that the α sub-unit comprises 4 homologous domains (DI-IV) each one comprising six transmembrane segments (SI-VI). The intracellular portions comprise the N- and C-terminuses, plus three loops (Fig. 1.3).

VGSCs are gated by changes of the voltage across the membrane. They exist in three states: closed, opened and inactivated. At rest, the probability of VGSCs to open is minimal, and they are assumed to be in the closed state. VGSCs contain a voltage “sensor” domain. Upon membrane depolarisation, a conformational change, due to a movement of the “sensor” domain, leads to channel opening. At this stage, extracellular sodium ions are driven intracellularly through the VGSCs and further depolarise the cell. The force behind this migration is guaranteed by the sodium electrochemical gradient existing at the resting membrane potential, which is far from the sodium reversal potential. Upon sodium ions influx the cell is further depolarised and more VGSCs are engaged. This sodium flux encodes the rising phase of action potentials. When the membrane potential reaches a value close to the sodium reversal potential, VGSCs inactivate and neurons enter the refractory period (Errington et al., 2005; Kandel et al., 2000). Transmembrane segments S5-6 plus the linker regions (P-loop) in each domain are thought to form the conductive pore (Catterall, 2001). It is believed that the voltage “sensor” is the S4 transmembrane segment present into each domain (Fig.
Highly conserved positively charged amino acids (Lysine or Arginine) are found every three positions in the S4 and organised in a linear array (Wood and Baker, 2001). The “sliding-helix” model proposes that, upon membrane depolarisation, the S4 segment moves outwards perpendicularly to the plane of the membrane in a rotating fashion, determining a conformational change that opens the VGSCs (Catterall, 2010; DeCaen et al., 2009). An alternative model, still under investigation for sodium channels (paddle model), suggests that the voltage sensor lies in a horizontal orientation, compared to the plane of the membrane, and moves to a vertical position during voltage changes (Catterall, 2010; Long et al., 2005).

The fast inactivation mechanism, with kinetics in the order of milliseconds, relies on the intracellular loop between domain III and IV, and particularly on the tri-peptide sequence IFM. A slow inactivation mechanism is also known, with kinetic in the order of hundreds of microseconds / seconds, but its molecular determinant are still not completely understood. The “hinged-lid” mechanism is the current model to explain fast inactivation. The intracellular loop between domain III and IV undergoes a movement which occludes the cytoplasmic side of the sodium conducting pore and inactivate the channel. IFM residues are crucial for the activity of the loop as well as the docking sites of the molecular lid, which have been found in the S4-SS linker regions of D-III and D-IV (Goldin, 2003). VGSCs are highly selective for sodium ions. Mutagenesis experiments have revealed that each P-loop contributes to the selectivity filter, formed by four amino acidic residues (DEKA) (Fig. 1.3) (Lipkind and Fozzard, 2008). These residues create the favourable environment for sodium ions, but not other monovalent ions (e.g. K+), to lose their hydration shell and enter the channel (Hille, 2001).

VGSC are glycosylated proteins. After the peptide synthesis, sugar residues (mostly Sialic acid and N-acetylglucosamine) are linked to the peptide chain in the Golgi network. Glycosylation state can differ between the isoforms and within the same isoform. Glycosylation has been found to be developmentally regulated (for Na\textsubscript{v}1.9), to tune VGSCs biosynthesis/degradation, membrane expression, electrophysiological properties and binding to other proteins (Denac et al., 2000; Diss et al., 2004; Schmidt and Catterall, 1987). The putative glycosylation sites are in the extracellular linkers (Fig. 1.3). VGSCs are post-translationally modified by kinases and phosphatases. Typically the effect of phosphorylation is isoform specific and tunes trafficking and electrophysiological properties of the \( \alpha \) sub-unit. The sites of phosphorylation are within the intracellular loops between domain I-II and III-IV; both cAMP dependent kinase PKA and PKC have been demonstrated to phosphorylate the channels (Fig. 1.3) (Fitzgerald et al., 1999; Gold et al., 1998). VGSCs have distinct electrophysiological and pharmacological properties. A
widely used classification relies on their sensitivity to Tetrodotoxin (TTX). This toxin is isolated from symbiotic bacteria of the puffer fish family *Tetraodontidae*. TTX blocks the channel by binding to the external side of the pore and obstructs the ion pore. It is thought that TTX binds to residues in the P-loops in a two step-mode. Firstly, residues EDDD are engaged, and then the selectivity filter DEKA plays a role in stabilising the toxin-channel complex. TTX insensitivity is conferred by a single mutation. TTX resistant (TTX-r) channels, lack the aromatic residue (Phenylalanine or Tyrosine) in position 356 which is necessary for the toxin binding to the DEKA motif, and are substituted with Serine (NaV1.8-9) or Cysteine (NaV1.5) (Sivilotti et al., 1997) (Fig. 1.3). TTX sensitive (TTX-s) channels are blocked by nM concentration of TTX, while TTX-r channels are blocked by µM concentration of TTX (Wood et al., 2004).

**Figure 1.3** VGSC α sub-unit topology. The topology of the VGSCs α sub-unit is shown, highlighting the main features.

VGSCs are distributed throughout the nervous system and serve different functions, according to the intrinsic electrophysiological features, cellular background and developmental stage. We shall focus on VGSCs that are mainly expressed in DRG neurons, and particularly on NaV1.8,
since they represent the most relevant isoforms for the purpose of this thesis. **Table 1.1** summarises the main features of the different isoforms.

**Table 1.1. Main properties of VGSCs.** Properties of VGSCs are summarised, focusing on their role and expression in DRG neurons. Presence in DRG neurons, + = present, +++ = abundant.

<table>
<thead>
<tr>
<th>Name</th>
<th>Main tissue distribution</th>
<th>Presence in DRG neurons (size)</th>
<th>TTX sensitivity (IC₅₀)</th>
<th>Main electrophysiological role in DRG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naᵥ1.1</td>
<td>CNS</td>
<td>+ (Large)</td>
<td>YES (6 nM)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Naᵥ1.2</td>
<td>CNS</td>
<td>+</td>
<td>YES (12 nM)</td>
<td>May regulate frequency of firing in injured neurons</td>
</tr>
<tr>
<td>Naᵥ1.3</td>
<td>Embryonic</td>
<td>+++ after axotomy</td>
<td>YES (4 nM)</td>
<td>Ectopic firing in injured neurons</td>
</tr>
<tr>
<td>Naᵥ1.4</td>
<td>Skeletal muscle</td>
<td>-</td>
<td>YES (5 nM)</td>
<td>/</td>
</tr>
<tr>
<td>Naᵥ1.5</td>
<td>Hearth</td>
<td>-</td>
<td>NO (1-2 µM)</td>
<td>/</td>
</tr>
<tr>
<td>Naᵥ1.6</td>
<td>CNS-PNS</td>
<td>+++ (Large)</td>
<td>YES (1 nM)</td>
<td>Action potential propagation in myelinated fibres</td>
</tr>
<tr>
<td>Naᵥ1.7</td>
<td>PNS</td>
<td>+++ (Large and Small)</td>
<td>YES (4 nM)</td>
<td>Ramp currents</td>
</tr>
<tr>
<td>Naᵥ1.8</td>
<td>PNS</td>
<td>+++ (Small)</td>
<td>NO (60 µM)</td>
<td>Action potential generation and propagation</td>
</tr>
<tr>
<td>Naᵥ1.9</td>
<td>PNS</td>
<td>+++ (Small, IB4⁺)</td>
<td>NO (40 µM)</td>
<td>Setting resting membrane potential</td>
</tr>
</tbody>
</table>
1.3 Voltage gated sodium channel Na\textsubscript{v}1.8

1.3.1 \textit{Na\textsubscript{v}1.8 electrophysiological properties and expression in DRG neurons.}

Na\textsubscript{v}1.8 was identified in 1995 by a genetic screening of DRG transcripts by using a subtraction library (Akopian \textit{et al.}, 1996; Akopian and Wood, 1995). The coding region of Na\textsubscript{v}1.8 comprises 27 exons and encodes for the main α sub-unit. Na\textsubscript{v}1.8 α sub-unit is composed of 1957 amino acid residues with an apparent molecular weight of approximately 220 kDa (considering only the peptide chain, and not post-translational modifications, such as glycosylation) (Souslova \textit{et al.}, 1997). To date two splicing variants have been described in literature, one with the absence of a glutamine residue in the first intracellular loop (Kerr \textit{et al.}, 2004) and one with a duplication of exons 12-13-14 (coding part of domain II), which results to be up-regulated in response to NGF (Akopian \textit{et al.}, 1999a). The functional role of these isoforms is unknown. During development, Na\textsubscript{v}1.8 starts being expressed at E15, peaks at P7 and its expression in the adult stabilises at P21 (Benn \textit{et al.}, 2001).

Na\textsubscript{v}1.8 is a TTX-r VGSC which underlies action potential (AP) generation in C-type neurons in DRG. Studies conducted on Na\textsubscript{v}1.8 knock-out (KO) mice revealed that in these animals, compared to controls, resting potential, input resistance, action potential threshold, and current threshold of DRG neurons are unchanged. The striking difference was found in the ability to generate APs. Small DRG neurons in Na\textsubscript{v}1.8 KO mice only generated graded responses upon depolarising stimuli, in marked contrast with wild type cells where all-or-none APs were generated. In neurons from Na\textsubscript{v}1.8 KO mice both the peak of the graded response and the rate of depolarisation was found to be reduced, compared to wild type cells. Indeed, it has been quantified that Na\textsubscript{v}1.8 underlies the majority of sodium influx (approximately 90%) during the rising phase of APs, in small DRG neurons. Na\textsubscript{v}1.8 also determines the firing properties of unmyelinated DRG neurons. It allows repetitive firing upon a sustained stimulation. In neurons from KO animals an ongoing stimulation determined graded response that adapted more quickly than in control cells, where APs firing at high frequency was sustained (\textbf{Fig. 1.4}) (Renganathan \textit{et al.}, 2001).
Figure 1.4) Electrophysiological properties of Na\textsubscript{v}1.8. Wild type Na\textsubscript{v}1.8 neurons generate all-or-none action potentials when stimulated with depolarizing currents (For clarity, traces generated by 0-, 20-, 40-, 60-, 70-, 80-, 90-, 100-, 110-, 150-, and 250-pA current injection only are shown). Na\textsubscript{v}1.8 KO neurons generate smaller graded responses. Wild type Na\textsubscript{v}1.8 neurons also produce sustained repetitive firing in response to depolarizing stimuli of 150 pA. Na\textsubscript{v}1.8 KO neurons fail to sustain high-frequency firing. Adapted from Renganathan et al. 2001.

Na\textsubscript{v}1.8 displays different electrophysiological properties, compared to TTX-s channels expressed in DRG neurons; this shapes the excitability of the cells where it is expressed. In fact, in small DRG neurons TTX-r currents mediated by Na\textsubscript{v}1.8 are kinetically slower than TTX-s currents in terms of time to peak and to decay. Also, the peak of the I-V relationship, the mid-point of the normalized conductance curve and the mid-point of the steady-state inactivation curve is significantly more depolarized than the curves of TTX-s currents (Fig. 1.5).
Figure 1.5) Electrophysiological differences between Na\textsubscript{v}1.8 mediated TTX-r currents and TTX-s currents. A) Representative traces of TTX-r and TTX-s currents. B) The relationship between peak current amplitude and test potential (I-V curve) for TTX-r and TTX-s currents. C) The relationship between $g/g_{\text{max}}$ and test potential of TTX-r and TTX-s currents (voltage dependence of activation). D) Steady state inactivation curves for TTX-r and TTX-s currents (voltage dependence of steady-state inactivation). Circles are TTX-s currents, triangles are TTX-r currents. Adapted from Elliott et al. 1993.

TTX-r channels also recover from fast inactivation more rapidly than TTX-s channels. These features confer DRG cells expressing Na\textsubscript{v}1.8 the ability to fire action potential when depolarised (when TTX-s are inactivated) and to sustain high frequency firing (Elliott and Elliott, 1993).

A comparison between rat and human Na\textsubscript{v}1.8 electrophysiological properties has been made. It was found that activation curves were similar and that the human isoform displayed more hyperpolarised voltage-dependence than the rat channel, faster development of inactivation, slower recovery from the fast component of inactivation, and faster recovery from the slow inactivation. It is not clear yet if these differences are due to intrinsic differences of the $\alpha$ subunits, or to indirect modulation of other factors (the electrophysiological properties were analysed in a heterologous background) (Browne et al., 2009).
Different VGSCs isoforms contribute to APs electrogenesis in nociceptors. $\text{Na}_\text{V}1.8$ underlies the vast majority of sodium conductance, both at the beginning and during the upstroke of APs, with very little contribution from TTX-s channels, such as $\text{Na}_\text{V}1.7$. $\text{Na}_\text{V}1.7$ activates at voltages close to the resting membrane potential, displays fast activating and inactivating currents (Klugbauer et al., 1995). Because of its electrophysiological properties it yields to ramp currents in response to small depolarisations (Cummins et al., 1998). In general TTX-s channels, even though they activate earlier than $\text{Na}_\text{V}1.8$, are only active during the first phase of APs (Blair and Bean, 2002). The other TTX-r channel expressed in DRG neurons, $\text{Na}_\text{V}1.9$, does not contribute to APs; indeed, this channel displays very slow activation and inactivation kinetics. In addition, it has a large overlap between the activation and steady-state inactivation curves, centred at voltage values close to the resting membrane potential. It encodes a persistent current and its contribution in nociceptor excitability is to set the resting membrane potential and to enhance sub-threshold depolarisations. Hence, the current model for AP electrogenesis in nociceptors is that $\text{Na}_\text{V}1.7$ encodes ramp currents (boosts sub-threshold stimuli) to depolarise the cell to an extent sufficient to recruit $\text{Na}_\text{V}1.8$, which generate the APs (Rush et al., 2007).

$\text{Na}_\text{V}1.8$ in the nervous system is only expressed in the PNS in DRG neurons. Recent evidence showed that cardiomyocyte also express this channel (Chambers et al., 2010). Because of its restricted expression pattern, a lot of efforts have been put into the characterisation of the neurons expressing this channel. $\text{Na}_\text{V}1.8$ is in the majority of small diameter neurons and in a small proportion of medium and large size neurons. Most importantly, virtually the entire neuronal population positive for $\text{Na}_\text{V}1.8$ expression (89% of C-fibres, 93% of Aδ, 64% of Aα/β) displays nociceptor-like properties (broad APs and large overshoots). It was also found, in agreement with $\text{Na}_\text{V}1.8$ role in AP electrogenesis, that there is a positive correlation between $\text{Na}_\text{V}1.8$ expression and large AP overshoots in the cells expressing the channel (Djouhri et al., 2003a). Within the C-fibre population, $\text{Na}_\text{V}1.8$ is expressed in both IB4+ and IB4- cells (Fang et al., 2005). These cells have unique electric properties: strongly IB4+ cells have longer AP durations and rise times, slower conduction velocities and more negative resting membrane potentials than IB4- cells. $\text{Na}_\text{V}1.8$ contributes to these differences. Indeed, it was demonstrated that in IB4+ cells, compared to IB4- cells, $\text{Na}_\text{V}1.8$ enters the slow inactivation state more rapidly and recovers from it more slowly. This translates into differences in excitability, between IB4+ and IB4- cells, with the latter being able to sustain repetitive firing and to undergo less
adaptation upon a continuous depolarising stimulation, compared to the IB4+ population (Choi et al., 2007).

1.3.2. Na\textsubscript{v}1.8 and pain pathways.

All the VGSCs isoforms expressed in DRG neurons have been shown to play a role in pain pathways (Benarroch, 2007; Dib-Hajj et al., 2010; Ekberg and Adams, 2006; Lai et al., 2004; Momin and Wood, 2008; Rogers et al., 2006; Wood and Baker, 2001). For the purpose of this thesis this section will mainly focus on the role of Na\textsubscript{v}1.8. The evidence that Na\textsubscript{v}1.8 expression is restricted to nociceptors and that it is the major determinant for APs generation in these cells hints to a role of Na\textsubscript{v}1.8 in pain pathways. Indeed there is a vast amount of literature demonstrating how Na\textsubscript{v}1.8 plays a role in nociception. This role has emerged from the characterisation of Na\textsubscript{v}1.8 in pain conditions, from electrophysiological and pharmacological studies, and from the studies carried out in Na\textsubscript{v}1.8 KO animals. KO mice are viable, have similar cell profiles as wild-type animals (WT) and show a complete absence of TTX-r currents. Na\textsubscript{v}1.8 KO mice have been tested for acute pain behaviours. It was found that Na\textsubscript{v}1.8 absence resulted in an increased latency of paw withdrawal and tail flick following the exposure of radiant noxious thermal stimuli. Also, pain threshold to acute mechanical stimuli (tail pressure), but not to graded Von Frey filament stimulation, was markedly increased in KO animals. Inflammatory condition was also investigated and it was found that in KO animals Carrageenan induced thermal hyperalgesia was delayed compared to WT animals (Akopian et al., 1999b). By studying WDR neurons response to peripheral stimulation it has emerged that Na\textsubscript{v}1.8 KO mice show stimulus-dependent deficits in spinal neuronal activity. The interesting aspect of this experimental model is that it exploits anesthetised animals and supra-threshold stimuli can be delivered. In this set-up Na\textsubscript{v}1.8 KO mice displayed less electric activity in WDR neurons upon mechanical stimulation (but not thermal) of the hind paw, both in the noxious and non-noxious range with Von Frey filaments. Brushing, acute mechanical stimuli (pinching) and noxious cold also led to less excitability of WDR neurons (Matthews et al., 2006). Na\textsubscript{v}1.8 role in inflammatory pain has also been explored. In inflammatory conditions (CFA and Carrageenan models) Na\textsubscript{v}1.8 is up-regulated in DRG neurons (Lai et al., 2004). NGF systemic injection in mice is linked to the development of thermal hyperalgesia. In this model Na\textsubscript{v}1.8 KO mice show a clear reduction of thermal sensitivity, compared to WT animals. In the same set of experiments, neuropathic pain behaviours following partial ligation of the sciatic nerve were
analysed. Na\textsubscript{v}1.8 KO developed mechanical allodynia like WT animals and showed a partial analgesia to thermal stimuli at early time points (Kerr et al., 2001). In vivo PGE\textsubscript{2} dermal injection produces a robust mechanical hyperalgesia and Na\textsubscript{v}1.8 KO animals displayed decreased TTX-r currents and attenuated hypersensitivity (Khasar et al., 1998). In models of neuropathic pain Na\textsubscript{v}1.8 transcript is down-regulated. Indeed upon axotomy or nerve ligation, Na\textsubscript{v}1.8 is down-regulated by at least 50% at the level of DRG cell bodies in rodents (Okuse et al., 1997) and in humans (Coward et al., 2000). These results suggested that Na\textsubscript{v}1.8 does not take part in hypersensitivity that accompanies chronic pain models. In fact reports argued against a role of this channel in the development or maintenance of neuropathic pain. For example, Nassar et al. demonstrated that double KO animals for Na\textsubscript{v}1.8 and Na\textsubscript{v}1.7 developed neuropathic pain and mechanical allodynia normally. On the other hand, acute pain thresholds following noxious mechanical stimuli and radiant heat noxious stimuli were increased; spontaneous pain following formalin injection was also markedly reduced in double KO mice (Nassar et al., 2005). The ablation in mice of cells expressing Na\textsubscript{v}1.8 did not impede thermal and mechanical allodynia from developing in neuropathic pain models. On the contrary, inflammatory hypersensitivity was reduced in these mice, compared to wild-type, in CFA and Formalin models. In addition, acute nociception was impaired upon presentation of noxious mechanical stimuli and noxious cold (Abrahamsen et al., 2008). On the other hand, other reports directly pointed to a role of Na\textsubscript{v}1.8 in neuropathic pain. Sodium channels are necessary for the generation of ectopic firing in neuromas (Matzner and Devor, 1994) that contributes to trigger chronic pain and associated hypersensitivity (Gracely et al., 1992; Rizzo et al., 1996; Wall and Melzack, 2005). In agreement with this finding, a therapeutic approach to treat neuropathic pain is the administration of Lidocaine, a sodium channel blocker (Mao and Chen, 2000; O'Connor and Dworkin, 2009; Wall and Melzack, 2005). Na\textsubscript{v}1.8, even though its expression is down-regulated in DRG cell bodies, accumulates at the site of injury upon chronic constriction injury (CCI) or axotomy (Novakovic et al., 1998). Interestingly, its presence is necessary for the generation of ectopic firing in the nerve. Indeed, upon neuroma formation in control mice, A- and C-fibres spontaneously fire, with C-fibres firing at higher frequency. In Na\textsubscript{v}1.8 KO the ectopic discharge is completely absent (Roza et al., 2003). Notably, redistribution of Na\textsubscript{v}1.8 in the peripheral nerves has also been described in humans with hyperalgesic and allodynic conditions resulting from brachial plexus injury (Coward et al., 2000). Further evidence of a role for Na\textsubscript{v}1.8 in chronic pain conditions in human comes from a study where it was shown that Na\textsubscript{v}1.8 is up-regulated within the axons of painful neuromas
It has also been observed that spinal nerve ligation (SNL) leads to an increase of Na\textsubscript{v}1.8 along the uninjured C-fibres of the sciatic nerve, where it supports increased TTX-r AP conduction (Gold \textit{et al.}, 2003). Notably, oligodeoxynucleotide mediated down-regulation of Na\textsubscript{v}1.8 in L4, L5 and L6 DRG completely reversed tactile allodynia and thermal hyperalgesia in a model of chronic pain (L5/L6 spinal nerve ligation) (Lai \textit{et al.}, 2002) and prevented them from developing in a model of chronic inflammatory pain (Porreca \textit{et al.}, 1999). Other evidence for a role of Na\textsubscript{v}1.8 in neuropathic pain comes from studies carried out with a specific inhibitor for this channel. Administration of this compound \textit{in vivo} was reported to have profound effects on the excitability of DRG neurons and pain behaviour in several models of neuropathic pain. In fact, spontaneous activity of WDR neurons and firing upon mechanical stimulation was reduced in the presence of the inhibitor. Also, it attenuated mechanical and cold allodynia in SNL and CCI models and decreased thermal hyperalgesia in an inflammatory model (Jarvis \textit{et al.}, 2007; Joshi \textit{et al.}, 2009). Another report showed that cone snails µO-Conotoxin MrVIB inhibits Na\textsubscript{v}1.8, blocks action potential propagation in C fibres of the sciatic nerve and has an analgesic effect in pain models (Bulaj \textit{et al.}, 2006). Recently, it has been found that lesion of motor neurons only (leaving DRG neurons uninjured) triggers neuropathic pain-like behaviour. It has been found that in this model Na\textsubscript{v}1.8 and its currents are up-regulated in DRG neurons (both small and medium size), and that this increase is dependent on the increase of TNF\textsubscript{α} after nerve injury (He \textit{et al.}, 2010). Given these evidence in now clear that Na\textsubscript{v}1.8 plays an undisputed role in inflammatory and acute pain and the potential discrepancies about Na\textsubscript{v}1.8 role in neuropathic pain conditions have been suggested to arise from 1) different species used (rats vs. mice), 2) differences in pain models (e.g. axotomy, SNL, CCI), 3) outcome testing modalities (spontaneous pain, thermal vs. mechanical stimuli), 4) time course (initiation of neuropathic pain vs. maintenance), and 5) potential compensatory effect in KO mice, which may hide or lead to underestimation of Na\textsubscript{v}1.8 influence in neuropathic pain models. Indeed, Na\textsubscript{v}1.8 KO mice show a robust up-regulation of TTX-s channel Na\textsubscript{v}1.7 (Akopian \textit{et al.}, 1999b). Notably, Na\textsubscript{v}1.8 plays a fundamental role in the cold. Indeed, this is the only channel able to fire APs at low temperature. When nociceptors are progressively cooled the voltage-dependent slow inactivation of TTX-s channels is enhanced, while Na\textsubscript{v}1.8 inactivation properties are temperature independent (Zimmermann \textit{et al.}, 2007). Overall, the amount of literature to date demonstrates the Na\textsubscript{v}1.8 plays a key role in different pain modalities.
1.3.3 Na\textsubscript{v}1.8 sensitisation mechanisms.

Na\textsubscript{v}1.8 has been shown to be the target of several signaling pathways, which usually result in its enhanced activity and nociceptors peripheral sensitisation. This phenomenon contributes to pain related hypersensitivity. PGE2 is a pro-inflammatory compound which is released in the site of injury from mast cells, where it contributes to vasodilation. Several enzymes are involved in its biosynthesis and they include the COX enzymes (the target of NSAIDs). PGE2 sensitises the nociceptors by modulating Na\textsubscript{v}1.8 currents. Indeed, PGE2 by acting on its GPCR EP2 determines an increase of Na\textsubscript{v}1.8 mediated currents and alters its electrophysiological properties by shifting the activation curve and the steady-state inactivation curve to more hyperpolarised potentials (England et al., 1996). This effect is mediated by cAMP-PKA dependent phosphorylation of the channel in its second intracellular loop (Fitzgerald et al., 1999). While PKA activation potentiates Na\textsubscript{v}1.8 currents, it has an inhibitory action towards other VGSCs (e.g. Na\textsubscript{v}1.7 and Na\textsubscript{v}1.9) (Kakimura et al., 2010; Vijayaragavan et al., 2004a). PGE2 also leads to increased trafficking of Na\textsubscript{v}1.8 to the membrane (Liu et al., 2010). Adenosine and Serotonin have been reported to have similar effects on Na\textsubscript{v}1.8, which are mediated by PKA dependent phosphorylation (Gold et al., 1996b). Na\textsubscript{v}1.8 is also a target of PKCε. PKCε dependent phosphorylation determines Na\textsubscript{v}1.8 increased currents and a shift of the activation and steady-state inactivation curves in a hyperpolarizing direction (Gold et al., 1998; Khasar et al., 1999). Upstream signaling molecules activating PKCε include neuropeptide Substance P, which has been shown to act in an autocrine way on DRG neurons through GPCR NK1 (Cang et al., 2009), and Bradykinin. Bradykinin is a nonapeptide pro-inflammatory agent released in the site of injury. It signals through GPCRs receptors B1 and B2. Activation of these receptors leads to activation of PKC (through phospholipase Cβ) and sensitisation of Na\textsubscript{v}1.8 (Gold and Gebhart, 2010; Gold et al., 1996b). Furthermore, PKCε is downstream of NGF receptor TrkA (Okuse, 2007). It has been shown that Na\textsubscript{v}1.8 is also a target of p38 MAPK. Hudmon et al. demonstrated that p38 MAPK and Na\textsubscript{v}1.8 are expressed in the same DRG neurons. P38 MAPK is activated in inflammatory conditions and increases Na\textsubscript{v}1.8 currents in DRG neurons (without affecting the electrophysiological properties) by phosphorylating two serine residues, different from residues targeted by the PKA pathway, in its first intracellular loop (Hudmon et al., 2008). A recent study has investigated the potential involvement of local translation on Na\textsubscript{v}1.8 protein changes in a model on neuropathic pain. Upon sciatic nerve entrapment it has been found that compound action potential and Na\textsubscript{v}1.8 immuno-reactivity increased in the treated nerve. Concomitant with these findings, Na\textsubscript{v}1.8 mRNA level was up-regulated in the sciatic
nerve but remained unchanged in the DRG. The authors propose that specific and enhanced transport of Na\textsubscript{v}1.8 mRNA into the axons may account for this up-regulation and that local mRNA accumulation could contribute to increased axonal Na\textsubscript{v}1.8 protein levels (Thakor et al., 2009). Na\textsubscript{v}1.8 has also been found to be modulated by endogenous miRNA (Zhao et al., 2010).

### 1.3.4 Trafficking of Na\textsubscript{v}1.8.

The determinants of Na\textsubscript{v}1.8 trafficking and localisation in DRG neurons have been unknown for a long period. The first study that identified a regulatory trafficking mechanism for Na\textsubscript{v}1.8 was conducted by Okuse et al. It was found that Annexin II light chain sub-unit p11 is necessary for the functional expression of TTX-r currents mediated by Na\textsubscript{v}1.8 (Okuse et al., 2002). The role of p11 in Na\textsubscript{v}1.8 trafficking will be discussed in more details in Chapter 2 (see section 2.1.5). Together with p11, other proteins have been found to regulate Na\textsubscript{v}1.8 association with the membrane. PDZD2 is a scaffolding protein containing the PDZ motif. It interacts with the second intracellular loop of Na\textsubscript{v}1.8. This association correlates with increased TTX-r currents, compared to a scenario where Na\textsubscript{v}1.8 PDZD2 interaction is impaired. It is not known yet if PDZD2 is important for the trafficking of the channel to the membrane or for its retention on it (Shao et al., 2009). Contactin, a GPI anchored cell adhesion molecule, has been found to regulate Na\textsubscript{v}1.8 currents. In Contactin KO mice Na\textsubscript{v}1.8 mediated currents are reduced in small diameter neurons (Rush et al., 2005). In addition, Calmodulin has been found to bind to the C-terminus of Na\textsubscript{v}1.8 in a calcium independent manner. It is hypothesised that Calmodulin binding results in the stabilisation of the channel into the membrane, by masking sites on the C-terminus that would act as endocytosis signal (Choi et al., 2006). Related to the endocytic pathway, CAP-1 (Clathrin associated protein-1) specifically binds the C-terminus of Na\textsubscript{v}1.8. It may act as a bridging element with Clathrin and may regulate the rate of removal of the channel from the membrane by endocytosis (Liu et al., 2005). Na\textsubscript{v}1.8 contains also a PY motif (LPXY) in the C-terminus which acts as a docking site for the Ubiquitin ligase Nedd4-2. It is proposed that the interaction of Nedd4-2 to Na\textsubscript{v}1.8 promotes its removal from the membrane and proteasomal degradation (Fotia et al., 2004). More recently, it has been found that Na\textsubscript{v}1.8 contains an endoplasmic reticulum (ER) retention motif RXR (495RRR497) in the first intracellular loop. It is thought that the β3 sub-unit participates in the forward trafficking of the channel by binding to this motif. The binding event would mask the retention motif and promotes the translocation of Na\textsubscript{v}1.8 to the membrane (Zhang et al., 2008). Furthermore,
transmembrane segments from each domain act as retention signal in the ER, and the association of transmembrane acidic residues with Calnexin determines the recruitment of $\text{Na}_v 1.8$ into the endocytic pathway, leading to decreased membrane expression (Li et al., 2010). A $\beta 3$ sub-unit independent mechanism has also been described. It has been demonstrated that PGE2 directly promotes surface expression of $\text{Na}_v 1.8$ in a dose-dependent manner and that it is dependent of the PKA phosphorylation on the first intracellular loop (Liu et al., 2010).

The studies presented above have shed light on the dynamics regulating $\text{Na}_v 1.8$ trafficking (Swanwick et al., 2010c). Nevertheless, $\text{Na}_v 1.8$ precise sub-cellular localisation remains obscure. Indeed, evidence suggests $\text{Na}_v 1.8$ presence along the axons (Gold et al., 2003), peripheral terminals (Black and Waxman, 2002; Brock et al., 1998; Persson et al., 2010) and dorsal horn afferent endings (Amaya et al., 2000) but the exact sub-cellular distribution of this channel in vivo and in vitro has not yet been thoroughly investigated. Neuronal excitability is shaped by the cohort of ion channels expressed and by their localisation within the neurons. The knowledge of where $\text{Na}_v 1.8$ is localised within nociceptors would open new perspectives in the comprehension of the excitability of these cell.
1.4 Voltage gated sodium channel β sub-units

VGSCs β sub-units are single transmembrane proteins with a V-set immunoglobulin loop in their extracellular domain, homologous to neuronal cell adhesion molecules (NCAM). Four genes have been identified coding for the isoforms Na\textsubscript{v}β1-4, with an approximate molecular weight of 36 kDa. Na\textsubscript{v}β1-3 associate with the α sub-unit non-covalently, while Na\textsubscript{v}β2-4 are covalently bridged through a disulfide bond. The site of interaction with the α sub-unit has been proposed to be in the extracellular portion. The IgG loop seems to be important for the binding to other proteins of the extracellular matrix (Tenascin-C-R, Neurofascin, NrCAM) or other transmembrane proteins exposed by adjacent cells (Cusdin \textit{et al.}, 2008). β sub-units regulate cell adhesion, trafficking and are required to express the full native electrophysiological properties of the α sub-unit. These effects depend on many factors including, isoform specificity, cellular background, developmental stage (Isom, 2001). With regards to Na\textsubscript{v}1.8, it has been found that β3 sub-unit promotes trafficking of the channel towards the membrane, by masking an ER retention motif present on Na\textsubscript{v}1.8 (Zhang \textit{et al.}, 2008). Interestingly, β3 sub-unit is up-regulated in small diameter neurons upon nerve axotomy (Takahashi \textit{et al.}, 2003), CCI (Shah \textit{et al.}, 2000) and, in medium size neurons, in diabetic neuropathy models (Shah \textit{et al.}, 2001). Thus, the β3 sub-unit mediated Na\textsubscript{v}1.8 release from the ER could also account for increased excitability in pain states due to additional Na\textsubscript{v}1.8 being transported to the cell membrane. In heterologous system it has been found that β sub-units and their specific combination have an effect on the electrophysiological properties of Na\textsubscript{v}1.8. In particular, β1 alone or in combination with other β sub-units determines an increase in TTX-r current amplitudes, accelerates the kinetics of decay, and negatively shifts the steady-state curves. On the other hand β2, alone and in combination with β1, shifts the steady-state inactivation curve to more depolarized potentials (Vijayaragavan \textit{et al.}, 2004b). It has been reported that in Xenopus oocytes β3 caused a hyperpolarizing shift in the threshold of activation of Na\textsubscript{v}1.8, and a threefold increase in the peak current amplitude when compared with Na\textsubscript{v}1.8 expressed alone (Shah \textit{et al.}, 2000).
1.5 Lipid rafts

1.5.1 Lipid rafts: a historical overview.

Originally cellular membranes were described as a two dimensional fluid mosaic where all lipids and proteins randomly diffuse (Singer and Nicolson, 1972). This model has been extensively developed over the decades. To date, the current vision describes the cellular membrane as a heterogeneous environment, characterized by a lateral organization, with a liquid disordered (L_d) phase and a liquid ordered (L_o) phase, which differ in the composition of lipids and proteins, in the extent of lipids packing, degree of order and mobility of the constituents. The domains of the plasma-membrane characterized by L_o phase are called lipid rafts (Coskun and Simons, 2010). The existence of lipid rafts was proposed following the observation that in polarized epithelial cells the apical and basolateral portions of the membrane exhibit marked differences in terms of lipid species, particularly with glycosphingolipids highly enriched in the apical portion (van Meer and Simons, 1988). The first definition of rafts was suggested after the discovery that GPI-anchored proteins, confined in the apical membrane, could be recovered from the portion of membrane that was not lysed by non-ionic detergents at 4°C. This detergent-resistant membrane (DRM) was found to be enriched with glycosphingolipids and excluded proteins from the basolateral membrane (Brown and Rose, 1992). Concomitant with this, it was discovered that the physical base of the lipid L_o phase and resistance of these domains to lysis is the abundance of sphingolipids (with saturated fatty acid chains) and cholesterol (Schroeder et al., 1994). Since then the scientific interest towards lipid rafts has been constantly growing, as more than 3500 publications demonstrate, and the features of these domains have been analysed in details. To date, lipid rafts are defined as “dynamic, nanoscale, sterol–sphingolipids enriched, ordered assemblies of proteins and lipids, in which the metastable raft resting state can be stimulated to coalesce into larger, more stable raft domains by specific lipid–lipid, protein–lipid and protein–protein oligomerising interactions” (Coskun and Simons, 2010; Pike, 2006; Simons and Gerl, 2010).

1.5.2 Lipid rafts: lipid content and L_o phase.

Lipid rafts are highly enriched in phospholipids with saturated fatty acid chains, sphingolipids, and cholesterol. A lipid analysis of rafts has revealed that cholesterol and sphingolipids are five times more concentrated in rafts than in the bulk membrane. Rafts also contain more
glycosphingolipids, such as cerebrosides and gangliosides. On the other hand, glycerophospholipids, including the major membrane phospholipids, phosphatidylcholine and phosphatidylethanolamine, comprise less than 30% of raft lipids (compared to approximately 60% in non raft membranes) (Pike, 2003, 2004). Membrane lipids are amphipathic molecules, meaning that they contain one hydrophilic and one hydrophobic moiety. Glycerophospholipids are glycerol based phospholipids. The hydrophilic head comprises the phosphate group with its negative charges plus the substituent (which may be polar) and faces the intracellular and extracellular side of the cells. The hydrophobic portion contains diacylglycerol, with saturated and/or unsaturated (one or more C=C bond) acid chains of various lengths. Sphingolipids derive from the amino-alcohol sphingosine. Usually sphingosine is linked to an acyl group, like a saturated fatty acid (ceramide). Differently from glycerophospholipids, sphingolipids have longer and fully saturated fatty acid chains. According to the nature of the polar head, sphingolipids can be classified into different sub-groups. Sphingomyelin has a phosphocholine group, glycosphingolipids have sugar group/s and gangliosides are glycosphingolipids with terminal sialic acids. Cholesterol is the most abundant non polar lipid on the cell membrane. It is characterized by a rigid hydrophobic planar structure and a hydrophilic hydroxyl group (Fig. 1.6) (van Meer et al., 2008).
Figure 1.6) Membrane lipids. The figure shows a schematic representation of membrane lipids, and their name according to the different substituents, plus the structure of phosphatidylcholine, sphingomyelin, glycosphingolipids and cholesterol. Adapted from Fantini et al. 2000.

According to physical studies on membrane lipids, mixtures of lipids can adopt different phases: gel like ($L_g$), $L_d$ and $L_o$. The $L_g$ and $L_d$ phase represent the two extremes of a spectrum of possible conformations. In the $L_g$ phase lipids are in a crystalline solid state while in the $L_d$ phase lipids exist in a fluid state (Hancock, 2006). The $L_g$ does not exist in biological membranes (Veatch, 2008) and the bulk membrane is in the $L_d$ phase. The $L_o$ phase represents an
intermediate state, which co-exists in live cells with the \( L_d \) phase, and is characteristic of lipid rafts (Ahmed et al., 1997). The co-existence of different lipid phases and the ability of rafts to cluster at physiological temperatures was clearly demonstrated in plasma membrane spheres by Lingwood et al. (Lingwood et al., 2008). The parameters that describe the phase of membrane lipids are the configurational order (trans vs. gauche isomerisation of the C-C bonds in the acyl chains), translational order (the position of a lipid in the by-layer, e.g. lateral motility) and rotational order (rotation of a lipid around its axis, perpendicular to the plane of the membrane). It has been estimated that rafts have a high configurational order (high trans/gauche ratio), similar to the \( L_g \) phase. Compared to the \( L_d \), the \( L_o \) rafts have a moderately higher translational order, with the translational diffusion coefficient estimated to be two times lower (in the order of \( 10^{-8} \) cm\(^2\)/sec, compared to \( 10^{-11} \) cm\(^2\)/sec of the \( L_g \) phase) (Simons and Vaz, 2004).

The high abundance of sphingolipids and cholesterol in lipid rafts is at the base of their \( L_o \) phase. These molecules work in concert and determine the physical properties of rafts. In fact, sphingolipids, compared to the unsaturated chains of glycerophospholipids enriched in the bulk membrane, have long saturated acyl chains that interact through hydrophobic interaction and allow tight packing of the lipids in rafts. In addition, sphingolipids differ from glycerophospholipids because they have both donor (the hydroxyl group) and acceptor groups (the amide group) in the polar heads which can be engaged in H-bonds (Rietveld and Simons, 1998). Glycosphingolipids can extend the formation of H-bonds also to their sugar residues (Brown, 1998). These features allow an extensive network of H-bonds which stabilizes the sphingolipids aggregates. Cholesterol preferentially intercalates with its planar sterol plane between the saturated long chains of sphingolipids, rather than between the unsaturated phospholipids with kinks in their chains. Because of its intercalating activity it further promotes the \( L_o \) phase by constraining the fatty acid chains of neighbouring sphingolipids in a trans (linear) conformation. Also, it impedes the formation of \( L_g \) phase (van der Goot and Harder, 2001). When cholesterol is embedded within the sphingolipids it is partially shielded from the extracellular environment by the polar heads of the sphingolipids and is thought to interact with these through an H-bond, between its hydroxyl group and sphingolipids’ amide group (Cebeceauer et al., 2009; Simons and Vaz, 2004).

The combined effect of sphingolipids and cholesterol enrichment in rafts is that these domains are thicker, more densely packed and less fluid than the bulk membrane. These physical
features confer lipid rafts the ability to resist to non-ionic detergent lysis at 4°C. In fact it is proposed that in this condition the lipid-lipid interaction is stronger than the lipid-detergent interaction, therefore the creation of lipid-detergent micelles is energetically unfavourable (Brown and Rose, 1992; London and Brown, 2000). Lipid rafts yields to DRMs upon lysis with a detergent. DRMs, because of their high lipid-protein ratio and low density, can be separated from the rest of solubilised membranes by floatation on a sucrose density gradient, after detergent extraction. Detergent extraction has become the gold standard to purify them and to test if a protein is raft or non raft associated.

1.5.3 Lipid rafts: biological roles.

Lipid rafts have been traditionally investigated in epithelial and immune cells; concomitant with the growing body of evidence showing that rafts are implicated in many biological roles, more cell types have been investigated. We shall concentrate our introduction on neuronal cells, since the focus of this thesis is on this cell type. Lipid rafts have been implicated in many biological processes; the core role of rafts is to act as hubs on the cellular membrane where proteins can be sorted and functionally localized. In other words, rafts tune the spatial and temporal organization of proteins on the membrane. Rafts have been proposed to exert their function via multiple ways, which can overlap. In particular, rafts have been shown to 1) facilitate/impede the association of protein involved in given signaling pathways, 2) allow the formation and/or stabilization of protein clusters on the neuronal membrane, 3) act as sorting signals for cellular trafficking, regulating both forward trafficking and endocytosis rate, 4) modify the intrinsic properties of certain proteins (e.g. electrophysiological features of ion channels) (Allen et al., 2007; Golub et al., 2004). For the purpose of this introduction, examples of how rafts modulate neuronal functions will be described.

1.5.4 Lipid rafts and cell signaling.

With regards to the nervous system, rafts have been implicated in the modulation of cell signaling. A notable example to show how rafts laterally modulate cell signaling by allowing interactions between partner proteins is given by RET signaling. GDNF binds to RET and to GPI anchored co-receptor GFRα1. It has been discovered that RET associates with raft upon GDNF binding, and that raft associated GFRα1 is responsible for this translocation. Notably, this
dynamic association between RET and rafts is physiologically important. In fact, mis-targeting of RET, due to the presence of modified GFRα1 (soluble or transmembrane anchored forms) or raft disruption by cholesterol depletion, abolishes intracellular signaling without affecting the formation of the GDNF-RET-GFRα1 complex. It has been proposed that the detrimental effect of non-raft targeting of RET is due to the lack of association between RET and raft associated effectors, such as Src and PI3K kinases. The absence of association between RET and rafts is reflected in ineffective neuronal differentiation and survival (Tansey et al., 2000). Another group further explored the importance of rafts in GDNF signaling. RET shuttling between raft and non raft portions determines how different intracellular pathways are recruited. In non raft portions RET associates with SHC while in rafts it associates with raft associated FRS2 (Paratcha et al., 2001). These studies exemplify how rafts can influence cellular signaling by spatially organizing different proteins. Also, NGF receptor p75NTR translocates to lipid rafts upon phosphorylation. It has been found that, in neurons not expressing TrkA, NGF promotes p75NTR mediated PKA intracellular activation. PKA in turn, phosphorylate p75NTR determining its recruitment to lipid rafts. In these microdomains p75NTR inactivates raft resident Rho (Higuchi et al., 2003). Lipid rafts are also necessary for NCAM mediated neurite outgrowth. Disruption of NCAM association with rafts, by mutating its palmitoylation domain or by depleting cholesterol which leads to raft disassembly, abolishes the activation of NCAM target kinases and completely blocks neurite outgrowth (Niethammer et al., 2002). Related to studies focusing on pain, there is an interesting study showing that rafts are involved in pain pathways in vivo. It was found that PGE2 induced hyperalgesia is mediated by Integrin sub-units α1, α3, β1 while epinephrine induced hyperalgesia depends on α5, β1. α1 Integrin sub-unit is found in lipid rafts in vivo, and raft disruption reduced PGE2 induced mechanical hyperalgesia, without affecting epinephrine mediated hypersensitivity. The authors propose that lipid rafts play an essential role in facilitating the interaction of Integrins with second messenger elements (Adenylyl cyclase/cAMP/PKA) that mediate PGE2 induced hyperalgesia (Dina et al., 2005). Apart from these examples, many other signaling pathways are modulated by rafts and include immune synapse formation and T-cells activation (Luo et al., 2008; Magee et al., 2002), IgE-FceRI signaling (Sheets et al., 1999), and eNOS signaling (Patel and Insel, 2009). The common scheme is that receptors in rafts may behave in three ways: 1) receptor is stably associated with rafts and ligand activates the receptor in rafts, 2) individual receptors with weak raft affinity could oligomerise on ligand binding, and this would lead to an increased residency time in rafts, 3) activated receptors could recruit proteins residing in different rafts and promote
protein cross-linking and raft coalescence. The formation of large stable rafts would allow the concentration of signaling proteins (and exclusion of not related proteins) (Simons and Toomre, 2000). Rafts also regulate cell processes on a spatial scale. Proteins regulating cell adhesion, axonal extension and cell motility are found to be raft associated and their activity is tuned by such association. For example, Integrin signaling relates to raft distribution and to small GTP-binding protein Rac activity. It was demonstrated that Integrin engagement leads to the accumulation of rafts at the cell surface, which determines cell polarisation and stabilisation of Rac within lipid rafts on the cell membrane. Furthermore, in the absence of surface rafts, Rac fails to associate with the plasma membrane and to activate the downstream effector of Integrins FAK (del Pozo et al., 2004). It is also proposed that small rafts, upon certain signals, coalesce into larger and more stable platforms. The functional meaning of this clustering is to promote/enhance cell signaling by bringing in close vicinity partners proteins. In addition, larger rafts would provide a signal on the membrane to polarise the cell and to feed-back protein/raft clustering (Golub et al., 2004). A remarkable example is given by the polarised distribution of receptors/rafts in the growth cone of axons, which is at the base of chemotaxis. Guirland et al. showed that rafts selectively mediate the growth cone guidance (but not growth cone outgrowth) in concert with BDNF, Netrin-1 and Sema3A. Upon presentation of the chemical stimuli rafts and rafts-associated protein asymmetrically distribute and polarise the growth cone, posing the base for axon steering (Guirland et al., 2004). Lipid raft domains also spatially organize specific G proteins on the membrane. This regulated partitioning between lipid rafts and bulk membrane could facilitate or prevent G proteins interactions with their receptors and/or effectors, resulting in either the abolition or the promotion of cell signaling. It has been found that G proteins can be stably associated with rafts or undergo a stimulus-dependent segregation (Allen et al., 2007).

1.5.5 Lipid rafts and protein clustering.
Lipid rafts are important for efficient clustering of several classes of proteins. It has been found that lipid rafts are necessary for the efficient clustering of Acetylcholine Receptors (AChRs) at the level of the neuromuscular junction. Motor nerves trigger muscle contraction through the neuromuscular junction, a specialised synapse which connects neurons and skeletal muscles. AChRs bind to endogenous acetylcholine and allow movement of cations, which depolarise the post-synaptic cells. AChRs determine an excitatory post-synaptic potential in neurons and a
muscle contraction in the case of myofibres. A hallmark of the neuromuscular junction is the clustering of AChRs in the post-synaptic membrane, which is needed for the correct functioning of the synapses. Lipid rafts play a role in clustering the AChRs. It has been found that lipid rafts act as a platform that allows the dynamic molecular interactions between AChRs and their binding partners (Pato et al., 2008; Zhu et al., 2006). Pato and others proposed that AChR is targeted to micro-rafts on the cell surface with MuSK, a tyrosine kinase receptor, and binding to Agrin, a large extracellular proteoglycan, leads to MuSK phosphorylation. Activated micro-rafts subsequently recruit downstream MuSK effector Cdc42 as well as the Actin-nucleation factors N-WASP and Arp2/3. The activation of these factors drives the coalescence of micro-rafts, via Actin-based movements, and leads to the formation of stable AChR macro-clusters. Importantly, integrity of rafts is crucial for the maintenance of AChR clusters (Willmann et al., 2006). Rafts also participate in the clustering of AChRs in neuronal somatic spines, as in ciliary neurons (Bruses et al., 2001). It has also been found that hippocampal neurons contain lipid rafts in the dendritic tree and they are crucial for a normal morphology and for the maintenance of the correct number of dendritic spines. In addition, a portion of AMPA receptors localises in these domains, and the stability of rafts is important for the stabilisation of AMPA receptor clusters on the membrane. In fact lipid raft depletion leads to a reduction of AMPA receptor molecules on the membrane (Hering et al., 2003). Interestingly, a few studies have also shed light on a potential role for lipid rafts in the stabilisation of potassium channels at the paranodes along myelinated axons in vivo. The paranodes flank the nodes of Ranvier and are rich with clustered Shaker type potassium channels. A complex feedback between glial cells and neurons is the key for the development of the correct morphology of nodes and paranodes (Poliak and Peles, 2003) and lipid rafts are thought to participate in the maintenance of such structures (Schafer et al., 2004). It has been revealed, for example, that many paranode-associated proteins are clustered with lipid rafts in this region (Ogawa and Rasband, 2009). In addition, the paranodal junctions in mice lacking the β1, 4-N-acetylgalactosaminyltransferase, a key enzyme in the synthesis of gangliosides, have an altered composition of lipid rafts, display an abnormal morphology and mislocalisation of Kv1.2 (Susuki et al., 2007).
1.5.6 Lipid rafts and membrane excitability.

Lipid rafts have been shown to shape neuronal excitability. Ion channels, which determine the cell’s electric properties, have been demonstrated to partition into lipid rafts. The effect of rafts on membrane excitability is mediated by their influence on proteins trafficking and/or on the intrinsic electrophysiological properties of the ion channels themselves (Pristera and Okuse, 2011). Proteins from different classes have been described to reside in lipid rafts. For example, Sodium/Potassium-ATPase pump, which plays the main role in setting the resting membrane potential, is distributed in clusters on the cell membrane of cerebellar granule cells. It also partitions in Brij-98 detergent-resistant membranes suggesting that it is localised in lipid rafts. The physiological meaning of the association between Sodium/Potassium-ATPase pumps and lipid rafts has not yet been investigated (Dalskov et al., 2005). Although the precise rafts targeting mechanism of the Sodium/Potassium-ATPase pumps is unknown, the authors speculated that the pumps themselves recruit the surrounding membrane lipids and thus function as a core for rafts formation. Hartmann et al. provided a good example of how rafts modulate ion channel trafficking and contribute to determine cell excitability. Chloride co-transporters Potassium-Chloride (KCC2) and Sodium-Potassium-2Chloride (NKCC1) regulate Chloride concentration across the plasma membrane (Delpire, 2000). KCC2 and NKCC1 show different patterns of raft association in mature brain neurons: NKCC1 is enriched in lipid rafts while KCC2 is distributed both in lipid rafts and in the non-lipid raft fractions. This dual segregation seems to have a functional role as disruption of lipid rafts alters the transport activity of these proteins. In fact, raft disruption has opposite effects on the transporters, with KCC2 being potentiated and NKCC1 becoming less active. Raft disassembly also leads to a redistribution of the transporters into larger clusters at the level of the plasma membrane (Hartmann et al., 2009). This study clearly shows how protein function can be modulated by sorting the proteins between the lipid raft microdomains and the bulk membrane. In addition, another study demonstrated that KCC2 associates with lipid rafts in clusters in hippocampal neurons and that this association appears to be dependent on the integrity of lipid rafts and on the phosphorylation of KCC2, which occurs at Tyr-1087 (Watanabe et al., 2009). Several ligand gated ion channels have been found in neuronal lipid rafts. A few studies have demonstrated the role of the association of NMDA receptors with lipid rafts, and how this association is dynamically regulated. The partition of NMDA receptors between the post synaptic density and lipid rafts has been found to be developmentally regulated in the brain: the amount of both NR2A and NR2B subunits increases postnatally (during the first three weeks), with NR2A
rapidly associating with lipid rafts after birth and with NR2B gradually being transported to lipid rafts over three weeks (Besshoh et al., 2007). Interestingly, the partitioning of NMDA receptors is regulated during memory formation as shown by Delint-Ramirez et al., who demonstrated that NR1, NR2A and NR2B subunits quickly move to lipid rafts during the learning of a spatial task (Delint-Ramirez et al., 2008). It is proposed that NMDA receptors trafficking between the post-synaptic density and lipid rafts may be mediated by the association between the intracellular portion of NR2 subunits and the scaffolding protein PSD-95, which contains the PDZ domain (Delint-Ramirez et al., 2010). AMPA receptors have also been described to associate with rafts and a few groups have highlighted a role for lipid rafts in AMPA receptor functional sorting. Hippocampal neurons contain lipid rafts in the dendritic tree and a portion of AMPA receptors localises in these domains. Importantly, the stability of rafts is crucial for the stabilisation of AMPA receptor clusters on the membrane. In fact, lipid raft disruption determines a reduction of AMPA receptor molecules on the membrane, both in basal and AMPA stimulated neurons, due to their engagement into the endocytic pathway (Hering et al., 2003). Hou et al. found that AMPA receptors association with rafts is modulated by NMDA receptors. This finding provides a regulatory loop between the two receptors, which segregate to lipid rafts. When NMDA receptors are activated, more AMPA receptors are trafficked to the lipid rafts and this effect is mediated by the nitric oxide synthase pathway. The integrity of lipid rafts is important for the correct insertion of the AMPA receptors into the membrane. Indeed this group showed that lipid raft disruption reduces the amount of AMPA receptors on the membrane; in contrast to the other study, this effect was found to be determined by a reduction in the rate of translocation of the molecules to the membrane, rather than an increased rate of endocytosis (Hou et al., 2008). Of particular interest for pain pathways, there is the evidence that serotonin receptor 5-HT₃, P2X3, ASIC, TrpV1 and TrpM8 partition into rafts. Serotonin receptor 5-HT₃ is widely distributed in the nervous system (Barnes et al., 2009) and when expressed in a heterologous system it is trafficked to lipid rafts (Eisensamer et al., 2005). This association has been demonstrated to regulate the receptor’s activity. Indeed, cholesterol depletion and raft disruption leads to reduction of the serotonin induced currents and to an alteration of the channel’s kinetics. Interestingly, raft disruption only alters the native properties of the channel, and leaves serotonin-mediated currents unaffected in the presence of antidepressant drugs (Notdhurfter et al., 2010). P2X3 is a nucleotide gated ion channel expressed in primary sensory neurons where it is thought to sense tissue damage, by binding to Adenosine-S'- triphosphate (ATP) released by
necrotic/apoptotic cells. Its association with rafts has been analysed in different tissues and cell types, and P2X3 has been shown to partition into raft domains in cerebellar granule neurons and DRG neurons, but the biological meaning of this partition is yet to be determined (Vacca et al., 2004). ASIC3 is a cation channel gated by low pH, and localises at nerve terminals where it is thought to act as a transducer of acidosis. It has been implicated in pain perception and may play a role in mechanosensation (Alvarez de la Rosa et al., 2002). It has been found that both ASIC3 and PSD-95 associate with lipid rafts, and either the disruption of rafts or the mistargeting of PSD-95, by mutating its palmitoylation domain, alters the properties of ASIC3 (Eshcol et al., 2008). TrpV1 is expressed in the peripheral nervous system in DRG and trigeminal ganglia. It is a polymodal receptor involved in the transduction of noxious heat and can be activated by the endogenous lipid anandamide, pH lower than 5.9 and capsaicin, the component that confers chilli peppers their hot taste. Interestingly, it has been found that in DRG neurons cholesterol, an essential component of lipid rafts, is needed for the correct functioning and trafficking of the channel. Cholesterol depleted DRG neurons display less TrpV1 trafficked to the membrane and both capsaicin and protons evoke smaller currents, when compared to control neurons (Liu et al., 2006). The functional interaction between TrpV1 and lipid rafts has been further investigated in trigeminal ganglia neurons. In these cells, upon raft depletion, capsaicin induced calcium fluxes were markedly reduced, when compared to untreated trigeminal ganglia neurons (Szoke et al., 2010). TrpM8, like TrpV1, contributes to the transduction of thermal stimuli. TrpM8, unlike TrpV1, shows higher ion permeability at temperatures lower than 25°C and is activated by substances like menthol and icilin, which produce a sensation of cold. TrpM8 also associates with and is modulated by lipid rafts. In particular, TrpM8 co-segregate with Flotillin-1 in the lipid raft fractions of mouse DRG and HEK cells. Lipid raft disruption increases the responsiveness of TrpM8 to menthol and cooling stimuli, suggesting that lipid rafts have an inhibitory role towards this channel. Interestingly the trafficking of the channel is unaffected in cholesterol depleted cells, suggesting the lipid rafts directly modulate the gating properties of the channel rather than its amount on the membrane. In fact, when the electrophysiological properties of the channel in raft depleted cells were analysed it was found that TrpM8 temperature activation threshold was shifted to warmer temperatures, and the voltage dependence of activation shifted to a less positive value (Morenilla-Palao et al., 2009). Members of the voltage gated potassium channels are also raft associated. Kv2.1 is expressed in the brain and localises preferentially to the dendritic compartment of neurons (Lim et al., 2000). Kv2.1 is mostly associated with planar lipid rafts.
and rafts directly modulate its electrophysiological features. Depletion of membrane cholesterol disrupts \( \text{K}_v2.1 \)-raft association and shifts its steady state inactivation curve towards more hyperpolarised values without altering its current density and activation kinetics (Martens et al., 2000). It has been proposed that the \( \text{K}_v2.1 \)-raft complex facilitates the interaction between \( \text{K}_v2.1 \) and signaling molecules such as kinases. The authors also conjectured that the transmembrane regions of \( \text{K}_v2.1 \) may contain the information that determines lipid rafts association per se (Scheiffele et al., 1997). In another report, \( \text{K}_v1.4 \) and \( \text{K}_v4.2 \) were examined. The former is localized in axons and contribute to action potential repolarisation (Debanne et al., 1997), while the latter is confined to the soma and dendrites where it contributes to set the excitability of the postsynaptic compartments (Hoffman and Johnston, 1998). \( \text{K}_v1.4, \text{K}_v4.2 \) and the scaffolding protein PSD-95 were all found in lipid rafts in rat brain membranes. Although both channels (in a heterologous system) have the ability to traffic to lipid rafts in the absence of PSD-95, co-expression of PSD-95 further promoted forward trafficking of \( \text{K}_v1.4 \) and its association with lipid rafts but it did not affect the trafficking of \( \text{K}_v4.2 \). In addition, the PDZ binding motif present in both channels seems to play a key role in the channel trafficking, since its deletion leads to a robust decrease in rafts association (Wong and Schlichter, 2004).

### 1.5.7 Lipid raft targeting mechanisms.

Several mechanisms have been shown to determine raft targeting (Brown, 2006; Levental et al., 2010) (Fig. 1.7). Proteins can be targeted to rafts by directly binding cholesterol, which is highly enriched in these microdomains. The most notable case is given by the Caveolin group, which directly binds cholesterol buried in the plasma membrane and promotes the formation of caveola-type lipid rafts (Parton and Simons, 2007). Proteins in the extracellular side usually bind to raft domains by a glycophsatidylinositol (GPI) anchor. GPI is post-translationally attached to the C-terminus of proteins and consists of an oligosaccharide core with a phosphatidylinositol group, with the latter carrying two saturated fatty acid chains that intercalates the sphingolipids present in rafts (Michael AJ Ferguson, 2009). Proteins targeting rafts via GPI include receptors GFRα1, adhesion protein NCAM-120, Thy-1 and F3/Contactin (Tsui-Pierchala et al., 2002). Intracellular proteins target rafts by the addition of fatty acid moieties to the peptide chain; in fact, it is believed that the addition of saturated fatty acid chains increases the affinity of the proteins carrying such modification for the \( L_o \) environment.
The most common modifications are palmitoylation and N-myristoylation. Palmitoylation is the reversible post-translational addition of palmitate to Cysteine residues, while myristoylation is a co-translational irreversible addition of a myristate to the N-terminus (Bijlmakers and Marsh, 2003; Boutin, 1997). Proteins targeting rafts via these motifs include lipid rafts marker Flotillins, Src-family protein kinases (Fyn, Lck, Lyn), MAP kinases, RAS family of small GTPase and G proteins (Tsui-Pierchala et al., 2002). On the other hand, acylation with unsaturated fatty acid chain (e.g. farnesylation) most likely determines a non-raft association (Levental et al., 2010). Proteins can also directly bind rafts without fatty acid modifications. The most notable example is transmembrane protein Haemagglutinin (HA). It was reported that amino acidic residues in the transmembrane segment were sufficient to target HA to rafts (Takeda et al., 2003). Some proteins are targeted to rafts in an indirect way, by binding to proteins that reside in rafts (Pike, 2004). The mechanisms described above have been found to promote raft partitioning, but they may not be sufficient for this process, pointing to the fact that other factors may contribute to traffic proteins to lipid rafts.

**Figure 1.7) Lipid rafts and raft targeting signals.** The figure shows a schematic representation of a lipid raft platform, with proteins partitioning to this domain through different ways. Non raft portion of the membrane and non raft protein are also shown. $L_o =$ Liquid ordered, $L_d =$ Liquid disordered.
1.6 Aims

Our hypothesis is that lipid rafts may act as platforms in the cell membrane of primary sensory neurons where Na\textsubscript{v}1.8 can be trafficked and functionally expressed.

The aims of this study are to:

1) Investigate the sub-cellular localisation of Na\textsubscript{v}1.8 in DRG neurons.

2) Investigate the association between Na\textsubscript{v}1.8 and lipid rafts.

3) Investigate the association between Na\textsubscript{v}1.8 and other proteins (raft markers and proteins involved in pain pathways).

4) Interfere with raft integrity and to explore the functional meaning of the association between Na\textsubscript{v}1.8 and lipid rafts.
Chapter 2: Investigation of the sub-cellular localisation of Na\textsubscript{v}1.8 and its association with lipid rafts in DRG neurons
2.1 Introduction

2.1.1 Synopsis.

The methodology to study lipid rafts and the proteins that we have investigated together with Na\textsubscript{v}1.8 in our study will be introduced in the introduction of this chapter. Na\textsubscript{v}1.8 partitioning into lipid rafts and its relationship with other molecules will be described and discussed. In the general introduction we have highlighted the known mechanisms and unknown issues about Na\textsubscript{v}1.8 trafficking and cellular distribution (See section 1.3.4). In the present chapter the results focusing on Na\textsubscript{v}1.8 sub-cellular localisation will be also presented and discussed.

2.1.2 Lipid raft methods of study.

Detergent extraction, combined with ultracentrifugation in a density gradient, is the most widely used technique to study lipid rafts and raft associated proteins (Allen et al., 2007; Coskun and Simons, 2010; Golub et al., 2004; Hancock, 2006; Simons and Ikonen, 1997; Simons and Toomre, 2000). Lipid rafts, due to their physical properties, can be extracted from the bulk membrane with non-ionic detergent (e.g. TritonX-100) at 4°C. Usually, the cells or tissue of interest are lysed with a non-ionic detergent in the cold; since rafts are resistant to lysis, compared to the bulk membrane, they are spared from the detergent action. Therefore, the membranes obtained with this method are defined detergent resistant membranes (DRMs). To separate DRMs from the lysed membranes a gradient ultracentrifugation is carried out. DRMs, because of their light density, float away from the solubilised portion and can be recovered from the top of the density gradient. The recovered fraction can be analysed by western blotting to determine if the protein of interest partitions or not with lipid rafts. Even though detergent extraction is the gold standard to study raft association caution must be taken when interpreting the results. The lipid and protein analysis carried on DRMs strongly suggests that this fraction represents lipid rafts on live cells (Brown and Rose, 1992; Schroeder et al., 1994; Simons and Gerl, 2010). Nevertheless, reports show that potential artefacts due to detergent extraction could lead to misinterpretation of protein-raft association. In fact, weak raft
associations could be disrupted by the detergent extraction. Also, the notion that different detergents may lead, for certain proteins, to equivocal results supports the hypothesis that rafts are heterogeneous (Pike, 2004), but also does not rule out that detergent-dependent artifacts could be created (Magee and Parmryd, 2003). This evidence led during the early period of the lipid raft field to mixed results and inconsistencies. Another point of strong debate was the inability to visualise intact small rafts in live cells. The small size and the dynamic nature of small rafts were proposed to be in the range of 20-50 nm. This size is below the detection limit of light microscopy (200 nm) which is determined by light diffraction (Shaw, 2006). Also, the mechanisms of raft biosynthesis were not investigated and they are still not fully understood. The current view proposes that rafts are most abundant on the plasma membrane and they may originate in the late Golgi network, where the enzymes to synthesise sphingolipids are present. The absence of cholesterol in the ER, and its gradual enrichment along the exocytosis pathway support this hypothesis (Simons and Toomre, 2000; van Meer et al., 2008). Another unclear aspect is how the outer leaflet of rafts (enriched in sphingolipids and cholesterol) communicates with the inner leaflet (enriched in cholesterol but lacking sphingolipids). It has been proposed that the long chains of the sphingolipids in the outer leaflet interdigitate with the inner part and bridge the two components; also, transmembrane proteins are hypothesised to serve communication between inner and outer leaflet. Nevertheless, strong experimental data supporting this hypothesis are still missing (Munro, 2003). These initial uncertainties and a technical impasse, led to controversies in the raft field. With the introduction of new methods of study, apart from the detergent extraction, and the development of powerful imaging techniques more features about the detailed nature of lipid rafts have been explained and all the initial uncertainties have been challenged. A fundamental advantage in the study of lipid rafts was the discovery that cross-linking of the small highly dynamic rafts leads to their patching and direct visualisation (Hammond et al., 2005; Owen et al., 2007). Both protein patching (Friedrichson and Kurzchalia, 1998; Harder et al., 1998) and lipid patching (Janes et al., 1999) have been demonstrated to determine raft coalescence in live cells. This method gives the advantage of analysing, by microscopy based techniques, the degree of association between rafts and the protein of interest, over the limit of microscopy resolution. Also, it gives important information on the spatial distribution of rafts and proteins. Notably, this process is known to be of physiological relevance in nature. In fact, in activated T-cells small dynamic rafts are clustered into an ordered stable platform, which is necessary for the correct cell signaling and creation of the immunological synapse (Harder and Sangani,
2009; Kabouridis, 2006). Also, the clustering of AchR at the neuro-muscular junction is mediated by the coalescence of small rafts into a stable platform (Pato et al., 2008). Furthermore, stimulation of EphrinB1 leads to the formation of large raft-patches that are thought to act as multiprotein signaling platform downstream of EphrinB1 (Bruckner et al., 1999). It has also been described that rafts coalesce during HIV-virus budding from the host cells (Fantini et al., 2002; Simons and Gerl, 2010). Recent studies, by exploiting cutting edge microscopy techniques, have revealed rafts existence in live cells (Day and Kenworthy, 2009; Duggan et al., 2008). These techniques involve Förster (fluorescence) resonance energy transfer (FRET) (Varma and Mayor, 1998), fluorescence lifetime imaging (FLIM) (Hofman et al., 2009; Hofman et al., 2008), Atomic force microscopy (AFM) (Nicolini et al., 2006; Yuan et al., 2002), fluorescence correlation spectroscopy (FCS) (Bacia et al., 2004; Lasserre et al., 2008), stimulated emission depletion (STED) (Eggeling et al., 2009), far-field fluorescence nanoscopy, single molecule tracking (Drbal et al., 2007) super resolution microscopy (van Zanten et al., 2009) and electron microscopy (Prior et al., 2003). The common feature of these techniques is that they overcome the resolution limit set by the physic nature of light and allow investigation of rafts at a molecular level. Another way to assess lipid rafts’ nature in live cells is to combine microscopy with fluorescent probes, whose spectral properties are dependent on the surrounding lipid phase. LAURDAN (6-acyl-2-dimethylamino-napthalene) represents the classic dye used for this purpose. LAURDAN is a ratiometric dye which shows a 50 nm blue shift of its emission spectra upon the transition between Ld to Lo phase. This behaviour has been attributed to differential penetrability of water molecules in the lipid phases, which affects the dissipation of energy from excited LAURDAN and its emission spectra (Gaus et al., 2006). By using LAURDAN it was found, for example, that phase separation of lipids occurs in live cells and that cholesterol dependent Lo domains are enriched in the filopodia of macrophages, positive for raft marker Caveolin-1 (Gaus et al., 2003). The major drawback of this dye is that it is excited in the UV range, which is toxic for the cells. 4-Di-ANEPPDHQ (amino-naphthylethenyl-pyridinium) represents a newer dye with improved characteristics. Similarly to LAURDAN, it readily partitions into the membrane of cells and shifts its emission spectra upon phase transition. Importantly, it has novel interesting features, compared to LAURDAN. It can be efficiently excited by blue light, has a greater signal/noise ratio, has low cellular toxicity and can be used both in single photon microscopy as well as with two-photon and FLIM (Jin et al., 2006; Owen et al., 2007). This dye proved to give a high contrast in the analysis of phase separation in live cells (Owen et al., 2007). A different approach in the study of lipid rafts
consists in interfering with raft stability and to assess the behaviour of the protein hypothesised to reside in these domains. This approach will be introduced in more detail in the next chapter (see section 3.1.2). Recently, advantages in lipid mass spectrometry (Shevchenko and Simons, 2010) and mathematical modeling supported by physical studies (Richardson et al., 2007) are contributing to understand fundamental properties of protein rafts. The data collected from all these approaches gave us a more detailed view of lipid rafts and raft associated proteins.

In conclusion, the strategy to study lipid rafts and raft associated proteins is based on complementary techniques. The recovery of a certain protein from the DRMs can be considered as a strong indicator of raft association but it must be supported by other data. For example, powerful methods to investigate protein-raft association are provided by microscopy based techniques such as phase sensitive dyes, induction of raft patching and high-resolution microscopy. Approaches to interfere with raft stability, which lead to the dissociation of the protein of interest from rafts, should be also considered. Moreover, functional assays, performed to understand the physiological meaning of protein-raft association, must be carried out.

### 2.1.3 Planar and Caveola type lipid rafts.

Lipid rafts are heterogeneous (Hancock, 2006; Pike, 2004). A planar form and a caveola-type lipid raft have been described. The former lacks a distinguishable morphological feature while the latter is a flask shaped invagination of the membrane (50-100 nm diameter) which can be readily observed with electron microscopy (Parton and Simons, 2007). Protein markers for these different rafts are Flotillins (Bickel et al., 1997; Stuermer and Plattner, 2005) and Caveolins (Parton and Simons, 2007), respectively.

Two Flotillin isoforms, with a predicted molecular weight of 49 kDa, have been described: Flotillin-1 (Reggie-2) and Flotillin-2 (Reggie-1). They are widely expressed in the adult organism (even though a detailed tissue expression map is missing) (Langhorst et al., 2005). With regards to the nervous system, they co-localise in non-caveola type rafts in neurons (Lang et al., 1998; Stuermer and Plattner, 2005). It has been reported that they can exist as homo-hetero-oligomers, where the presence of Flotillin-2 is required for the stability of Flotillin-1 (Solis et al.,
2007). They co-localise along the processes of DRG neurons in micro-clusters (approximately 2.5 µm²) and are associated with GPI-anchored proteins (Thy-1 and CAM F3) in stable raft platforms (Lang et al., 1998). These microdomains (raft platforms) can be efficiently visualised by confocal microscopy and electron-microscopy with gold particles (Kokubo et al., 2003; Stuermer and Plattner, 2005). Flotillins lack a transmembrane segment and are juxtaposed to the intracellular side of the plasma membrane, with both N- and C-terminus facing the intracellular space. They target lipid rafts by acylation via a Golgi independent pathway; Flotillin-1 is palmitoylated (Morrow et al., 2002), while Flotillin-2 is both myristoylated and palmitoylated (Langhorst et al., 2005). Due to the paucity of data from KO animals, the exact functions of Flotillin are still unknown but their widespread expression suggests they may be implicated in several biological processes. Flotillins co-localise and co-immunoprecipitate with Src kinases and are thought to be implicated in signal transduction (Stuermer et al., 2001). They have been described to control cytoskeletal dynamics by forming signaling raft platforms on the plasma membrane. They are crucial for a balanced and controlled cytoskeletal remodelling, which occurs during neuronal differentiation. Indeed, Flotillins are necessary for the recruitment of adaptor proteins to the membrane and the engagement of signaling pathways which determine Actin remodelling in neuronal cells (Langhorst et al., 2008). Also, they are up-regulated during axon regeneration (Schulte et al., 1997). Flotillin-1 and membrane rafts have been demonstrated to be necessary for efficient development and branching of hippocampal neurons. It has been proposed that it associates with membrane rafts and acts as a nucleation site for other proteins. The formation of a multi-protein complex on the membrane (including Actin and Exocysts) drives the events of branching and neurite elongation (Swanwick et al., 2010a). Furthermore, Swanwick et al. showed that the NMDA receptor subunit NR2B binds to lipid raft markers Flotillin-1 and Flotillin-2, while NR2A binds only to Flotillin-1 (Swanwick et al., 2009). This group also demonstrated that in the hippocampus Flotillin-1 drives the formation of glutamatergic synapses, co-localises with synaptic NMDA receptors, and regulates the excitability of hippocampal neurons by tuning the firing frequency of these synapses (Swanwick et al., 2010b). Flotillin-1 seems also to define a clathrin-independent endocytic pathway (Glebov et al., 2006).

Four Caveolin isoforms have been discovered: Caveolin-1 (α and β), -2 and -3. Caveolin-1β results from an internal translation and lacks thirty-one amino acid residues, compared to Caveolin-1α. Caveolin-1 and -2 are ubiquitously expressed, with the highest level observed in
adipocytes, endothelial cells, smooth muscle cells and pneumocytes. Caveolin-3 is a muscle specific isoform (Williams and Lisanti, 2004). In the nervous system Caveolin-1 and -2 mRNA have been detected in the peripheral nervous system, in the spinal cord and in several parts of the brain (with the highest expression in the subthalamic nucleus and substantia nigra). Galbiati et al. described the presence of Cavolin-1 and -2 in DRG neurons. Both isoforms were detected in the insoluble fraction of cultured DRG neurons. Imaging techniques were employed to analyse the sub-cellular localisation of Caveolin-1 and -2. It was found that both proteins are present on the entire surface of DRG neurons and caveola-like structures were identified by electron microscopy (Galbiati et al., 1998). Caveolin-2 has also been found in cortical neurons where it defines rafts involved in dendritogenesis (Takemoto-Kimura et al., 2007). Caveolin-1 expression in neurons has been proven to be dynamic. In hippocampal neurons Caveolin-1 is expressed in a punctuate fashion and glutamate stimulation of these neurons, via AMPA receptors, increases Caveolin-1 expression in a time and concentration dependent manner (Bu et al., 2003). Caveolins predicted size is 23 kDa. Caveolin-1 and -2 are thought to form hetero-oligomers (up to 16 units) in caveolae and Caveolin-1 is necessary for Caveolin-2 expression and membrane targeting. The predicted topology of Caveolins indicates that these proteins have a highly hydrophobic stretch which is buried into the membrane. For this reason Caveolins are thought to assume a hairpin structure, with both N- and C-terminus in the intracellular environment. Caveolins are the structural determinant of caveola-type lipid rafts and heterologous expression of Caveolins is sufficient to drive the formation of caveolae (Lipardi et al., 1998). Caveolin-1 and -3 KO mice lack distinguishable caveolae and have impaired endothelial nitric oxide synthase (eNos) and Calcium signaling in the cardiovascular system (Drab et al., 2001; Galbiati et al., 2001). Caveolin-2 KO mice do not show apparent loss of caveolae but are characterised by abnormal lung morphology and exercise intolerance (Razani et al., 2002). Caveolins directly bind cholesterol (1-2 molecules) and are palmitoylated. As expected, caveolae are enriched in cholesterol and sphingolipids and cholesterol depletion leads to their disruption (Parton and Simons, 2007). In line with rafts’ role in biological processes, caveolae and Caveolins have been implicated in signal transduction, protein clustering and trafficking (Stern and Mermelstein, 2010). They are thought to compartmentalise and concentrate signaling molecules. It has been proposed, for example, that Caveolin-1 modulates the ERα-mGluR1/5 pathway in hippocampal neurons by facilitating the interaction between these proteins (Boulware et al., 2007). Caveolins also scaffolds and coordinates protein complexes involved in NMDA receptor signaling and it has been
demonstrated that such interaction is necessary for NMDA receptor mediated neuronal protection from ischemic cell death (Head et al., 2008). In addition, various classes of signaling molecules, including G-protein subunits, receptor and non-receptor tyrosine kinases, eNOS, and small GTPases have been found associated with Caveolins (Williams and Lisanti, 2004). Caveolins also modulate trans-endocytosis and define a Clathrin independent pathway, but its regulation is still poorly understood (Cohen et al., 2004; Shmuel et al., 2007).

### 2.1.4 Nerve Growth Factor and TrkA.

NGF is a neurotrophic factor of the family of neurotrophin, which includes BDNF, Neurotrophin-3 and Neurotrophin-4/5 (Kandel et al., 2000). It was discovered more than fifty years ago as the molecule responsible for the survival and differentiation of neurons in the peripheral nervous system (Levi-Montalcini, 1987). NGF is a 13 kDa protein which exists as a homo-dimer. The active form is the result of a proteolytic cleavage of a precursor which is expressed by different cell types (Sofroniew et al., 2001). NGF has pleiotropic functions ranging from a role in differentiation, cell survival, regulation of gene expression, sensitization and regeneration. NGF acts through two receptors: the low affinity p75 and the high affinity TrkA. TrkA is a 87 kDa transmembrane tyrosine kinase (Sofroniew et al., 2001). TrkA is mostly expressed in CGRP positive, IB4 negative, DRG neurons which account for approximately 40% of the total DRG neuronal population (Averill et al., 1995), and it has been demonstrated the TrkA expressing DRG neurons are nociceptors (Fang et al., 2005). NGF-TrkA signaling plays a key role in many biological processes. In fact, during development NGF is crucial for differentiation and survival of primary sensory neurons. Mice lacking NGF or TrkA lack unmyelinated small diameter neurons and display hypoalgesia (Wall and Melzack, 2005). NGF, through TrkA, acts also in adult animals and on DRG neurons in vitro. In adult animals it is not necessary for cell survival but promotes neurite outgrowth in cultured DRG neurons (Lindsay, 1988). Upon nerve injury NGF is expressed by non-neuronal cells in the site of injury. NGF in this case has a neuroprotective role, by decreasing apoptosis, promoting regeneration and correct nerve targeting (Petruska and Mendell, 2004). On the other hand, NGF is also a pro-nociceptive factor. It modulates DRG neurons excitability upon trauma and in pain states and determines thermal and mechanical allodynia when injected intradermally (Gould et al., 2000; Kerr et al., 2001). NGF acts directly on TrkA expressing neurons and sensitise them through the
activation of intracellular pathways. It has been found that NGF increases TrpV1 mediated currents through PLC activation, by determining the removal of tonic inhibition maintained by phosphatidylinositol 4-5-bisphosphate (Chuang et al., 2001), and by increasing its trafficking to the membrane (Zhang et al., 2005). It has been proposed that NGF, via the ceramide pathway, also determines an increase of Na\textsubscript{v}1.8 TTX-r mediated currents (Zhang et al., 2002). NGF, by acting on non-neuronal cells (e.g. mast cells), triggers the release of pro-inflammatory compounds from these cells and indirectly sensitises nociceptors (Wall and Melzack, 2005). TrkA mediates NGF effects by homo-dimerising, by auto-phosphorylating and by phosphorylating target proteins. The intracellular pathways that are engaged are multiple and include cAMP-PKA, PLC-PKC, MAPK p38, RAS and ERK1/2 (Sofroniew et al., 2001). TrkA-NGF complex can also undergo endocytosis, be trafficked and trigger signaling at the level of soma (Zweifel et al., 2005). NGF also modulates nociceptor excitability by regulating gene expression of several classes of proteins including neuropeptides (e.g. Substance P) and molecular transducers (e.g. TrpV1) (Okuse, 2007; Petruska and Mendell, 2004). In relationship to Na\textsubscript{v}1.8, basal NGF expression in adult animals is necessary to maintain the channel's tonic expression in nociceptors (Fjell et al., 1999b). Na\textsubscript{v}1.8 expression is up-regulated in vivo during inflammatory conditions (CFA, Carrageenan) (Lai et al., 2004), when elevated concentrations of NGF are circulating (Donnerer et al., 1992), and NGF increases Na\textsubscript{v}1.8 expression in vitro (Black et al., 1997; Fjell et al., 1999a). TrkA represents a protein of particular interest in studies focusing on Na\textsubscript{v}1.8 and pain pathways because it mediates NGF effects on Na\textsubscript{v}1.8 and because NGF-TrkA signaling shapes nociceptors phenotype.

2.1.5 Annexin II light chain S100A10 (p11).

Annexins are a large group of proteins (Annexin I-XI, XIII) characterised by two common features: the presence of a conserved annexin domain and the ability to bind negatively charged phospholipids in a Calcium dependent manner. Annexins, by virtue of their ability to bind phospholipids, are involved in several biological processes, including vesicle trafficking, scaffolding of membrane proteins and exo-endocytosis. Annexins exist as a hetero-tetramer of two heavy chains plus two light chains (Seaton and Dedman, 1998). The complex Annexin II-S100A10 (p11) shows unusual features compared to the other members of the Annexin family. P11 has two EF hand motif which carries two deletions and mutations, which make the
Calcium binding site non functional. Indeed, this mutation makes Annexin II-p11 constantly locked in an “active” state related to the binding of its target proteins (Rescher and Gerke, 2008). Notably, Annexin II-p11 is acylated at its N-terminus and localises in lipid rafts (Babiychuk and Draeger, 2000; Gerke and Moss, 2002; Oliferenko et al., 1999; Rescher and Gerke, 2004). Since p11 only exists in an active state, modulation of Annexin II-p11 dynamics in trafficking proteins to the membrane may be regulated by Annexin II-p11 interaction. It has been found that PKC, by phosphorylating Annexin II modulates its affinity for p11 (Rescher and Gerke, 2008). During a yeast two-hybrid genetic screening it was found that p11 binds to the N-terminus of Na\textsubscript{v}1.8 (Malik-Hall et al., 2003). Virtually all DRG neurons expressing Na\textsubscript{v}1.8 are positive for p11 expression. Importantly, p11 promotes Na\textsubscript{v}1.8 localisation onto the membrane. In CHO cells (heterologous system where exogenous Na\textsubscript{v}1.8 currents are undetectable) co-expression of p11 with Na\textsubscript{v}1.8 is sufficient to promote channel insertion and detection of TTX-r currents. Furthermore, in DRG neurons antisense treatment for p11 induces a loss of Na\textsubscript{v}1.8 currents (Okuse et al., 2002). P11 expression is up-regulated by NGF in DRG neurons. It has been suggested that NGF increases Na\textsubscript{v}1.8 mediated TTX-r currents by increasing p11 amount and by promoting forward trafficking of the channel into the membrane (Okuse, 2007; Okuse et al., 2002). P11 binding is specific for Na\textsubscript{v}1.8 and occurs between Na\textsubscript{v}1.8 residues 74-13 and p11 residues 33-78 (Poon et al., 2004). Consistent with the role of Na\textsubscript{v}1.8 in pain pathways and p11 key role in trafficking it to the membrane, p11 conditional KO mice display deficits in pain behaviours. When p11 is ablated in nociceptors only, total Na\textsubscript{v}1.8 expression is not affected, but there is a marked decrease of membrane bound Na\textsubscript{v}1.8 in DRG neurons; this also correlates with a 50% reduction of TTX-r currents. These mice display deficits in WDR neurons activity upon presentation of mechanical and thermal stimuli. Furthermore, an increase in pain threshold following a mechanic stimulus (Randall-Selitto test) was observed. Deletion of p11 from nociceptors also reduced mechanical allodynia following L5 spinal nerve injury (Foulkes et al., 2006). The well documented effect of p11 on Na\textsubscript{v}1.8 makes this auxiliary protein an interesting target for studies focusing on the trafficking and cellular localisation of Na\textsubscript{v}1.8.
2.1.6 Aims.

The studies presented in this chapter were aimed at the investigation in vivo and ex vivo of the association between Na\textsubscript{v}1.8 and lipid rafts in DRG neurons, with biochemistry and imaging techniques. As we outlined in the introduction, there is a paucity of data about Na\textsubscript{v}1.8 localisation within DRG neurons. Therefore we also investigated Na\textsubscript{v}1.8 sub-cellular localisation in vitro and ex vivo. In addition, the localisation of Na\textsubscript{v}1.8 in DRG neurons was analysed in relation to other proteins of interest (TrkA, Flotillin-1, and Caveolin-2).
2.2 Materials and Methods

2.2.1 Detergent resistant membranes (DRMs) preparation and purification.

Cervical, thoracic, lumbar DRGs and sciatic nerves were extracted from female Wistar rats (6 weeks old; 150 grams) and homogenized, by using a glass pestle and mortar, in solution B (150 mM NaCl, 5mM dithiothreitol (DTT), 5mM EDTA, 25 mM Tris-HCl pH 7.4) supplemented with 1:1000 protease inhibitor cocktail set III (Merck). The homogenate was centrifuged at 3600 rpm for 10’ at 4°C to pellet chromatin, the supernatant recovered and adjusted with 10% Triton X-100 (Sigma) in Solution B to give a final concentration of 1% Triton X-100. When DRG cultures were used as the initial source, cells were recovered by scraping the cells in solution D (Solution B with 1% Triton X-100). DRMs were prepared by incubating the samples 30’ on ice. The lysate was mixed with 60% OptiPrep® (Iodixanol; Sigma) to obtain a final concentration 40% OptiPrep® (100 µl OptiPrep® and 50 µl sample). 120 µl of the 40% fraction was layered with 360 µl of 30% OptiPrep® in solution D and 120 µl of solution D in a 5/16 x 1 3/8 inch Beckman ultracentrifuge tube (Fig. 2.1). All the described procedures were carried out on ice in a cold room at 4°C with pre-cooled solutions. Following layers preparation, samples were centrifuged at 36000 rpm at 4°C for 4.5 hrs in a Beckman Optima MAX ultracentrifuge with a fixed angle TLA 120.1 rotor. After centrifugation the whole gradient was recovered from the tubes by collecting nine fractions (66.6 µl each). Fractions were stored at -20°C.
Figure 2.1) Schematic diagram of DRMs preparation. The diagram shows the steps involved in DRMs extraction.

2.2.2 Western blotting and dot blotting.

Fractions recovered from ultracentrifugation were mixed with 5X Laemmli buffer (10% SDS, 50% glycerol, 20% β-mercaptoethanol, 0.01% bromphenol blue, 0.312 M Tris-HCl, pH 6.8) in the presence of 20 mM and incubated 45’ at room temperature (R.T.). Equal volumes were subjected to SDS-PAGE at constant voltage (80 V for migration through the stacking gel, 100 V for migration through the resolving gel) till the proteins of interest were efficiently separated. In particular, for the detection of Na\textsubscript{v}1.8 150 μl of sample (112.5 μl of each fraction recovered from the ultracentrifugation step plus 37.5 μl 5X Laemmli buffer) were loaded in each well of the gel. For the detection of all other protein 25 μl of sample (20 μl of each fraction recovered from the ultracentrifugation step plus 5 μl 5X Laemmli buffer) were loaded in each well of the gel. For the detection of Na\textsubscript{v}1.8, a large electrophoresis apparatus (Bio-rad) was used in combination with an 8% poly-acrylamide gel. For other proteins, small electrophoresis apparatus (Bio-rad) and 12% poly-acrylamide gels were used.

After electrophoresis proteins were transferred onto Polyvinylidene fluoride (PVDF) membrane (Amersham) using either a wet system (for the detection of Transferrin receptor and Flotillin-1; 2.5 hours at 200 mA) (Bio-rad) or the iBlot\textsuperscript{TM} (Invitrogen) dry method (for the detection of Na\textsubscript{v}1.8; Program 3; 9 mins transfer period). Non specific sites were blocked with 5% dried fat-free milk dissolved in phosphate buffered saline (PBS) supplemented with 0.1% Tween-20 (Sigma) (PBS-T) over-night (o.n.) at 4°C. After washes (1x15’, 2x5’) in PBS-T membranes were incubated with the following primary antibodies: rabbit anti Na\textsubscript{v}1.8 (from K.Okuse), mouse anti Flotillin-1 (DB Biosciences), mouse anti Transferrin receptor (Zymed; Invitrogen). All antibodies were used 1:1000 for 1 hr at R.T. Following washes (1x15’, 2x5’) the membranes were incubated with the appropriate secondary antibodies conjugated to Horseradish Peroxidase (HRP), with the following conditions: anti-rabbit IgG-HRP (Dako) 1:200 1 hr at R.T., anti-mouse IgG-HRP (Dako) 1:1000 1 hr at R.T. Antibodies were diluted in 10% goat serum (GS) in PBS-T.
For dot blot analysis, 1 µl of each layer recovered from the centrifugation step was applied to a nitrocellulose membrane (0.45 µm; Amersham) and dried at R.T. Non specific interactions were blocked by incubating the membranes with 5% Bovine Serum Albumin (BSA) diluted in PBS for 1 hr at R.T. Membrane was probed with biotinylated Cholera toxin sub-unit B (CTB; Invitrogen) at a final concentration of 0.1 µg/ml in PBS for 20’ and, after three washes, was incubated with HRP conjugated Streptavidin (Dako) diluted 1:10,000 in PBS for 20’.

All membranes were washed (1x15’, 2x5’) before developing chemiluminescence with the enhanced chemiluminescence western blotting detection system kit (Applichem). Chemiluminescence was detected with Fujifilm LAS-3000 Imaging System and images stored as 8 bit TIFF files. Signal intensities were quantified using freeware image analysis software ImageJ (http://rsbweb.nih.gov/ij/).

When needed membranes were stripped and re-probed with the appropriate antibody. In particular, membranes were incubated in stripping buffer (2% SDS, 100 mM β-mercaptoethanol added fresh, 50 mM Tris-HCl, pH 6.8) at 50 °C for 30’ with gentle shaking in a sealed box. Membranes were subsequently thoroughly washed in PBS-T (3x15’) and blocked overnight in 5% dried fat-free milk dissolved in PBS-T.

### 2.2.3 DRG culture.

Female Wistar rats (6 weeks old; 150 grams) were culled by CO₂ asphyxiation. DRGs (40-50 per dissection) were harvested and placed in cold sterile Dulbecco’s Modified Eagle Medium (DMEM) (GIBCO; Invitrogen). Excess of dorsal roots and spinal nerves were trimmed under a stereo microscope with micro-dissection Vannas scissors (Fine science tool). After a quick wash in DMEM, DRGs were incubated with 0.125% Collagenase XI (Sigma) and 0.1 mg/ml DNase II (Sigma) in DMEM for 90’ at 37°C. After enzymatic digestion, DRGs were triturated with a cut 1 ml tip until a cloudy cell suspension was obtained (approximately 15 strokes). Cells were spun down, resuspended in pre-warmed DMEM and filtered through a 70 µm mesh (BD Biosciences). DRG neurons were recovered by using BSA (PAA) cushions. 1 ml BSA cushions were prepared to a final concentration of 10% BSA in DMEM. Filtered cell suspension was divided in 1 ml aliquots, gently overlaid on top of the cushions and spun for 8’ at 700 rpm. After the centrifugation debris were aspirated from the interphase and DRG neurons
recovered from the pellet. DRG neurons were pooled and washed in pre-warmed DMEM. Cells were plated on 13 mm glass coverslips coated with poly-L-Lysine and Laminin in complete media (DMEM, 10% fetal bovine serum (FBS), Penicillin/Streptomycin (1:100; Sigma), NGF (50 ng/ml; Peprotech) and Aphidicolin (10 µM; Sigma). Cell yield was calculated by counting cell number of an aliquot of the cell suspension in a Neubauer hemocytometer. 10000 and 15000 cells per coverslips were plated for immunofluorescence and biochemistry purposes, respectively. Cells were incubated in a 95% air/5% CO₂ humidified incubator. Media was changed every two days with pre-warmed complete media. Prior to plating, glass coverslips were treated with 0.1 mg/ml poly-L-Lysine (Sigma) for 2 hours at R.T. After three washes with deionised distilled water they were incubated with 10 µg/ml Laminin (Invitrogen) in DMEM for 90’ at R.T.

2.2.4 ND7/23 cell culture.

ND7/23 clone (Wood et al., 1990) was cultured in DMEM with 10% FBS in 10 cm diameter dishes. The cell line was trypsinised and sub-cultured at a 90% confluence. When transfection was needed, 15,000 cells were seeded onto poly-L-Lys covered glass coverslips the day before transfection.

2.2.5 Ganglioside GM1 staining.

DRG neurons were quickly washed with sterile PBS and incubated with 1 µg/ml biotinylated CTB (Invitrogen, Molecular Probes) in PBS for 20’ at R.T. CTB specifically binds to ganglioside GM1 (Merritt et al., 1994; Schon and Freire, 1989) which is a marker of lipid rafts (Hammond et al., 2005; Harder et al., 1998; Janes et al., 1999; Yuan et al., 2002). After three washes with PBS, DRG neurons were incubated with 1:1000 Streptavidin-488 (Invitrogen) for 20’ at R.T. Cells were washed three times in PBS, fixed with 4% paraformaldehyde (PFA) (Sigma) in PBS for 10’ at R.T. and processed for immunofluorescence if needed.
2.2.6 Immunofluorescence on cultured DRG neurons.

DRG neurons were washed in PBS three times and fixed with 4% PFA for 10’ at R.T. Following washes in PBS (3 x 10’ each) DRG neurons were incubated with primary antibodies. All the antibodies (Table 2.1) were diluted in 10% GS for 1 hour at R.T. After washes (3 x 10’ each), cells were incubated with fluorescently labeled secondary antibodies or Streptavidin (Table 2.1), diluted in 10% GS for 1 hour at R.T. in the dark. Absence of background fluorescence was shown by omitting the primary antibodies (Fig. 2.2).

Figure 2.2) Secondary antibodies testing. The images show the immunofluorescence carried out with secondary antibodies only. Scale bar is 20 µm.

When nuclear counterstain was needed, cells were incubated with Hoechst 33342 (Invitrogen) diluted 1:10000 in PBS for 10’ at R.T. After washes (3 x 10’ each) cells were quickly rinsed twice in deionised water and coverslips were mounted on glass slides with anti fade agent AF1 (Citifluor LTD) and sealed with nail varnish. If storage was needed samples were left at 4°C in the dark (or -20°C for long term storage). Samples were analysed on a widefield Nikon 80i microscope and pictures acquired with an ultrahigh-quality digital Nikon DXM1200F camera controlled with LUCIA G software. Post-acquisition processing was carried out with Adobe Photoshop 7.
Table 2.1. Antibody used for immunofluorescence. M=mouse, R=rabbit, C=chicken, FITC=Fluorescein isothiocyanate, TRITC=tetramethylrhodamine isothiocyanate.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution Cultured DRG</th>
<th>Dilution Teased sciatic nerve</th>
<th>Company</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>α Na\textsubscript{v}1.8</td>
<td>1:200</td>
<td>1:100</td>
<td>K.Okuse</td>
<td>Before first use spun down 5’ 13k rpm at 4°C</td>
</tr>
<tr>
<td>α p11</td>
<td>1:100</td>
<td>-</td>
<td>Neuromics</td>
<td>-</td>
</tr>
<tr>
<td>α TrkA</td>
<td>1:100</td>
<td>-</td>
<td>BD Biosciences</td>
<td>Biotinylated</td>
</tr>
<tr>
<td>α Peripherin</td>
<td>1:200</td>
<td>1:100</td>
<td>Chemicon</td>
<td>-</td>
</tr>
<tr>
<td>α NF200</td>
<td>1:500</td>
<td>-</td>
<td>Chemicon</td>
<td>-</td>
</tr>
<tr>
<td>αM-FITC</td>
<td>1:200</td>
<td>1:100</td>
<td>Dako</td>
<td>-</td>
</tr>
<tr>
<td>αM-TRITC</td>
<td>1:1000</td>
<td>-</td>
<td>Dako</td>
<td>-</td>
</tr>
<tr>
<td>αR-TRITC</td>
<td>1:1000</td>
<td>1:100</td>
<td>Dako</td>
<td>-</td>
</tr>
<tr>
<td>αC-TRITC</td>
<td>1:200</td>
<td>-</td>
<td>Dako</td>
<td>-</td>
</tr>
<tr>
<td>Streptavidin-488</td>
<td>1:200</td>
<td>-</td>
<td>Invitrogen</td>
<td>-</td>
</tr>
</tbody>
</table>

Antibody anti Na\textsubscript{v}1.8 has already been validated and widely described in literature (Djouhri et al., 2003a; Fang et al., 2005; Malik-Hall et al., 2003; Okuse et al., 1997; Okuse et al., 2002; Stirling et al., 2005). We further confirmed the specificity of the antibody against Na\textsubscript{v}1.8. We exploited the features of the DRG-like neuroblastoma cell line ND7/23 (Wood et al., 1990). ND7/23 cells express TTX-s channels, display TTX-s currents and lack Na\textsubscript{v}1.8 (John et al., 2004; Leffler et al., 2007). We transfected ND7/23 cells with plasmid encoding GFP, Na\textsubscript{v}1.8 or Na\textsubscript{v}1.8-Dronpa (construct with fluorescent protein Dronpa fused to the C-terminus of Na\textsubscript{v}1.8). We delivered 1 µg total plasmid DNA with Lipofectamine\textsuperscript{TM} 2000 (Invitrogen), following manufacturer’s instruction. Twenty-four hours after transfection, ND7/23 cells were processed for immunofluorescence by using anti Na\textsubscript{v}1.8 antibody (Fig. 2.3). Non transfected cells and ND7/23 cells expressing GFP did not display any immunoreactivity for Na\textsubscript{v}1.8, showing that antibody anti Na\textsubscript{v}1.8 does not bind to TTX-s VGSCs expressed by ND7/23. ND7/23 transfected with plasmids expressing GFP and Na\textsubscript{v}1.8 (in a ratio 1:9) showed immunoreactivity for Na\textsubscript{v}1.8. Also, all the cells expressing the fusion protein Na\textsubscript{v}1.8-Dronpa were positive for
immunoreactivity against Na\textsubscript{v}1.8. These results show that the antibody we use to detect Na\textsubscript{v}1.8 is specific for this channel and does not cross-react with other VGSCs.

![Untransfected and GFP](image1)

![GFP/Na\textsubscript{v}1.8 (1:9) and Na\textsubscript{v}1.8-Dronpa](image2)

**Figure 2.3) Anti Na\textsubscript{v}1.8 antibody testing.** ND7/23 cells have been transfected with plasmid expressing GFP, Na\textsubscript{v}1.8 and Na\textsubscript{v}1.8-Dronpa. Control cells have not been transfected. The images show the immunofluorescence for Na\textsubscript{v}1.8 in fixed ND7/23 cells. Only Na\textsubscript{v}1.8 and Na\textsubscript{v}1.8-Dronpa transfected cells display immunoreactivity for Na\textsubscript{v}1.8. The insets show the magnification of cells expressing Na\textsubscript{v}1.8 and Na\textsubscript{v}1.8-Dronpa positive for Na\textsubscript{v}1.8 immunoreactivity.

### 2.2.7 Electroporation of DRG neurons.

Exogenous DNA was delivered into DRG neurons by electroporation with Neon\textsuperscript{TM} transfection system (Invitrogen). Electroporation was carried out before plating the cells. DRG neurons were prepared as described above (see section 2.2.3). The cell suspension was washed in 4 mls of sterile PBS (Gibco; Invitrogen) and spun down. The pellet was resuspended in 27 µl buffer R (Invitrogen) plus 3 µl of plasmid DNA. DNA was prepared by using Plasmid Midi Kit (Qiagen) and used at a final concentration higher than 1 µg/µl and with a 260/280 nm reading not lower than 1.8. The electroporation chamber was filled with 3 ml of buffer E. The cell suspension was aspirated in a 10 µl gold tip and the electric pulses delivered (2 pulses, 1200 volts, 20 msec).
For electroporation purposes, 200,000 cells were electroporated in four rounds of electroporation. Gold tips were used for three times and electroporation chambers ten times before being discarded. After the electric pulses neurons were quickly plated in pre-warmed DMEM with 10% FBS without antibiotics. Two hours after plating, NGF was added to the cells. The day after plating, media was replaced with complete DMEM and media changed every two days. Expression of the exogenous constructs was monitored daily by fluorescence microscopy.

2.2.8 Plasmids.

\(\text{Na}_v 1.8\) cDNA with a mutated stop codon (TGA \(\rightarrow\) KpnI site) was sub-cloned from expression vector pRK7-Na\(_v\)1.8 (K. Okuse) by using unique HindIII and KpnI restriction sites into pDsRed2-N1 (Clontech). Na\(_v\)1.8 cDNA was cloned upstream of DsRed2 sequence to generate an in-frame fusion product (Na\(_v\)1.8-DsRed2). Flotillin-1 cDNA was amplified from a DRG neuron expression library (K. Okuse). EcoRI site was introduced upstream of the starting codon and NotI was used to replace TGA stop codon. Flotillin-1 cDNA was cloned into pDG1-MN1 vector (Amalgaam; MBL) in frame with cDNA coding for CoralHue™ Dronpa-Green to generate a Flotillin-1 C-terminus fusion product (Flotillin-1-Dronpa). Caveolin-2 cDNA was amplified from rat adipocytes. EcoRI and HindIII sites were introduced upstream of the starting codon and to eliminate the TGA stop codon, respectively. Caveolin-2 cDNA was cloned into pDG1-MN1 vector by using EcoRI and HindIII restriction sites. The cDNA was cloned in frame with Dronpa cDNA to generate a C-terminus fusion product (Caveolin-2-Dronpa) (gift from S. Shepherd). All the coding regions described above are specific for rat (\textit{Rattus norvegicus}).

2.2.9 Teased fibre preparation from sciatic nerve and immunofluorescence.

Sciatic nerves were harvested and placed on top of a 2% gelatin (Sigma) coated glass coverslip. Under a stereomicroscope nerve fascicles were freed from the epineurium by pulling them with fine tips tweezers (Fine science tools). The fascicles were dissociated in fibre bundles and single fibres by gently pulling them with forceps. The sciatic nerve fibres were washed in PBS and fixed in 4% PFA. After washes (3x10') fibres were permeabilised with 0.1% Triton X-100 in
PBS 10’ at R.T. Following washes, samples were blocked in 10% GS in PBS for 30’ at R.T. and incubated with primary antibodies (Table 2.1) diluted in 10% GS/PBS for 2 hrs at R.T. After washes with PBS samples were incubated with fluorescently labelled secondary antibodies (Table 2.1) diluted in 10% GS in PBS for 1 hour at R.T. Samples were mounted in anti fading agent AF1 (Citifluor LTD) on glass slides, sealed with nail varnish and analysed under the microscope (same equipment as for DRG culture immunofluorescence).

2.2.10 Statistical analysis.

Data were computed and analysed with OriginPro 8.5 software (Originlab Corporation). For the comparison of the degree of colocalization between endogenous NaV1.8 and Flotillin1-Dronpa and Caveolin2-Dronpa Fisher’s exact test was used. Data are expressed as mean ± SEM. P value < 0.05 was considered statistically significant.
2.3 Results

Investigation of Na\(_v\)1.8 association with lipid rafts by biochemical techniques.

Lipid rafts, due to their biophysical features, are resistant to non-ionic detergents at 4°C. This property is exploited to separate them from the soluble portion of the membrane (non-lipid raft). After the detergent treatment, the membrane lysate, which includes intact lipid rafts and solubilised membrane, is centrifuged in a density gradient; lipid rafts, because of their light density, float towards the top of the gradient, where they can be recovered. We asked if Na\(_v\)1.8 associates with lipid rafts, therefore we analysed its partition between lipid rafts and the soluble fraction from three different sources: DRG cell body (tissue), sciatic nerve (tissue) and cultured DRG neurons. The following sections will describe the results from these experiments.

2.3.1 Na\(_v\)1.8 association with lipid rafts in DRG neurons ex vivo.

In adult animals Na\(_v\)1.8 is mostly expressed in DRGs (Akopian et al., 1996). Anatomically DRGs contain the cell bodies of sensory neurons and a small proportion of axons which originates from these cells and project to the dorsal horn (through the dorsal root) and to the peripheral tissues (through spinal nerves). We sought to investigate Na\(_v\)1.8 partition between lipid rafts and the soluble fraction in this tissue. We first assessed if our protocol allows us to efficiently separate lipid rafts from the soluble membrane. In our study, we used a protein and a lipid marker, Flotillin-1 and ganglioside GM1 (GM1) respectively, to define the floating, low density, raft fraction. In our preparation, we have found that Flotillin-1 is present as two pools: one associated with the bottom fractions (lanes 7, 8 and 9) and one associated with the top fractions (lanes 2, 3 and 4) (Fig. 2.4 A, B). GM1 is highly enriched in the top fraction (lanes 2 and 3) and a minor amount is associated with the soluble fraction. To define the non-lipid raft portion of the membrane we used Transferrin Receptor, which is widely used as a non-raft marker (Harder et al., 1998). Transferrin receptor was only found in the bottom fractions (7, 8 and 9). Given the distribution of these markers, we defined fractions 2 and 3 as the lipid raft fraction and fraction 7, 8 and 9 as the soluble portion of the membrane (Fig. 2.4 A, B). Blots in
**Fig. 2.4** represent n=1 experiment. This result indicates that we can efficiently separate lipid rafts from the bulk membrane by using DRGs as a source.

![Image of western blot and band intensity graph](image)

**Figure 2.4) Lipid raft separation from DRGs ex vivo.** A) Fractions from a density gradient were analysed by western blotting (Flotillin-1, Transferrin Receptor) and dot blotting (GM1) to test the separation of lipid rafts from the soluble portion. Different fractions are labelled from 1 to 9, with 1 being the top fraction and 9 the bottom fraction. M is the protein ladder. B) Shows the bands intensity of the different markers, plotted against the fraction number, of the blots shown in A.

We next analysed how NaV1.8 partitions between lipid rafts and the soluble fraction in DRGs. For this purpose, we probed with an antibody against NaV1.8 the same sample used for the raft preparation presented above. We have found that NaV1.8 was present in lane 8, and only by extending the acquisition exposure time (and saturating NaV1.8 signal in the lower fraction), a minor amount of NaV1.8 could be detected in the lipid raft fraction (**Fig. 2.5 A, B**). Blots in **Fig.
2.5 represent n=1 experiment. This data indicates that in fresh DRGs the great majority of Na\textsubscript{v}1.8 does not associate with lipid rafts, and is restricted to the soluble fraction.

![Image](134x428 to 491x722)

**Figure 2.5** Na\textsubscript{v}1.8 is mostly associated with the non lipid raft fraction in fresh DRGs. A) Same fractions as in Fig. 2.4 were analysed by western blotting with an antibody against Na\textsubscript{v}1.8. Different fractions are labelled from 1 to 9, with 1 being the top fraction and 9 the bottom fraction. M is the protein ladder. The two blots represent the same membrane, with different exposure times. B) Shows the bands intensity from the membrane labelled as “Na\textsubscript{v}1.8 long exposure”, plotted against the fraction number, of the blot shown in A.

### 2.3.2 Na\textsubscript{v}1.8 association with lipid rafts in sciatic nerve ex vivo.

Sciatic nerve is a spinal nerve which contains the axons of sensory and motor neurons, whose cell bodies are located in the DRGs and ventral horn of the spinal cord, respectively. Since Na\textsubscript{v}1.8 is localised in the unmyelinated axons of the sciatic nerve (Gold *et al.*, 2003) and Fig. 2.14 (see section 2.3.5), we tested if Na\textsubscript{v}1.8 associates with lipid rafts in the sciatic nerve. We
defined the raft compartment in the sciatic nerve with the same markers we used for the DRG cell bodies. In this preparation we found that raft marker Flotillin-1 is present in the bottom (lanes 8 and 9) and in the floating fractions (lanes 2, 3 and 4). GM1 is concentrated in the top fractions (2, 3 and 4), while Transferrin Receptor is excluded from the buoyant rafts and enriched in the soluble fraction (lanes 8 and 9) (Fig. 2.6 A, B). According to these profiles we defined the raft fraction the one recovered from lanes 2, 3 and 4. Blots in Fig. 2.6 are representative of at least n=2 experiments.

Figure 2.6) Lipid raft separation from sciatic nerve ex vivo. A) Fractions from a density gradient were analysed by western blotting (Flotillin-1, Transferrin Receptor) and dot blotting (GM1) to test the separation of lipid rafts from the soluble portion. Different fractions are labelled from 1 to 9, with 1 being the top fraction and 9 the bottom fraction. M is protein ladder. B) Shows the bands intensity of the different markers, plotted against the fraction number, of the blots shown in A.

We next evaluated Na\textsubscript{v}1.8 partitioning on this preparation and found a completely opposite finding compared to Na\textsubscript{v}1.8 distribution in DRG neurons cell bodies. Remarkably, in the sciatic
nerve Na\textsubscript{v}1.8 was only found in the raft fraction (lanes 2 and 3) and no Na\textsubscript{v}1.8 was associated with the soluble fraction (Fig. 2.7 A, B). Interestingly, Na\textsubscript{v}1.8 was present as slightly different sizes (Fig. 2.7 A). The predicted sizes of these bands (as determined by Rf measures) were 222 kDa, 201 kDa (in lane 2) and 247 kDa (band in lane 3). Blot in Fig. 2.7 is representative of n=2 experiments.

Figure 2.7) Na\textsubscript{v}1.8 is only associated with the lipid raft fraction in the sciatic nerve. A) Same fractions as in Fig. 2.6 were analysed by western blotting with an antibody against Na\textsubscript{v}1.8. Different fractions are labelled from 1 to 9, with 1 being the top fraction and 9 the bottom fraction. M is protein ladder. B) Shows the bands intensity of Na\textsubscript{v}1.8 bands, plotted against the fraction number, of the blot shown in A.

2.3.3 Na\textsubscript{v}1.8 association with lipid rafts in DRG neurons in vitro.

DRG neurons can be dissociated and cultured in vitro. In this condition they are able to extend neurites from the cell bodies. Similarly to what we did for DRGs and sciatic nerve ex vivo, we separated lipid rafts from 15000 DRG neurons maintained for two days in vitro (DIV). From this
source we found that the majority of Flotillin-1 was recovered from the low density fractions (lanes 2 and 3). GM1 associated with top (lanes 2 and 3) and bottom fractions (lanes 7, 8 and 9). Transferrin receptor, as expected, did not display raft-like properties and was retained in the soluble fraction (lanes 7, 8 and 9) (Fig. 2.8 A, B). Blots in Fig. 2.8 are representative of at least n=2 experiments. These data clearly show that cultured DRG neurons, similarly to DRG neurons in vivo, contain DRMs which can be extracted from the soluble fraction. Hence, from plated DRG neurons, by density gradient, lipid rafts were defined by floating fractions 2 and 3.

Figure 2.8) Lipid raft separation from DRG neurons in vitro. A) Fractions from a density gradient were analysed by western blotting (Flotillin-1, Transferrin Receptor) and dot blotting (GM1) to test the separation of lipid rafts from the soluble portion. Different fractions are labelled from 1 to 9, with 1 being the top fraction and 9 the bottom fraction. M is protein ladder. B) Shows the bands intensity of the different markers, plotted against the fraction number, of the blots shown in A.
DRG neurons, when plated, express functional Na\textsubscript{v}1.8 (Fjell \textit{et al.}, 1999a). We investigated Na\textsubscript{v}1.8 association with rafts after 2 DIV. At this stage both cell bodies and axons are present in culture (see section 2.3.4, Fig. 2.10). We probed the same sample as in Fig. 2.8 with the antibody against Na\textsubscript{v}1.8 and found that in this condition Na\textsubscript{v}1.8 was clearly associated both with the lipid raft (lane 2) and the non lipid raft (lane 8) fractions (Fig. 2.9 A, B). Blot in Fig. 2.9 is representative of n=2 experiments. The predicted sizes of these bands (as determined by Rf measures) were 226 kDa (band lane2), 246 kDa and 285 kDa (bands in lane 8).

\textbf{Figure 2.9} Na\textsubscript{v}1.8 is associated with the lipid raft fraction and the soluble fraction in DRG neurons \textit{in vitro}. A) Same fractions as in Fig. 2.8 were analysed by western blotting with an antibody against Na\textsubscript{v}1.8. Different fractions are labelled from 1 to 9, with 1 being the top fraction and 9 the bottom fraction. M is protein marker. B) Shows the bands intesity of Na\textsubscript{v}1.8 bands, plotted against the fraction number, of the blot shown in A.
Investigation of Na\textsubscript{v}1.8 sub-cellular localisation and association with lipid rafts by imaging techniques.

We further explored Na\textsubscript{v}1.8 association with lipid rafts by using microscopy techniques. We first determined the sub-cellular localisation of Na\textsubscript{v}1.8 in DRG neurons and then correlated it to the localisation of lipid rafts, by visualising GM1. Also, we analysed the localisation of other protein of interests: p11, TrkA, Flotillin-1 and Caveolin-2.

2.3.4 Na\textsubscript{v}1.8 sub-cellular localisation \textit{in vitro}.

We first analysed the sub-cellular distribution of Na\textsubscript{v}1.8 in DRG neurons \textit{in vitro} by immunocytochemistry. DRG neurons, once plated, exhibit the remarkable feature to extend neurites from the cell body, if growth factors are supplemented. The great majority of Na\textsubscript{v}1.8 is known to be expressed in small diameter neurons. Nevertheless, a small percentage of medium and large diameter neurons have been found to express the channel (Djouhri \textit{et al.}, 2003a). After 2 DIV, DRG neurons already show an extensive neurite outgrowth and we found that Na\textsubscript{v}1.8 displays a dual pattern of localisation. In small diameter neurons (< 25 µm), most likely to be nociceptors, Na\textsubscript{v}1.8 is found both at the level of the cell body and along the neurites in a clustered fashion (Mean length of the clusters ± SEM = 3.56 ± 0.37 µm; n=3; Total clusters counted = 255; \textbf{Fig. 2.10 A, B}). In large diameter neurons (> 25 µm), Na\textsubscript{v}1.8 localises at the cell body and along the neurites as large patches or in an evenly distributed fashion (Mean length of the patches ± SEM = 11.76 ± 1.55 µm; n=3; Total clusters counted = 194; \textbf{Fig. 2.10 C, D}). We also analysed the sub-cellular distribution of Na\textsubscript{v}1.8 after 4 DIV. Similarly to what we found after 2 DIV, Na\textsubscript{v}1.8 clusters on the neurites of small diameter neurons (\textbf{Fig. 2.11 A, B}), and show an even distribution along the neurites of large diameter neurons (\textbf{Fig. 2.11 C, D}).
Figure 2.10) Sub-cellular localisation of Na\textsubscript{v}1.8 in DRG neurons at 2DIV. A) Endogenous Na\textsubscript{v}1.8 is localised at the cell soma and in clusters along the neurites of a small diameter neuron. B) Same neuron as in A is shown here, superimposed on the phase contrast image to show the cell soma and the full extent of neurites. C) Endogenous Na\textsubscript{v}1.8 is localised at the cell soma of a large diameter neuron. Na\textsubscript{v}1.8 is distributed along the neurites in large patches. D) Same neuron as in C is shown here, superimposed on the phase contrast image to show the cell soma and the full extent of neurites. Scale bars are 20 µm.
Figure 2.11) **Sub-cellular localisation of Na\textsubscript{v}1.8 in DRG neurons at 4DIV.** A) Endogenous Na\textsubscript{v}1.8 is localised at the cell soma and in clusters along the neurites of a small diameter neuron. B) Same neuron as in A is shown here, superimposed on the phase contrast image to show the cell soma and the full extent of neurites. C) Endogenous Na\textsubscript{v}1.8 is localised at the cell soma of a large diameter neuron. Na\textsubscript{v}1.8 is distributed along the neurites in large patches. D) Same neuron as in C is shown here, superimposed on the phase contrast image to show the cell soma and the full extent of neurites. Scale bars are 20 µm.
To further confirm Na\textsubscript{v}1.8 sub-cellular distribution we performed a double immunocytochemistry between Na\textsubscript{v}1.8, Peripherin and NF200 after 2DIV. Peripherin is a cytoskeletal protein, specifically a sub-unit of class III intermediate filaments, and is widely used as a marker for small diameter, unmyelinated fibres (Goldstein et al., 1991). In contrast, NF200, which is a sub-unit of intermediate filaments IV, is a common marker of large diameter neurons (Perry et al., 1991). Similarly to what we found previously, in small diameter neurons, expressing Peripherin, Na\textsubscript{v}1.8 is localised along the neurites in distinguishable puncta (Fig. 2.12 A, B, C). On the other hand, in large diameter neurons, expressing NF200, Na\textsubscript{v}1.8 is evenly distributed along the neurites (Fig. 2.12 D, E, F).

![Figure 2.12](image)

**Figure 2.12** Sub-cellular localisation of Na\textsubscript{v}1.8 in small and large diameter DRG neurons. A) In small diameter neurons, positive for Peripherin, Na\textsubscript{v}1.8 is localised at the cell soma and in clusters along the neurites. B) Same neuron as in A is shown here, with Peripherin signal omitted. C) Same neuron as in A is shown here, superimposed on the phase contrast image. D) In large diameter neurons, expressing NF200, endogenous Na\textsubscript{v}1.8 is localised at the cell soma and distributed along the neurites in large patches. E) Same neuron as in D is shown here, with NF200 omitted, to highlight the even distribution of Na\textsubscript{v}1.8 along the neurites. F) Same neuron as in D is shown here, superimposed on the phase contrast image. Scale bars are 20 µm.
2.3.5 Na\textsubscript{v}1.8 sub-cellular localisation \textit{ex vivo}.

We thought to investigate Na\textsubscript{v}1.8 distribution along the axons of small diameter, unmyelinated fibres \textit{ex-vivo}. The sciatic nerve contains a heterogenous mix of axons which originate from different sources: DRG neurons and motor neurons. Na\textsubscript{v}1.8 is mostly expressed in unmyelinated fibres originating from the DRG neurons. Myelinated fibres can be easily identified by their morphology (myelin envelope and nodes of Ranvier) in a preparation of teased sciatic nerve fibres. On the other hand, small unmyelinated fibres due to their small dimension and lack of myelin show a low contrast in white light illumination. We have develeped a protocol to separate and visualise unmyelinated axons in the sciatic nerve. In our preparation, both myelinated and unmyelinated fibres are present. In phase contrast, myelinated fibres are identified as large axons (Fig. 2.13 A, *) with nodes of Ranvier (Fig.2.13 A, arrow heads), while unmyelinated axons are smaller fibres which display a low contrast. When the preparation is processed by immunofluorescence against peripherin (Fig. 2.12 B), bundles of unmyelinated fibres (Fig. 2.13 C, #) and single axons (Fig.2.13 C, arrow) can be readily identified.
Figure 2.13) Visualisation of small diameter fibres ex vivo. A) Shows a preparation of teased fibres from sciatic nerve, imaged by phase contrast. Large myelinated fibres (*) with nodes of Ranvier (arrow heads) are present with unmyelinated fibres, associated in bundles (#) and as single axons (arrow). B) Shows the Peripherin positive, unmyelinated fibres, present in the sample. C) Phase contrast image and immunofluorescence against Peripherin are merged in this image to show unmyelinated axons present as a bundle (#) and as single axons (arrow). Framed area are also shown at higher magnification. Scale bars are 20 µm.

Since this method allowed us to efficiently separate and visualise unmyelinated fibres, we used this technique to investigate Na\(_{v}1.8\) distribution along these fibres ex vivo. In small diameter fibres, identified both by morphology (Fig. 2.14 A, arrow) and by the expression of peripherin (Fig. 2.14 D), Na\(_{v}1.8\) showed a clustered distribution (Fig. 2.14 B, E), similar to what we found in an in vitro preparation.

Figure 2.14) Visualisation of Na\(_{v}1.8\) clusters along small diameter fibres ex vivo. A) Shows a preparation of teased fibres from sciatic nerve. Arrow indicates a small diameter fibre, identified by morphology. B) Shows Na\(_{v}1.8\) immunoreactivy along the unmyelinated axons,
distributed in clusters. C) Shows the phase contrast image and the immunofluorescence superimposed. D) Shows a preparation of teased fibres from sciatic nerve. Small diameter fibre, in this case, is identified by Peripherin staining. E) Shows Na\(_v\)1.8 immunoreactivity along the unmyelinated axons, distributed in clusters. C) Shows the Peripherin and Na\(_v\)1.8 immunofluorescences superimposed. Scale bars are 20 µm.

### 2.3.6 Ganglioside GM1 visualisation and Na\(_v\)1.8 immuno-localisation in vitro.

GM1 ganglioside is highly enriched in lipid rafts and is considered a standard marker of these micro-domains (Harder et al., 1998). To investigate the association between Na\(_v\)1.8 and lipid rafts in vitro we have visualised GM1 and localised Na\(_v\)1.8 by immunocytochemistry. CTB is a pentameric protein which directly binds GM1 on the cell surface. We used biotinylated CTB and a fluorescent derivate of Streptavidin to visualise GM1 on the neuronal plasmamembrane. We found that 77.3% ± 4.4 of DRG neurons expressed GM1 ganglioside. Also, we found that 54.6% ± 8.0 of DRG neurons expressed GM1 ganglioside are positive for Na\(_v\)1.8 immunoreactivity and that 82.3% ± 12.2 of DRG neurons that expressed Na\(_v\)1.8 were positive for GM1 ganglioside labelling. At the sub-cellular level we have found that GM1 is present as puncta on the cell surface of the cell body and along the axons of DRG neurons after 2 DIV (Fig. 2.15 A, B, C). Absence of background staining due to Streptavidin-488 was demonstrated by performing the staining with omission of CTB (Fig. 2.15 D). At the level of the cell bodies Na\(_v\)1.8 and GM1, did not show a clear co-localisation. At high magnification is possible to distinguish clear puncta for GM1, but Na\(_v\)1.8 immunoreactivity is evenly distributed and occasionally aggregates in brighter patches (Fig. 2.15 A). However, along the neurites, GM1 and Na\(_v\)1.8 show an interesting pattern of association. We found that the great majority of Na\(_v\)1.8 clusters co-localised with clusters of GM1 (Fig. 2.15 B, C, arrows), but not all GM1 clusters were positive for Na\(_v\)1.8 (84.6% ± 3.6 of Na\(_v\)1.8 clusters positive for GM1 from eleven different neurons; Total number of clusters = 182). Even though some of the clusters were associated with knobs along the axons, it is worth to note that both the clusters for Na\(_v\)1.8 and GM1 could be readily visualised in portions of the neurites with a linear morphology in the absence of knobs, varicosities, growth cones and branches (Fig. 2.15 C).
Figure 2.15) Association between Na\textsubscript{v}1.8 and GM1 along the neurites of DRG neurons. A) At the level of the cell body Na\textsubscript{v}1.8 and GM1 do not show clear co-localisation. The framed area is shown at higher magnification. B) Along the neurites the clusters of Na\textsubscript{v}1.8 co-localise with GM1. The framed area is shown at higher magnification; Two neurites crossing each other are imaged. Regions of co-localisation between Na\textsubscript{v}1.8 and GM1 are indicated by arrows. C) A linear neurite is shown, with clusters of Na\textsubscript{v}1.8 co-localising with GM1 indicated by arrows. D)
shows that Streptavidin-488 does not bind to the cells (identified by staining nuclei with Hoechst). Scale bars are 20 µm.

We also studied the pattern of association between Na\(_v\)1.8 and GM1 in small and large diameter neurons. Small neurons showed, along the axons, co-localisation between the clusters of Na\(_v\)1.8 and GM1 (Fig. 2.16 A, arrows); in large diameter neurons, even though occasional co-localisation occurred, this association was less evident (Fig. 2.16 B, dashed arrow).

**Figure 2.16** Different degree of association between Na\(_v\)1.8 and GM1 along the neurites in small and large diameter neurons. A) GM1 and Na\(_v\)1.8 co-localise in a small diameter neuron. The framed area is magnified to show regions of co-localisation indicated by arrows. B) GM1 and Na\(_v\)1.8 are do not co-localise in a large diameter neuron. The framed area is magnified to show a region lacking co-localisation indicated by a dashed arrow. Scale bars are 20 µm.
2.3.7 Ganglioside GM1 visualisation and Na\textsubscript{v}1.8-DsRed2 in vitro.

The techniques involved so far to localise endogenous Na\textsubscript{v}1.8 have been based on a specific antibody against this channel and on fixed cells. We sought to confirm Na\textsubscript{v}1.8 sub-cellular localisation and association with lipid rafts by using a fluorescent version of Na\textsubscript{v}1.8. We cloned the fluorescent tag DsRed2 to the C-terminus of Na\textsubscript{v}1.8. DsRed2 is a red fluorescent protein, derived from the coral reef *Discosoma Striata*. By mean of microporation we delivered exogenous DNA into the DRG neurons and monitored DsRed2 fluorescence. Primary neurons, and in particular DRG neurons, are notoriously hard cells to transfect. We achieved around 20% transfection efficiency with DsRed2 alone, whose expression was readily observed one day after transfection (Fig. 2.17 A, B). DsRed2 didn’t show any clear sign of sub-cellular targeting/compartmentalisation and was evenly distributed in the cell cytoplasm along the neurites (Fig. 2.17 B).

![Figure 2.17](image)

**Figure 2.17** DsRed2 expression in live DRG neurons. A) Shows a low magnification image of live DRG neurons expressing red fluorescent protein DsRed2. The fluorescent signal is merged with the phase contrast image to visualise the transfection efficiency B) Shows DsRed2 fluorescence only; the framed region is magnified and shows a neuron expressing DsRed2, which is evenly distributed along the axons. Scale bar is 100 µm.
Na\textsubscript{v}1.8-DsRed2 proved to be a challenging construct to be delivered and/or expressed. Nevertheless, we managed to express it in a few neurons, in separate independent experiments, and to monitor Na\textsubscript{v}1.8-DsRed2 sub-cellular localisation by live cell imaging. Remarkably, we found that Na\textsubscript{v}1.8-DsRed2 showed a clustered localisation in live cells along the axons, similar to what we described by using antibody based technique on fixed cells (Fig. 2.18 A, arrows). At the level of the cell body, Na\textsubscript{v}1.8-DsRed2 was highly accumulated intracellularly (Fig. 2.18 A, arrow head), most likely in the endoplasmic reticulum which is known to contain the majority of the channel in the neurons expressing it (Djouhri et al., 2003a; Okuse et al., 2002; Zhang et al., 2008).
Figure 2.18) \( \text{Na}_V\text{1.8-DsRed2} \) is clustered along the axons of live DRG neurons. A) Shows \( \text{Na}_V\text{1.8-DsRed2} \) distributed in clusters (arrows) along the neurite of a neuron. B) The fluorescence image is superimposed on the phase contrast image, to show the integrity of the cell process. C) The discontinuous distribution of \( \text{Na}_V\text{1.8-DsRed2} \) along the axons has been mapped by quantifying pixels intensity along the neurite by using ImageJ software. The pixels quantified are represented in the inset as a yellow line superimposed on the cell process. Clusters are represented as peaks of fluorescence in the intensity fluorescence plot. Scale bar is 20 \( \mu\text{m} \).
We also used Na\textsubscript{1.8}-DsRed2 construct to further investigate the co-localisation of Na\textsubscript{1.8} with lipid rafts along the neurites. We have electroporated the DRG neurons with the plasmid expressing Na\textsubscript{1.8}-DsRed2 and have performed the CTB staining to visualise the GM1. We have found that the clusters of Na\textsubscript{1.8}-DsRed2 co-localised with GM1 (Fig. 2.19), confirming the previous finding, and showing that the addition of a fluorescent tag to Na\textsubscript{1.8} neither disrupts its ability to cluster nor to co-localise with GM1.

![Image](GM1_Na1.8-DsRed2_Merge)

Figure 2.19) Na\textsubscript{1.8}-DsRed2 co-localises with GM1 along the neurites of cultured DRG neurons. The co-localisation between Na\textsubscript{1.8}-DsRed2 and GM1 is shown as a yellow signal in the merged picture. Scale bars is 20 µm.

2.3.8 Effect of NGF on the association between Na\textsubscript{1.8} and GM1 in vitro.

NGF is a pleiotropic cytokine involved in several biological processes. Specifically related to sensory neurons, it is a crucial factor for cell survival. In fact, in the absence of NGF DRG neurons expressing TrkA and p75 undergo apoptosis. It also promotes axonal regeneration and neurite outgrowth in vitro (Lindsay and Harmar, 1989). NGF modulates Na\textsubscript{1.8} electrophysiological properite in the short term, through the PKCe pathway, and in the long term by increasing its expression (Okuse, 2007). Given the multiple roles of NGF we asked whether it also regulates the pattern of association between Na\textsubscript{1.8} and lipid rafts. We cultured 5000 DRG neurons in the presence of 50 ng/ml NGF or without NGF plus an antibody against NGF, to minimise the effect of circulating NGF released from non neuronal cells. After 2 DIV we found, as expected, that the absolute number of neuronal cells was reduced in the
sample without NGF (total count of neuronal cells, based on morphology, from 40 random fields of view per sample = 322 neurons in NGF treated samples, 181 neurons in samples without NGF). Also, the percentage of small diameter neurons, identified by Peripherin staining, was reduced in the samples without NGF (from 40 random fields of view per sample: 66.1% Peripherin positive neurons in NGF treated sample vs. 45.3% Peripherin positive neurons in samples without NGF). In addition, NGF treated sample showed extensive neurite outgrowth, while the depletion of NGF determined a less developed neurite network (Fig. 2.20 A). In the population of cells, which still presented neurites and expressed \( NaV1.8 \) after the depletion of NGF, the association between \( NaV1.8 \) and lipid raft was impaired. In control cells, along the neurites, \( NaV1.8 \) and GM1 co-localised; On the contrary, in the samples without NGF, \( NaV1.8 \) and GM1 did not show a clear co-localisation (Fig. 2.20 B).

**Figure 2.20** Effect of NGF on the association between \( NaV1.8 \) and GM1. A) Peripherin stained neurons are shown (note that the saturated cell body is due to allow visualisation of the neurites). Neuron treated with NGF shows longer neurites than neurons not treated with NGF. B) In NGF treated samples \( NaV1.8 \) and GM1 co-localise on the neurites, while in samples depleted from NGF, the association between \( NaV1.8 \) and GM1 was not evident. Framed region
are magnified to show co-localisation (arrows) and lack of co-localisation (dashed arrow). Scale bars are 20 µm.

### 2.3.9 \(\text{Na}_\text{V}1.8\) and TrkA immuno-localisation *in vitro*.

TrkA is a transmembrane tyrosine kinase receptor, which binds to NGF. TrkA is expressed in DRG neurons and has been reported to translocate into lipid rafts when it binds to NGF, to initiate intracellular pathways (Limpert *et al.*, 2007). One of the well established roles of lipid rafts is to act as platforms on the membrane, where they regulate the physical association between signaling proteins and their targets. Since, 1) we found that \(\text{Na}_\text{V}1.8\) co-localises with lipid raft marker GM1, 2) \(\text{Na}_\text{V}1.8\) is known to be modulated by NGF, through TrkA (Okuse, 2007), and 3) TrkA itself partitions to lipid rafts (Limpert *et al.*, 2007), we studied the distribution of these proteins in DRG neurons. We used immunocytochemistry after 2DIV, to visualise the proteins of interest. For the purpose of this analysis we focused our interest on cells that were both expressing \(\text{Na}_\text{V}1.8\) and TrkA. We have found that TrkA was distributed on the cell body and along the neurites, and that a high percentage of \(\text{Na}_\text{V}1.8\) clusters co-localised with TrkA (85.2% ± 8.6 of \(\text{Na}_\text{V}1.8\) clusters positive for TrkA from four different neurons; Total number of clusters = 67) (Fig. 2.21 A, B, arrows).
2.3.10 \( \text{Nav} \text{1.8, Flotillin-1-Dronpa and Caveolin-2-Dronpa in vitro.} \)

Lipid rafts are heterogeneous microdomains. Traditionally, we can refer to two distinct types of rafts: planar and caveola-type. Planar lipid rafts lack a distinguishable morphology, while caveola-type are identified as 50-100 nm diameters flask shaped invagination of the cell membrane, which can be observed by electronic microscopy. Apart from morphological features, planar lipid rafts and caveola-type can be distinguished by protein markers. Flotillin-1 has been shown to be enriched in planar lipid rafts (Langhorst et al., 2005), while Caveolin-2 is present in caveola-type lipid rafts (Parton and Simons, 2007). Since valid antibodies against these proteins for immunocytochemistry are unavailable, we created fluorescent forms of Flotillin-1 and Caveolin-2. We delivered the constructs into DRG neurons to monitor their expression and localisation, related to endogenous \( \text{Na}_\text{v} \text{1.8}. \) We tagged the C-terminus of Flotillin-1 and Caveolin-2 with Dronpa. Dronpa is a photochromic protein derived from GFP. Similarly to GFP, it emits fluorescence in the green region of the electromagnetic spectrum when excited with blue light, and compared to GFP it is brighter and shows the ability to undergo rounds of activation and de-activation (Ando et al., 2004; Wilmann et al., 2006). We found that Flotillin-1-Dronpa is localised both in the soma and along the neurites. This construct was evenly distributed, with brighter puncta of fluorescence along the axons (Fig. 2.21) Co-localisation between \( \text{Na}_\text{v} \text{1.8 and TrkA.} \) A) Shows a neuron expressing both \( \text{Na}_\text{v} \text{1.8 and TrkA.} \) The framed region is magnified to show co-localisation between the proteins (arrows). B) Shows another example of co-localisation between \( \text{Na}_\text{v} \text{1.8 clusters and TrkA along a neurite.} \) The framed region is magnified to show co-localisation between the proteins (arrows). Scale bars are 20 µm.
Interestingly, when we correlated the signal of Flotillin-1-Dronpa to the localisation of endogenous Na$_v$1.8 (visualised by immunocytochemistry), we found that a high percentage of Na$_v$1.8 clusters co-localises with the brighter spots of Flotillin-1-Dronpa along the axons (Fig. 2.22 A, B, arrows) (70.8% of Na$_v$1.8 clusters positive for Flotillin-1-Dronpa; Total number of clusters counted = 24).

**Figure 2.22** Co-localisation between Flotillin-1-Dronpa and Na$_v$1.8. A) Shows a neuron expressing Flotillin-1-Dronpa. The framed area is magnified to highlight the brighter patches of
Flotillin-1-Dronpa (arrowheads). The same magnified area is shown in the merged image, to show co-localisation with endogenous Na\textsubscript{v}1.8 clusters (arrows). B) Shows another example of co-localisation (arrows) between Na\textsubscript{v}1.8 and patches of Flotillin-1-Dronpa characterised by high fluorescence along a neurite. Scale bars are 20 µm.

Caveolin-2-Dronpa showed a distinct clustered organisation along the neurites (Fig. 2.23 A, B), even though a small percentage of neurons characterised by a very high expression of the construct displayed Caveolin-2-Dronpa distributed along the entire length of the neurite (Fig. 2.23 C). In neurons with a clustered distribution of Caveolin-2-Dronpa we found that few of Na\textsubscript{v}1.8 clusters were associated with Caveolin-2-Dronpa puncta, with the majority being excluded from it (29.4% of Na\textsubscript{v}1.8 clusters positive for Caveolin-2-Dronpa, Total number of clusters counted = 43) (Fig. 2.23 B). In conclusion, endogenous Na\textsubscript{v}1.8 showed a higher percentage of co-localisation with Flotillin-1-Dronpa, compared to Caveolin-2-Dronpa (70.8% vs. 29.4% *, respectively. * = p <0.05, Fisher’s exact test).
Figure 2.23) Co-localisation between Caveolin-2-Dronpa and Naᵥ1.8. A) Shows a neuron expressing Caveolin-2-Dronpa. The framed region is magnified to show Caveolin-2-Dronpa puncta. B) Shows a neuron expressing Caveolin-2-Dronpa. Naᵥ1.8 is visualised by immunofluorescence. The framed regions are magnified to show Caveolin-2-Dronpa puncta co-localising (arrow) and not co-localising (dashed arrow) with Naᵥ1.8. C) Show a representative neuron characterised by a high expression of Caveolin2-Dronpa construct. Scale bars are 20 µm.
2.3.11 P11 and lipid rafts *in vitro*.

P11 is the light chain sub-unit of Annexin II. P11 was discovered in a genetic screening to identify novel interactors of Na$_V$1.8 (Malik-Hall *et al.*, 2003). Subsequent studies demonstrated that P11 binds the N-terminus of Na$_V$1.8 and plays a key role in its trafficking, since p11 expression is necessary but not sufficient for Na$_V$1.8 functional expression on the cell membrane. Also, the complex Annexin II-p11 has been reported to partition into lipid rafts (Oliferenko *et al.*, 1999; Rescher and Gerke, 2004, 2008). Given this evidence, we investigated its sub-cellular localisation in DRG neurons. By using immunocytochemistry on fixed cells we found that p11 mirrors Na$_V$1.8 sub-cellular localisation. Indeed, it is expressed on the cell bodies and along the axons in clusters, where it co-localises with GM1 clusters (Fig. 2.24 A, B, arrows). This finding indirectly supports Na$_V$1.8 sub-cellular localisation and partition into lipid rafts, given the evidence that p11 binds to channel and it has the ability to move into lipid rafts.
Figure 2.24) Co-localisation between p11 and GM1. A) Shows a neuron expressing p11. The framed area is magnified to show co-localisation between p11 and GM1 (arrows). B) Shows another example of co-localisation between p11 clusters and GM1 (arrows) along a neurite. Scale bars are 20 μm.
2.4 Discussion

2.4.1 Na\textsubscript{v}1.8 association with lipid rafts: biochemistry techniques.

Lipid rafts were firstly discovered thanks to their biophysical characteristics, which are different from the characteristics of the bulk membrane. Indeed, they are enriched in cholesterol, sphingolipids and lipids with saturated fatty acid chains. They exist in a liquid ordered phase and are resistant to non-ionic detergent lysis at 4°C. This feature was also proved to be a valuable property that can be used to separate lipid rafts from the rest of the membrane. A classic method to isolate lipid rafts is to lyse the tissue/cells of interest at 4°C, in a lysis buffer containing a non-ionic detergent. Since lipid rafts are resistant to lysis, during this process, they will be spared from the detergent action while the bulk membrane will be lysed. A second step is necessary to biochemically isolate rafts: a centrifugation on a density gradient. Typically, the membrane lysate, which contains intact rafts associated with raft proteins, and the lysed bulk membrane are mixed with an agent (e.g. sucrose or iodixanol) and overlaid with fractions, containing less amount of the chosen agent. During the centrifugation step, lipid rafts, due to their low density, will migrate towards the top of the centrifuge tube, while solubilised lipids and non-raft proteins will remain at the bottom of the tube. A common strategy to ascertain if a protein partitions into lipid rafts is to test whether it can be recovered from the low density, buoyant, detergent-resistant membrane fraction. Because the purpose of the ultracentrifugation on a density gradient is to separate rafts (and rafts associated proteins) from the rest of the membrane, after the centrifugation step usually the same volume, rather than the same amount of protein, from the different fractions is subjected to SDS-PAGE. This allows assessing the relative degree of separation between lipid rafts and solubilised membranes. The absence of raft markers between the buoyant fraction and the soluble fraction provides the evidence that rafts were efficiently separated.

In our experiments we have found that in DRGs, sciatic nerve and DRG neurons \textit{in vitro}, lipid raft can be efficiently isolated from the rest of the membrane. The detergent of choice in our case was Triton X-100. Many different detergents have been reported in literature to extract lipid rafts, including CHAPS, Brij96, Brij98, and Lubrol WX (Pike, 2004). We choose Triton X-100 because this detergent, compared to the others listed before, has the highest detergent power.
and is the most stringent (Chamberlain, 2004; Schuck et al., 2003). It is also the most commonly used detergent in biochemical preparations of membrane rafts (Bessho et al., 2007; Bruses et al., 2001; Davies et al., 2006; Martens et al., 2001; Morenilla-Palao et al., 2009; Simons and Ikonen, 1997). The potential inconvenient, by using less harsh detergents, is to only partially digest the cell membrane. In this scenario, non-raft portions of the membrane could be under digested, contaminate the raft fraction and lead to misinterpretation of the results.

We have used different markers to define the raft and non raft fractions of the cell. Transferrin receptor is a recognised marker of the soluble portion of the membrane and does not display raft properties (Harder et al., 1998). On the other hand, lipid rafts can be identified by protein and lipid markers. We exploited the known association of Flotillin-1 with rafts (Langhorst et al., 2005) and the high abundance of GM1 (Brown, 1998; Parton, 1994) in these domains to define lipid rafts in our biochemical preparations. Since the association of a protein with the detergent resistant portion of the membrane is proved to be a very powerful indication of raft association, we looked whether Na\textsubscript{v}1.8 co-migrates with Flotillin-1 and/or GM1. We efficiently separated rafts from different sources: DRG cell bodies and sciatic nerve \textit{ex vivo} and DRG neurons \textit{in vitro}. In DRG cell bodies and sciatic nerve we found similar pattern of raft extraction. In both tissues, Transferrin receptor was retained in the bottom fractions, which indicates that the detergent effectively solubilised the non-raft portion of the membrane. Therefore we defined the fractions containing Transferrin receptor as non-lipid rafts. The partition of GM1, which is highly enriched in lipid rafts, in the top fractions, suggests that in our preparation lipid rafts are efficiently extracted and separated from the soluble portion of the tissues. Flotillin-1 profiles also supported this. In fact, Flotillin-1 showed both in DRG cell bodies and in the sciatic nerve samples, to be associated with two pools; one is floating in the top layers and the second one, separated from the top one, associated to the non-raft portion of the membrane. The existence of these two pools could reflect either Flotillin-1 physiological trafficking between the membrane and the intracellular compartments or an over-digestion due to the detergent. Flotillin-1 is known to exist in two pools: one on the membrane, fully raft associated, and one retained in the intracellular compartments (Langhorst et al., 2005). During the detergent extraction, only the pool of Flotillin-1 associated with rafts is spared from lysis, while the intracellular pool, not associated with rafts is subjected to detergent mediated degradation. For this reason, only the raft associated Flotillin-1 will float with rafts and will be
recovered from the top layers of the density gradient. On the other hand, the intracellular pool, not raft associated, will be restricted to the soluble fraction. Alternatively, the soluble pool of Flotillin-1 could arise from an excessive lysis of the membrane, which could impair raft integrity, and Flotillin-1 migration towards the top of the gradient. This hypothesis is not likely since GM1 is highly enriched in the top fractions. If over digestion occurred, we would have expected also an enrichment of GM1 in the bottom fractions. Therefore, in our preparations we can conclude that the floating detergent resistant membranes are *bona fide* lipid rafts and that the soluble fraction is not contaminated with over-digested lipid rafts.

In DRGs and sciatic nerve we found a different pattern of association of Na\(_V\)1.8 with lipid rafts. Interestingly, at the level of the cell bodies the great majority of Na\(_V\)1.8 is in the soluble fraction (non-raft). At the level of the sciatic nerve, we found a completely opposite scenario: all Na\(_V\)1.8 is raft associated. DRGs contain the cell bodies of sensory neurons, glial cells, and short axonal processes which arise from the cell bodies. It is known that Na\(_V\)1.8 at the level of DRG is selectively expressed in the neuronal population, and more specifically, mostly in the small diameter, unmyelinated, nociceptors (Djouhri *et al.*, 2003a). Also, the great majority of Na\(_V\)1.8 is retained intracellularly (Zhang *et al.*, 2008) and it is not functional in terms of cell excitability. The biological meaning of this intracellular retention is not fully understood. One possibility is that a reservoir of the channel is stored internally in the ER, ready to be trafficked to the membrane, upon excitatory stimuli. In fact, inflammatory compounds determine a rapid increase of TTX-r currents mediated by Na\(_V\)1.8, which is consistent with a rapid traffic of the channel rather than an increase in the transcription rate, which could account for increased TTX-r currents at later stages (Liu *et al.*, 2010). Na\(_V\)1.8 association with the soluble fraction in DRG neurons that we have reported perfectly matches the known evidence that this channel is mostly retained in the ER. In fact, during raft extraction, the bulk plasma-membrane and the ER, which devoid of rafts (Simons and Ikonen, 1997), are lysed. In this condition, Na\(_V\)1.8 not associated with rafts, will not float to the top of the density gradient and will be recovered from the soluble fraction. By extending the exposure time of our blot, we found that a minor proportion of the channel is indeed associated with rafts. This pool can be of different origins; a potential source could be from the minority of the channel which is inserted into the membrane, and be raft associated. Another source may be the pool of channels which is trafficked to the axons and exposed onto the membrane where it underlies sodium currents during action potential propagation. The other source we used to investigate Na\(_V\)1.8-raft
association was the sciatic nerve and we found that Na\textsubscript{v}1.8 is fully raft associated in this tissue. Sciatic nerve is a spinal nerve which contains efferent axons of motor-neurons and afferent axons of sensory neurons, plus Schwann cells which contribute to the myelination of the fibres. In this tissue Na\textsubscript{v}1.8 is expressed along the axons of unmyelinated DRG neurons where it contributes to the propagation of action potentials, originated in the peripheral tissues, towards the spinal cord by encoding TTX-r currents (Jeftinija, 1994b; Quasthoff \textit{et al}., 1995). Also, Na\textsubscript{v}1.8 must be inserted in the membrane to be able to underlie sodium currents and evoke action potentials (Okuse \textit{et al}., 2002; Renganathan \textit{et al}., 2001). Hence, it is assumed that functional Na\textsubscript{v}1.8 is localised on the plasma-membrane of axons \textit{in vivo}. It is worth to mention that sciatic nerve may also contain intracellular vesicles, which traffic the channel towards its final destination. The biosynthesis of lipid rafts is still unknown and it is hypothesised the intracellular rafts may exist in the late trans-Golgi network apparatus (van Meer and Sprong, 2004), where both cholesterol and sphingolipids are present (Ikonen, 2001; van Meer \textit{et al}., 2008). Given this evidence, a fraction of raft associated Na\textsubscript{v}1.8 from this source cannot be excluded \textit{a priori}. Nevertheless, it is important to stress that our result clearly shows that in the sciatic nerve total Na\textsubscript{v}1.8 is associated with lipid rafts. This means that the functional, membrane bound, Na\textsubscript{v}1.8 pool which mediates action potentials is localised on membrane rafts and is laterally excluded from the non-lipid raft, bulk portion, of the plasma-membrane. Interestingly, we found Na\textsubscript{v}1.8 to be present in different sizes. Na\textsubscript{v}1.8 predicted size (as calculated by using the algorithm at http://expasy.org/tools/pi_tool.html) is 220 kDa, which could considerably vary due to glycosylation states (Goldin \textit{et al}., 2000; Schmidt and Catterall, 1987; Yu and Catterall, 2003). In our preparation, we found Na\textsubscript{v}1.8 as three different sizes: 201 kDa, 222 kDa and 247 kDa. The heaviest form could arise from a highly glycosylated form or from the splicing variant that has been reported to have a duplication of exons 12-14 (Akopian \textit{et al}., 1999a), while the lowest molecular weight could represent one of Na\textsubscript{v}1.8 shorter isoforms. In fact, one isoform has been reported to lack a portion of the first intracellular loop, which could account for the reduced apparent molecular weight (Dr. K. Okuse, personal communication). Also, we recovered Na\textsubscript{v}1.8 from lipid rafts being associated with two different fractions (lane 2 and 3 in Fig. 2.7). One hypothesis to explain the different motility of the channel is to consider raft heterogeneity. Rafts do share the same biophysical characteristics, nevertheless they can display subtle differences in terms of density (and when subjected to centrifugation they float to different position (Roper \textit{et al}., 2000; Schuck \textit{et al}., 2003), protein markers (e.g. planar vs. caveola-type) and detergent extraction (Pike, 2004). In
our hands, Na\textsubscript{v}1.8 recovered from lanes 2 and 3 could account for pools of the channel associated with rafts that are both extracted by Triton X-100 but characterised by slightly different density, which leads to a different migration pattern. In this scenario, the highly glycosylated form of Na\textsubscript{v}1.8 would associate with the denser rafts while the lower molecular weight forms with the lower density rafts. A study supporting a correlation between glycosylation state and raft trafficking has already been reported. This report focused on TrpM8, a cation channel expressed in DRG neurons which transduces cold stimuli into cell depolarisation that, in turn, triggers action potentials. TrpM8, similarly to Na\textsubscript{v}1.8, is a highly glycosylated protein. TrpM8 associates with rafts and it has been found that its glycosylation pattern correlates with the extent of raft trafficking. In particular, the highly glycosylated form is raft associated while the low glycosylation form tends to partition into the soluble fraction (Morenilla-Palao \textit{et al.}, 2009). Furthermore other studies have also identified glycosylation as a targeting signal determining the raft association of membrane proteins (Alfalah \textit{et al.}, 1999; Scheiffele \textit{et al.}, 1995). In conclusion, from fresh tissues we found that Na\textsubscript{v}1.8 raft association is different in DRGs and sciatic nerve. In DRGs cell bodies the majority of Na\textsubscript{v}1.8 is non-raft associated (most likely to be retained intracellularly) and along the axons in the sciatic nerve it is trafficked to lipid rafts. Also, different isoforms/glycosylation pattern could correlate to the targeting of these forms to different rafts.

We have also analysed Na\textsubscript{v}1.8 raft association in DRG neurons in culture. Once plated, DRG neurons display features that they express \textit{in vivo}. For this reason they are commonly exploited as a model to investigate DRG protein function (Gold \textit{et al.}, 1996a; Malin \textit{et al.}, 2007). The advantage of a DRG culture, compared to an \textit{ex vivo} source, is that the population of cells is virtually composed only by neuronal cells, as non-neuronal cells are not present due to the presence of Aphidicolin which inhibits their proliferation. We thought to determine if rafts could be extracted from DRG neurons \textit{in vitro} and whether Na\textsubscript{v}1.8 associates with them. Our lipid raft preparation, displaying floating fractions enriched in Flotillin-1 and GM1, clearly demonstrate that DRG neurons do contain raft domains and these can be efficiently extracted. Interestingly, Na\textsubscript{v}1.8 is present as two pools; one raft associated and one that is not. This result can be interpreted considering that, differently from the \textit{ex vivo} sources where it’s possible to anatomically separate DRG cell bodies from the axons, \textit{in vitro} both the neuronal cell bodies and the axons are present in culture. Given this condition, both intracellular and membrane bound Na\textsubscript{v}1.8 is present. Hence, the most likely sources of Na\textsubscript{v}1.8 are from the ER, which
appear in the soluble fraction in the biochemical extraction, and from lipid rafts. This latter pool is likely to be represented by the channels inserted into the membrane along the axons (see next section of the discussion, 2.4.2). Thus, we have found in vitro a situation that recapitulates what we have found ex vivo, by using DRGs and sciatic nerve separately: Na\textsubscript{v}1.8 associates with rafts along the axons and the soluble pool, non raft linked, is likely to be derived from the intracellular stores of the channel.

2.4.2 Na\textsubscript{v}1.8 sub-cellular localisation and association with lipid rafts: imaging techniques.

We explored Na\textsubscript{v}1.8 sub-cellular localisation and further confirmed its association with lipid rafts in DRG neurons by using imaging techniques.

Firstly, we investigated Na\textsubscript{v}1.8 localisation by immunocytochemistry in cultured DRG neurons. We have found an interesting pattern of localisation of Na\textsubscript{v}1.8, with a different distribution between small and large diameter neurons. In particular, in small diameter neurons, which we identified both by morphology and expression of Peripherin, Na\textsubscript{v}1.8 was expressed in clusters along the length of the axons. This distribution was evident for unmyelinated fibres, but not for large diameter neurons. In fact, these latter cells, which we identified by morphology and expression of NF200, showed Na\textsubscript{v}1.8 being either evenly distributed or associated in large patches along the axons. Interestingly, this dual pattern was already evident after 2 DIV and conserved up to 4 DIV. This suggests that the clustered distribution of Na\textsubscript{v}1.8 in small diameter neurons most likely represents a terminal organization of the channel, already established after 2DIV, rather than a temporary distribution, due to the channel being trafficked to its final destination. In this preparation, the localisation of the channel which we identify by immunocytochemistry cannot be unequivocally determined, since we cannot distinguish between intracellular and membrane bound channels. DRG neurons, once plated, undergo a “regeneration” process and extend axons, if growth factors are supplemented (Campenot, 1977). Hence, the distribution of Na\textsubscript{v}1.8 could also be attributed to a dynamic process that happens in vitro, which does not occur in adult animals. Strongly supporting the evidence that Na\textsubscript{v}1.8 is present in clusters as a terminal organization, we found a clustered appearance of the channel along the unmyelinated axons in the sciatic nerve in vivo. It is also worth to
underline that the biochemical sciatic nerve lipid raft preparation showed that all Na\textsubscript{v}1.8 is raft associated. Hence, the Na\textsubscript{v}1.8 channels we found along the axons ex vivo in clusters are most likely exposed on the membrane. Thus, the clustered appearance along unmyelinated teased fibres confirms the distribution we found in vitro; this one mirrors a situation which is established in vivo, rather than being an artifactual localisation, due to regenerative processes in vitro. The meaning of this clustered distribution is unknown at the moment. The spatial localisation of ion channels and the electrophysiological properties of the channels themselves are of paramount importance in shaping the electric excitability of neuronal cells. In fact, compartmentalisation of ion channels defines the electric properties not only among different neurons, but also between different regions of the same neuron. The most evident example is supported by the nodes of Ranvier along the myelinated fibres which guarantee saltatory, high speed, conduction. In these structures sodium channels are clustered at high density and potassium channels are spatially segregated, and this contributes to the ability of the nodes to boost and regenerate action potentials (Kandel et al., 2000). The clustered appearance of Na\textsubscript{v}1.8 could have a functional role in terms of action potential propagation along the axons where this channel is expressed. As discussed before Na\textsubscript{v}1.8 plays a key role in action potential generation, by underlying the majority of sodium influx during the rising phase (see section 1.3.1). It is tempting to speculate that in unmyelinated fibres Na\textsubscript{v}1.8 clusters represent sites on the membrane where action potential can be actively generated. In the portion of membrane lacking Na\textsubscript{v}1.8 the electric signal would spread following passive cable properties, and before dissipating it could be re-generated at the sites of Na\textsubscript{v}1.8 clustering. In this hypothetical view, unmyelinated fibres would generate action potentials only in discrete portions of the membrane, like myelinated fibres, where action potentials are regenerated only at the nodes of Ranvier. In unmyelinated fibres we found that the clusters are a few µm apart, differently from myelinated fibres, where sodium clusters can be separated by hundreds/thousand of µm (Hille, 2001). It could be hypothesised that the close vicinity of the clusters we have found in our condition represents a distance short enough for the passively conducted depolarisation to not be dissipated and to be propagated from cluster to cluster, where it reaches a threshold to recruit Na\textsubscript{v}1.8, which actively further depolarises the membrane. Interestingly, classic studies support a way of action potential conduction in unmyelinated fibres similar to the one that we hypothesise. In fact, upon demyelination sodium channels redistribute along the unmyelinated region (England et al., 1990; Meiri et al., 1985; Waxman, 2006a). Electrophysiological analysis reported that in demyelinated axons (Lysophosphatidyl-choline method) action potential
conduction is restored before re-myelination occurs. In this condition conduction was found to be discontinuous and proceeded via “new foci of inward membrane current”. The authors hypothesise that these foci represent clusters of sodium channels that precede the formation of nodes of Ranvier and saltatory conduction, and allow action potential conduction in the absence of myelin (Smith et al., 1982). The biological meaning of clusters could be also attributed to the fact that clusters of NaV1.8 increase its local concentration, which could be needed to efficiently generate action potentials. In fact, it is has been demonstrated that high concentration of sodium channels is needed for an efficient action potential generation. Apart from the example discussed before about the nodes of Ranvier, where sodium channels are highly concentrated (> 1200/µm²) (Poliak and Peles, 2003), sodium channels also cluster at high concentration in the axonal initial segment (AIS). The AIS is an unmyelinated region of the axon, which is flanked by the cell body and the myelin wrap of the axon. AIS integrates the depolarisation propagating from the cell body and triggers an action potential which travels along the axon and back propagates in the soma (Lai and Jan, 2006). It has been demonstrated in pyramidal cells that a high concentration of sodium channels (approximately 50 fold greater than ion channels concentration at the level of the soma and dendrites - most likely NaV1.6) is needed to generate action potentials (Kole et al., 2008). Another example of clusters of sodium channel at high density has been reported by Engel et al. Remarkably, this study demonstrated that in the hippocampal mossy fibre pathway (which connect the dentate gyrus to the C3 region) sodium channels are at high concentration in the en passant boutons along the unmyelinated fibres (calculated in 2000 units per bouton). The authors conclude that the high density of sodium channels is needed to amplify action potential at the pre-synaptic sites and boost neurotransmitter release. Also, by computation modelling, it has been predicted that the clusters of sodium channels at the boutons along the axons influence the reliability and velocity of action potential propagation (Engel and Jonas, 2005). Very interestingly, a clustered distribution of sodium channels, similar to what we have reported, has also been described in the unmyelinated axons of animal model Aplysia californica (Johnston et al., 1996). In this invertebrate the distribution in clusters has been proposed to be more energetically efficient in terms of cell metabolism and action potential propagation. The authors predict that by clustering channels along the unmyelinated fibres, fewer channels are needed to conduct action potentials, compared to a scenario where sodium channels are evenly distributed. This would imply less channels being transcribed, translated and transported, with an overall reduction of energetic expenditure. In conclusion, since neuron
excitability is dependent on many factors, including ion channel sub-cellular localisation and local density, Na\textsubscript{v}1.8 clusters may be at the base of action potential generation and conduction and be important to shape the excitability of unmyelinated axons of nociceptors.

We next exploited cultured DRG neurons and imaging techniques to further investigate Na\textsubscript{v}1.8 raft association. Imaging techniques represent complementary methods to study lipid rafts and raft associated proteins, which support data obtained by conventional biochemistry techniques. Most of the detailed features of rafts nature have been, in fact, provided by using these optical methods. FRET allowed the direct visualisation of nano-clusters of GPI-anchored proteins. FRET is based on the energy transfer between a donor and an acceptor molecule. Since this phenomenon only occurs in the range of 1-10 nm is suited to study rafts dynamics. Analysis of these GPI-anchored proteins nano-clusters showed that they are immobile, cholesterol dependent and that GPI-anchored proteins follow a non random distribution (Goswami et al., 2008). By using FRET, Silvius et al. showed that at physiological temperature membrane lipids do partition into L\textsubscript{o} and L\textsubscript{d} phases (Silvius, 2003). Zacharias et al. showed that acylated, but not prenylated, GFP and YFP partition and cluster into lipid rafts in live MDCK cells. Notably, clustering was dependent on cholesterol presence and raft partition correlates with their extraction from DRMs (Zacharias et al., 2002). FRET was also used to investigate G proteins association with rafts (Abankwa and Vogel, 2007). In addition, FRET analysis gave us information about the diffusion kinetics of raft residing proteins. It was found that clustered Src in rafts are less motile that Src in the non raft portion of the membrane and in the cytoplasm (Lu et al., 2008). FLIM measures the average time that an electron spends in the excited state of a fluorochrome before relaxing back to the ground state. By FLIM microscopy the mechanism behind epidermal growth factor receptor (EGFR) signaling has been unveiled. It was found that EGFR co-localises at a nano-scale level with ganglioside GM1 (raft marker) but not with Transferrin receptor (non raft marker). Furthermore, it was found that heterogeneous resting small rafts are induced to coalesce upon EGF binding to the EGFR, leading to the formation of a signaling platform (Hofman et al., 2009; Hofman et al., 2008). AFM, which relies on the “scanning” of a surface with a mechanical probe controlled by a piezo-electric element, proved to be a valuable tool in studying artificial membranes (Nicolini et al., 2006). Rafts size has been determined in model membranes to be in the range of 20-100 nm (Yuan et al., 2002). In FCS microscopy the temporal fluorescence fluctuations, due to diffusion kinetics, of a single fluorescent molecule are monitored through a small detection volume. FCS was found to be a
powerful tool to discriminate between diffusion coefficients of raft markers (GM1 ganglioside) and non raft markers, both in live cells and model membranes. In this study it was found that cholesterol depletion plus cytoskeletal disruption altered GM1 motility, suggesting the existence of a raft population stabilized by interaction with intracellular elements (Bacia et al., 2004). A more recent study monitored rafts in live cells by FCS of raft fluorescent protein markers (GFP-Thy1, Lck1,12-GFP). It was demonstrated that in live cells rafts exist in the outer and inner leaflet of the membrane, that they are dependent on cholesterol and sphingolipids presence and their stability is crucial for Akt recruitment and signaling (Lasserre et al., 2008). STED allows to excite a small area (smaller than conventional confocal microscopy) and to lower the resolution limit. STED microscopy allowed single molecule tracking of raft markers (GM1 and GPI-anchored proteins) as well as non raft markers. The analysis of the traces and fluctuations in live cells revealed that unlike phosphoglycerolipids, sphingolipids and GPI-anchored proteins are transiently (10–20 ms) trapped in cholesterol-mediated molecular complexes, consistent with the idea of small dynamic rafts (Eggeling et al., 2009). Super resolution microscopy (resolution 3 nm) allowed the direct visualisation of raft enriched nano-clusters and that the formation of “hot-spots” on the cell surface triggers cell-adhesion. It was found that in quiescent cells, Integrin LFA-1 and GPI-anchored proteins pre-organize in separate nano-compartments forming “hot-spots” sites on the cell surface. Activation of LFA-1 by ligand binding drives the formation of larger supramolecular platforms that serve as nucleation sites for nascent cell adhesion (van Zanten et al., 2009). Fluorescence photoactivation localisation microscopy (FPLAM) unveiled that raft associated transmembrane protein haemagglutinin is distributed in nano-cluster (40 nm/> 100 µm diameter) in live cells (Hess et al., 2007).

We investigated raft NaV1.8 association with a microscopy based technique by visualising GM1 on the neuronal surface with CTB. CTB is a 60 kDa pentameric protein which is implicated in the pathogenesis of Vibrio Cholerae. CTB, by binding to GM1, allows the internalisation of the toxin which drives its toxic effects intracellularly by releasing the A sub-unit. Because of the specificity of the CTB to GM1 (Merritt et al., 1994), and being GM1 highly enriched in lipid rafts, CTB is used as a common marker to visualise lipid rafts (Bruses et al., 2001; Harder et al., 1998; Janes et al., 1999; Zhu et al., 2006). The protocol we use to visualise lipid rafts employs live cells incubated with a biotinylated form of CTB. The toxin itself is visualised by a fluorescent conjugate of Streptavidin, which binds to the biotin moiety harbouried by the toxin.
Since we use live cells, which are impermeable to Streptavidin, all the GM1 detected with this method is exposed on the membrane. Small dynamic rafts, which are not stabilised in larger platforms, are 50-100 nm in diameter. This size, being below the limit of optic detection (200 nm with conventional microscopes) hinders a direct visualisation of the smaller lipid rafts. CTB is a pentameric protein, which bids to five GM1 molecules (Merritt et al., 1994) and induces rafts patching (Janes et al., 1999). Streptavidin, which binds to four molecules of biotin, further enhances the patching process. This method is widely used to induce coalescence between rafts, and to bring them above the limit of light detection (Hammond et al., 2005; Harder et al., 1998; Owen et al., 2007). We correlated the immunoreactivity of Na\textsubscript{v}1.8 to the signal for lipid rafts in our system. We have found that GM1 is present as puncta on the cell surface, both at the level of the cell body and along the neurites. Interestingly, the degree of co-localisation between GM1 and Na\textsubscript{v}1.8 was very high along the axons, suggesting the clusters of Na\textsubscript{v}1.8 do co-localise with rafts in these cell processes. On the contrary, at the level of the cell body this co-localisation was not as clear as along the axons. This result could contribute to elucidate the sources of the different pools of Na\textsubscript{v}1.8 which we identified with biochemistry techniques in vitro. As we discussed beforehand, it is known that the majority of Na\textsubscript{v}1.8 is retained intracellularly at the level of the cell body. We found that at the level of the soma, by imaging, there wasn’t a clear co-localisation between Na\textsubscript{v}1.8 and membrane bound GM1. Hence, it is highly possible that the soluble pool of Na\textsubscript{v}1.8, extracted with biochemistry techniques, is derived from the intracellular stores. On the other hand, along the axons, we found co-localisation between GM1 and Na\textsubscript{v}1.8. Thus, it is likely that the raft associated pool of the channel that we extracted with the detergent method is derived from the axonal compartment. We also found a different pattern of co-localisation between GM1 and Na\textsubscript{v}1.8 in small and large diameter neurons. In fact, in large diameter neurons, where Na\textsubscript{v}1.8 is either evenly distributed or associated in patches along the axons, the channel and GM1 do not co-localise. This pool could contribute as a potential source of non-raft associated Na\textsubscript{v}1.8 which we detected with biochemistry techniques. This finding also suggests that the process of Na\textsubscript{v}1.8 raft targeting is specific for the unmyelinated nociceptors. Rafts play a role in trafficking, and they have been shown to act as signal to drive channel insertion on the membrane (Pristera and Okuse, 2011). It is possible that rafts in unmyelinated neurons are needed for an efficient translocation of Na\textsubscript{v}1.8 on the membrane. In myelinated neurons a complex feed-back between neurons and glial cell is at the base of channel clustering at the nodes of Ranvier or AIS. In unmyelinated neurons, less is known about the mechanisms.
regulating channel trafficking and insertion into the plasma-membrane. Given rafts established role in trafficking (Echarri et al., 2007; Helms and Zurzolo, 2004; Ikonen, 2001) it is possible to hypothesise that rafts may play a role in Na\textsubscript{v}1.8 insertion and/or clustering into the membrane.

We confirmed Na\textsubscript{v}1.8 clustered distribution and raft association by using a fluorescent construct. We tagged the C-terminus of Na\textsubscript{v}1.8 with DsRed2. The choice to tag the C-terminus of Na\textsubscript{v}1.8 is due to the fact that the N-terminus of Na\textsubscript{v}1.8 is a docking site for p11, a chaperon protein which promotes Na\textsubscript{v}1.8 membrane association by directly binding it. We thought that the addition of a fluorescent tag to this end may have altered the ability of the channel to bind to p11. In addition, it has already been demonstrated that the fusion of a fluorescent protein to this end does not alter the Na\textsubscript{v}1.8 electrophysiological properties (Schofield et al., 2008).

DsRed2 is a new generation red fluorescent proteins which, compared to the wild-type and first generation of engineered red fluorescent proteins DsRed1, has more advantages. Compared to DsRed1, DsRed2 has been genetically modified to form a variant which is brighter, faster maturing (Terskikh et al., 2002) and importantly, less prone to aggregation (Yanushevich et al., 2002). This construct has been widely used to tag protein of interests (Maruyama et al., 2004; Traweger et al., 2003; Yamaguchi et al., 2006) and, in neurons, to analyse trafficking of membrane associated protein (Stan et al., 2010). The major advantages of this method, compared to antibody based techniques, are that an analysis of live cells and a direct visualisation of Na\textsubscript{v}1.8 are possible. Moreover, potential artifacts due to cell fixation are avoided. A large scale analysis was hindered by the very low transfection efficiency of this construct by microporation. Primary neurons are very hard cells to transfect in first instance (Dib-Hajj et al., 2009), and the large size of our construct further complicated its delivery and/or expression. Nevertheless, in the neurons we managed to transfect we found that Na\textsubscript{v}1.8-DsRed2 does cluster and co-localises with GM1 along the axons. This finding confirms the results we have achieved by using antibody against Na\textsubscript{v}1.8. It also shows that the addition of a tag at the C-terminus of Na\textsubscript{v}1.8 does not alter the gross distribution of the channel.

Given the results showing that Na\textsubscript{v}1.8 associated with lipid rafts we tested if this association can be modulated. Our choice was to investigate the potential role of NGF in the association between rafts and Na\textsubscript{v}1.8. There are several reasons why we choose NGF. Lipid rafts act as signaling platform on the neuronal membrane, where they regulate the spatial and temporal dynamics of different set of proteins. In fact, they can allow molecular interaction and act as
permissive factors for given pathways, or impede proteins from interacting and therefore act as an inhibitory signal (Allen et al., 2007). We found that TrkA, NGF receptor, is expressed along the axons and that Na\textsubscript{v}1.8 clusters co-localise with TrkA. TrkA, upon binding to NGF, translocates and is retained into lipid rafts, through the association with Flotillin and CAP (c-Cbl associated protein), where it initiates intracellular signaling (Limpert et al., 2007). Also, NGF increases Na\textsubscript{v}1.8 mediated currents by different routes (Okuse, 2007); in the short term, through PKC\textepsilon, by directly phosphorylating the channel (Gold et al., 1998; Khasar et al., 1999), and interestingly in neuronal cells PKC has also been reported to translocate into lipid rafts upon activation (Botto et al., 2007). In the long term, NGF increases Na\textsubscript{v}1.8 currents by augmenting its expression (Fjell et al., 1999a; Okuse, 2007). Given these circumstantial evidence, we tested if NGF modulates Na\textsubscript{v}1.8 raft association. We found that the co-localisation between GM1 and Na\textsubscript{v}1.8, which is evident in small diameter neurons in control cells, is impaired in cells not treated with NGF. We investigated long term effects of lack of NGF. In this experimental model, the lack of association between GM1 and Na\textsubscript{v}1.8 could be the results of different causes. NGF is a pleiotropic cytokine, which is fundamental for DRG neurons survival and to promote neurites extension (Sofroniew et al., 2001). Therefore, many factors may contribute to mis-colocalisation between Na\textsubscript{v}1.8 and GM1 in the prolonged absence of NGF. A direct effect of the lack of NGF in properly trafficking Na\textsubscript{v}1.8 to lipid rafts can be hypothesised as well as more generic factors, like decreased expression of Na\textsubscript{v}1.8, increased cell apoptosis, cellular stress, lack of an extensive neurite outgrowth. It would be interesting to test an acute effect of lack of NGF, or the effect of the application of NGF to short-term deprived neurons, on the association between Na\textsubscript{v}1.8 and lipid rafts, both by imaging and biochemical techniques.

We also investigated the sub-cellular localisation of p11. P11 (Annexin II light chain) has been demonstrated to act as a key role for the functional expression of Na\textsubscript{v}1.8 into the membrane. Annexins are a group of proteins which regulate protein trafficking to the membrane in a calcium dependent fashion. P11 is the only member that has suffered mutations into its calcium binding site, and exists in a permanent activated state (Gerke and Moss, 2002). P11 binds the N-terminus of Na\textsubscript{v}1.8 (residues 74-103) (Poon et al., 2004) and is necessary, but not sufficient, to allow Na\textsubscript{v}1.8 localisation on the membrane. Moreover, the complex Annexin II-p11 resides into Triton X-100 resistant membrane (Oliferenko et al., 1999; Rescher and Gerke, 2004). In our system, we found co-localisation of p11 with GM1, suggesting raft association in
agreement with other groups’ findings (Oliferenko et al., 1999). Also, p11 displayed a clustered localisation along the axons in vitro, similar to the distribution of Na\textsubscript{v}1.8. Given the evidence that p11 specifically binds to Na\textsubscript{v}1.8 and it resides in lipid rafts, this result indirectly confirms the sub-cellular localisation of Na\textsubscript{v}1.8 and the channel’s raft association in DRG neurons. To summarise the data discussed so far, we have found that Na\textsubscript{v}1.8 does associate with lipid rafts in vitro, and this was confirmed both with biochemical techniques and by imaging.

One pitfall of biochemical extraction is that this is not the best method to ascertain which type of raft the proteins of interest is targeted to. Lipid rafts can be distinguished by morphology and protein content, but since the lipid content is very similar, detergent extraction and density gradient does not unequivocally distinguish between planar and caveola-type lipid rafts. For this purpose, we used imaging techniques to explore this point. At the time of performing the experiment, valid antibodies for immunocytochemistry for Flotillin-1 and Caveolin-2 were not available. For this reason, we cloned these two proteins with a fluorescent tag, to visualise them in DRG neurons and to correlate their localisation with endogenous Na\textsubscript{v}1.8. We used Flotillin-1 as a marker of planar lipid rafts and Caveolin-2 as a marker of caveola-type lipid rafts, given the established roles of these proteins as markers of the respective raft types. In neuronal cells the expression of Flotillins has been extensively documented. In fact, Flotillin-1 has been found in several tissues, including brain (Kokubo et al., 2003), hippocampal (Swanwick et al., 2010b) and DRG neurons (Lang et al., 1998). Expression of Caveolins has also been showed to exist in DRG neurons (Galbiati et al., 1998), hippocampal neurons (Bu et al., 2003) and cortical neurons (Takemoto-Kimura et al., 2007); nevertheless reports argue against the existence of caveolae and Caveolins in neuronal cells (Lang et al., 1998; Scherer et al., 1997). The tag of choice was fluorescent protein Dronpa. This construct is a modified form of GFP, which exhibits useful features. It is more than two-fold brighter than GFP (Chapman et al., 2005) and has the remarkable property to undergo rounds of photoactivation/deactivation (Ando et al., 2004; Habuchi et al., 2005; Wilmann et al., 2006). We tagged the C-terminus of Flotillin-1 and Caveolin-2 and found different localisation pattern: evenly distributed with brighter puncta and distributed in discrete puncta, respectively. Fluorescent forms of these proteins have already been described. In particular, in hippocampal neurons, an EGFP C-terminus fusion of Flotillin-1 tag appears distributed in puncta at the cell surface with a lower fluorescence signal between the puncta (Swanwick et al., 2010a). We have found a very similar distribution, with the difference that in our hands the fluorescence
between the puncta was higher compared to the reported Flotillin-1-EGFP construct. Possible explanation of this discrepancy could be attributed to the different cellular background which could partly affect Flotillin-1 trafficking, different fluorescent tag (Dronpa vs. EGFP) and imaging apparatus. The nature of the brightest puncta cannot be described with certainty by our data. Given the existence of a heterogeneous population of rafts (small, highly dynamic vs. large, stable) (Simons and Gerl, 2010; Simons and Ikonen, 1997) it is tempting to speculate that the brightest puncta of Flotillin-1-Dronpa are associated with large and stable raft platforms which serve as hubs on the membrane regulating trafficking and cell signaling (Lingwood and Simons, 2010; Simons and Gerl, 2010). A fluorescent form of Caveolin-2 has not been reported in neuronal cells. We have found that this construct localises in discrete clusters. We have correlated the localisation of Flotillin1-Dronpa and Caveolin-2-Dronpa with endogenous Na\textsubscript{V}1.8 and found that Na\textsubscript{V}1.8 preferentially associated with Flotillin-1-Dronpa, suggesting that planar lipid rafts are the micro domains that Na\textsubscript{V}1.8 is targeted to in DRG neurons.

In conclusion, we have found that Na\textsubscript{V}1.8 partitions into lipid rafts along the sciatic nerve ex vivo, while at the level of the DRG cell bodies it is mostly associated with non-raft membranes. In cultured DRG neurons it is associated both with lipid raft domains and non raft portions of the cells. More specifically, it co-localises along the axons with raft marker GM1 and preferentially targets planar lipid rafts. We have also shown that Na\textsubscript{V}1.8 is distributed in clusters along the axons of nociceptors ex vivo and in vitro.
Chapter 3: Investigation of the functional meaning of the association between $\text{Na}_V 1.8$ and lipid rafts in DRG neurons
3.1 Introduction

3.1.1 Synopsis.

In the previous chapter we demonstrated that Na\textsubscript{v}1.8 is trafficked to lipid rafts. In this chapter we shall investigate the biological relevance of Na\textsubscript{v}1.8 raft association by interfering with raft stability. Methods to disrupt the association between rafts and proteins of interest will be introduced. We shall also describe and discuss the functional assays (mechanical and chemical stimulation of DRG neurons to evoke action potentials) that we have exploited to test the functional meaning of the association between Na\textsubscript{v}1.8 and lipid rafts in nociceptors.

3.1.2 Methods to disrupt lipid rafts.

The strategy to unravel the biological meaning of the association between a certain protein and lipid rafts is to disrupt such association and to test the activity of the protein of interest. Several ways have been described to manipulate lipid rafts stability. These methods interfere with rafts by changing different aspects of their nature. Cholesterol depletion, inhibition of cholesterol synthesis, sphingolipids down-regulation and lipid phase change have been described. The most common method is to deplete the cell from membrane cholesterol. Indeed, raft stability is dependent on this sterol and its depletion leads to raft disruption. Cholesterol is a hydrophobic molecule and cyclic oligosaccharides are used to remove it from the membranes. The most common is Methyl-β-cyclodextrin (MBCD). MBCD is a seven 7-P (1-4)-glucopyranose unit, toroid shaped, oligosaccharide. The external surface renders MBCD hydrophilic while the interior surface is hydrophobic. This internal cavity has the ability to encapsulate hydrophobic molecules and allow them to become soluble in a water aqueous environment (Brewster, 1990). MBCD shows high affinity for cholesterol compared to other lipids and is the most effective, between cyclodextrins, in binding cholesterol (Christian et al., 1997; Yancey et al., 1996). When applied to the cells, MBCD promotes cholesterol depletion from the cell surface, by binding to it (Christian et al., 1997; Klein et al., 1995; Pottosin et al., 2007). This effect has been demonstrated to occur because MBCD substantially decrease the
activation energy for cholesterol efflux from the hydrophobic plasma membrane (Yancey et al., 1996). MBCD mediated cholesterol depletion leads to rafts disruption and represent the standard method to assess raft proteins function (Dart, 2010; Davies et al., 2006; Eshcol et al., 2008; Hartmann et al., 2009; Hering et al., 2003; Limpert et al., 2007; Morenilla-Palao et al., 2009; Oldfield et al., 2009; Simons and Toomre, 2000; Zhu et al., 2006). Consistent with the importance of cholesterol for the integrity of rafts, other methods have been used to reduce its amount on the membrane. Statins are small inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA), an enzyme involved in cholesterol and isoprenoids biosynthesis (Endo, 1992). Treatment with Fluvastatin disrupts lipid rafts and impairs FcγR signaling in macrophage, which is at the base of the inflammatory response (Hillyard et al., 2004). It has also been documented that rafts can be destroyed with Simvastatin and that this treatment reduces the serotonin evoked currents mediated by raft associated 5-HT3 receptor (Nothdurfter et al., 2010). Another valid approach that leads to raft disruption is to decrease the amount of sphingolipids. This has been achieved both by using enzymes that digest sphingolipids and by knocking down enzymes involved in their synthesis. Sphingomyelin synthase (SMSase) is the last enzyme involved in sphingomyelin synthesis. Inhibition (with potassium tricyclodecan-9-yl-xanthogenate) or siRNA mediated knock-down of SMSase determine a robust decrease of sphingomyelin on the plasma membrane and within lipid rafts (Li et al., 2007). With a complementary approach, it was found that sphingomyelin degradation (with Sphingomyelinase treatment) affected raft associated protein NKCC1 activity (Hartmann et al., 2009). The common feature of the strategies presented above is that they target the amount of certain lipids on the membrane to impair raft stability. A recent method to disrupt lipid raft has been reported and is based on the perturbation of the Lα lipid phase of rafts, rather than on the depletion of cholesterol or sphingolipids. This strategy relies on the delivery of 7-ketocholesterol (7KC) to the cell membrane. 7KC is an analogue of cholesterol, produced by the non-enzymatic oxidation of cholesterol and is abundant in the arteriosclerotic plaques (Jessup et al., 2002). 7KC differs from cholesterol only for a ketone group which protrudes perpendicularly from the sterol plane. 7KC intracellular trafficking is identical to cholesterol (Gaus et al., 2004). It has been demonstrated that 7KC incorporation into the plasma membrane decrease lipid order. Indeed, the ketone group limits the depth of 7KC insertion into the membrane and its interaction with phospholipids acyl chains. This results in 7KC being orientated with the two polar moieties of the oxysterol near the membrane-water interface. Importantly, the alignment of the sterol ring of 7KC with trans-configured saturated
acyl chains of sphingoglycolipids is impaired and this leads to decreased formation of ordered membrane domains (Massey and Pownall, 2005). Remarkably, it has been shown that 7KC preferentially partition into lipid rafts and does not alter non-raft portion of the membrane (Gaus et al., 2004). With this method Rentero et al. showed that 7KC, in T-cells during the development of the immunological synapse, decreased lipid order at the activation site and prevented raft platform formation. 7KC delivery to T-cells also determined less efficient signaling at the cell membrane, resulting in compromised downstream activation responses (Rentero et al., 2008).

3.1.3 Mechano-sensitivity.

DRG neurons have the ability to detect mechanical stimuli, ranging from innocuous light touch to painful stimuli. DRG neurons are heterogeneous (see section 1.1.2) and mechanical stimuli recruit different cell types. Anatomically, C- and Aδ neurons terminate in the epidermis as free nerve ending, or associated with hairs as in the case of some Aδ neurons (D-hair). On the contrary, Aβ neurons terminate deeper in the skin (deep layer of epidermis/dermis) and their endings are encapsulated in highly specialised structure such as Pacinian corpuscle (detecting rapid vibration), Meissner’s corpuscle (detecting low frequency vibration) (Kandel et al., 2000) and Merkel’s discs (detecting sustained light touch/pressure (Halata et al., 2003). C- and Aδ neurons, as discussed in Chapter 1 (see section 1.1.2), detect high threshold mechanical stimuli (noxious touch). Also, Aδ and Aβ neurons are thought to substantially contribute to the mechanical hyperalgesia and allodynia present in chronic pain conditions (Wall and Melzack, 2005). The different afferents can be classified according to their electrophysiological features and activity upon presentation of the stimulus. In fact, sub-groups of Aβ neurons have been found to be rapidly adapting or slowly adapting. The former, which includes D-hair, Pacinian and Meissner’s corpuscles, fires at high frequency (proportional to the stimulus intensity) upon the presentation and withdrawal of the stimulus and adapts rapidly. The latter, such as Merkel discs, fires for the duration of the stimulus with little adaptation (Halata et al., 2003). The polymodal nature of nociceptors (C-, Aδ neurons) complicates the classification of these neurons, in terms of mechano-sensation. In fact, rapidly, slowly and intermediate adapting neurons have been described. Furthermore, there are mechano-sensitive C-nociceptors that also respond to thermal stimuli and/or chemical stimuli (Lewin and Moshourab, 2004).
Amongst the senses, touch is the least understood. The molecular identity of the mechano-transducers is unknown to date (Ingber, 2006). The general assumption is that the molecule/s gated by touch is a non-specific cation channel which is gated by mechanical stimulation (e.g. touch, stretch). To date, there are few ion channels which have received attention from the scientific community: Degenerin/Epithelial sodium channels (DEG/ENaC), Transient Receptor Potential (Trp) channels and two-pore potassium (KCNK) channels (Nicolson, 2005; Tsunozaki and Bautista, 2009). *C. elegans* DEG/ENaC channels MEC-4 and MEC-10 associate in a macro molecular complex including integral membrane MEC2. It has been found that this complex transduces light touch in this animal model (Chalfie, 2009). Based on this assumption, mammalian homologue acid sensitive ion channels (ASICs) were proposed to act as mechano-transducers. ASICs are thought to form hetero-oligomers complexes with other accessory proteins (Lingueglia, 2007). In mice, deletion of ASICs did not reveal profound effect on mechano-sensitivity (Drew *et al.*, 2004). On the other hand, more profound effects are revealed in Aβ and Aδ, but not C neurons, when accessory protein SLP3 (mammalian homologue of MEC2) is missing (Wetzel *et al.*, 2007). Studies on Trp channel led to mixed results (Christensen and Corey, 2007). A recent study using single-fiber recordings in a skin-nerve preparation showed that Trpa1 KO animals have reduced responses to mechanical stimuli in C-fibers and slowly adapting Aβ fibers, but have increased responses in rapidly adapting Aβ fibers (Kwan *et al.*, 2009). On the other hand, KO mice do not show significant differences in terms of behaviour (Bautista *et al.*, 2006). Trpv4 has been linked to mechanical hyperalgesia (Chen *et al.*, 2007), but its involvement as a first mediator is still uncharacterised. The current view on Trp channels is that they are likely to contribute the excitability of mechano-sensitive neurons, rather than directly transducing mechanical stimuli (Chalfie, 2009). Potassium channels of the KCNK family have been linked to mechano-sensation. TREK-1 and TRAAK are mechano and temperature sensitive channels expressed in DRGs and KO mice show impairments in mechanical and thermal sensitivity (Noel *et al.*, 2009). It is yet to be determined if these channels are directly gated by mechanical stimuli or contribute to the excitability of DRG neurons towards mechanical stimulation.

The gating mode of mechano-sensitive channels is also not fully characterised. Indeed, multiple models have been proposed, such as channels that open upon membrane stretch, or channels that are indirectly opened and the gating mode depends on molecular bridges between the channel and the membrane/cytoskeleton/extracellular matrix (tether model).
(Christensen and Corey, 2007). To date, the unequivocal identification of mechanical transducers has not yet been achieved (Chalfie, 2009; Drew, 2009; Drew et al., 2007).

The ability of DRG neurons to retain the competence to respond to mechanical stimuli in vitro has given the scientific community a model to operate with, in order to find and characterise the molecular nature of touch sensation (Eilers and Schumacher, 2005). It has been described in literature that stimulation of DRG neurons evokes action potentials. These studies have been traditionally performed with electrophysiological techniques, which have allowed the quantitative analysis of several cellular electric parameters upon mechanical stimulation (e.g. nature and kinetics of inward currents, currents-voltage relations for mechanically activated currents). Concomitant with the development of microscopy and ion-sensitive dyes, live cell imaging (particularly Calcium imaging) has determined a valuable input into the field of mechanosensation. This method provides a rapid set-up and read-out of mechanostimulation assays. It allows qualitative and quantitative (upon calibration) information about Calcium ions during the experiment. In addition, imaging techniques combined with ion-indicators permit the visualisation of ions dynamics in real time within different compartments of the same cell, giving useful spatial information. An early report describing DRG mechanic stimulation dates back to 1999, when McCarter et al. showed that the mechanic stimulation of the DRG neuron cell bodies, but not sympathetic ganglia neurons, determine an inward current which depolarises the membrane and triggers firing of action potentials. The nature of the inward current has been further investigated and has been found that the transient mechanically evoked current in DRG neurons is mediated by large pore mechanically gated channels, non selective for cations (McCarter et al., 1999). Inward currents have been classified according to their kinetics into two broad categories, rapidly and slowly adapting. This difference has been shown to rely on the type of dynamic stimulus applied and it can be further influenced by other factors like kinetics of decay, ion channel cohort expression, and properties of the mechano-transducers themselves (Rugiero et al., 2010). The analysis of axon evoked depolarisations has been recently carried out, both with electrophysiological and imaging tools. It has been demonstrated that mechanical stimulation of the axons of cultured DRG neurons evokes action potentials at the level of the cell soma. Interestingly, neurite stimulation evoked rapidly and slowly adapting currents. A proportion of rapidly adapting and most of slowly adapting neurons were identified, by the presence of TTX-r currents and soma size, as nociceptors (Hu and Lewin, 2006). Calcium imaging has also been employed to study
mechanostimulation *in vitro*. By monitoring cell excitability with Fura-2, a ratiometric calcium indicator, it was found that stimulation of the cell bodies determines an increase of intracellular calcium. Depletion of extracellular calcium abolished the peak response (without altering the resting calcium concentration), and TTX had no effect on the calcium increase. The increase of calcium was also proposed to be directly mediated by mechano-sensors which are La\(^{3+}\)-insensitive, Gd\(^{3+}\)-sensitive and amiloride-sensitive (Gschossmann *et al.*, 2000). Axon mechanostimulation *in vitro* has been also carried out in combination with calcium imaging. It was found that it evoked an increase of fluorescence (e.g. intracellular calcium) which propagated away from the point of stimulation (Sanchez *et al.*, 2007). More recently it has been clearly demonstrated that axons of nociceptors, when stimulated with a static force, generate TTX-r action potentials that propagate to the soma. Soma activation was monitored with Fura-2 upon axonal stimulation and it was described that neurite stimulation evoked depolarisations at the level of the cell bodies which correlated with an increase of intracellular calcium (Usoskin *et al.*, 2010).

In conclusion, DRG neurons elicit inward currents and generate action potentials upon mechanical stimulation of the cell bodies (Gschossmann *et al.*, 2000; McCarter *et al.*, 1999) or the neurites (Hu and Lewin, 2006; Sanchez *et al.*, 2007). Nociceptors, which express Na\(_v\)1.8 (Djouhri *et al.*, 2003a) and are high-threshold mechanoreceptors *in vivo* (Wall and Melzack, 2005), also respond to mechanic stimuli *in vitro* (Drew *et al.*, 2007; Drew *et al.*, 2002). Because Na\(_v\)1.8 mediates action potential generation in nociceptors, mechanostimulation based assay can give insights into Na\(_v\)1.8 functions. These features make DRG neurons, and nociceptors, also good candidates for the study of mechano-sensation.

### 3.1.4 Chemical stimulation.

Nociceptors have the ability to fire action potential upon chemical stimulation. This process is at the base of nociception triggered by endogenous molecules, released upon cellular stress (e.g. injury, metabolic imbalance, cell death), or by irritant exogenous compounds. Several compounds have been described to excite and/or sensitise these cells, including anandamide, ATP, Bradykinin, Histamine, Serotonin (Basbaum *et al.*, 2009; Okuse, 2007), capsaicin (hot chilli peppers) (Caterina *et al.*, 1997), allicin (garlic), isothiocyanate compounds (wasabi, mustard,
horseradish), Acrolein (tear gas, vehicle exhaust) (Bautista et al., 2006; Jordt et al., 2004) and menthol (peppermint) (Bautista et al., 2007). These compounds, when they bind to their receptors, elicit an inward depolarising current which brings the membrane potential close to the activation threshold of VGSCs. If the depolarisation is strong enough, VGSCs are engaged and action potentials are generated (Kandel et al., 2000). In this section, the receptors recruited by capsaicin, Bradykinin and ATP (compounds that we have used to chemically stimulate DRG neurons for the purpose of this thesis) will be described.

TrpV1 is a member of the Transient Receptor Potential (Trp) channels family (Venkatachalam and Montell, 2007) and is the receptor for anandamide (vasodilation mediator in inflammatory condition) (Zygmunt et al., 1999) and capsaicin. TrpV1 is also gated by noxious temperature (higher than 43°C) and low pH enhances TrpV1 currents (Caterina et al., 1997). TrpV1 putative structure comprises six transmembrane segments with intracellular N- and C-terminuses which form a 95 kDa ion channel. TrpV1 is a non-selective cation channel with high permeability for Calcium (Caterina et al., 1997) expressed by nociceptive neurons (mostly C fibres) in both the IB4 positive and IB4 negative sub-populations (Caterina et al., 1997; Guo et al., 1999; Hwang et al., 2005). TrpV1, apart from being implicated in the transduction of acute noxious stimuli (e.g. high temperature) plays a role in hyperalgesic conditions (Caterina et al., 2000). Indeed, TrpV1 represent the convergent target of several intracellular pathways which result in its sensitisation and/or increased trafficking to the membrane. These effects determine an increased excitability of nociceptors endings. It has been found that PLC (Rohacs et al., 2008), Src kinase (downstream of NGF-TrkA pathway) (Zhang et al., 2005), PKCε (Numazaki et al., 2002) and CamKII (Jung et al., 2004) positively modulate TrpV1 currents. Capsaicin is routinely used as an activator of nociceptors, as its application and binding to TrpV1 elicits robust action potential generation (Urban and Dray, 1993) which result in pain sensation, if it is applied to the skin (Schmelz et al., 2000).

Bradykinin binds to GPCRs receptor B1 and B2. B2 is constitutively expressed (Wang et al., 2006) and B1 is expressed after injury and release of inflammatory compounds (e.g. GDNF) (Lee et al., 2002; Vellani et al., 2004). Bradykinin plays a role in inflammation states and is formed from kininogen precursors following the activation of plasma or tissue kallikrein enzymes by patho-physiological stimuli, such as inflammation and tissue damage (Calixto et al., 2000). Bradykinin receptors are expressed in nociceptors and application of this compound results in an evoked pain sensation (Beck and Handwerker, 1974). Bradykinin exerts its effect
in pain pathways through parallel mechanisms: direct activation and sensitisation of nociceptors. Bradykinin binding leads to the activation of PLC\(_{\beta}\) and PLA\(_2\). PLC\(_{\beta}\) determines the synthesis of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) from phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)). In turn, DAG activates PKC which gates sodium and calcium channels that depolarise the neuron and trigger action potentials (Burgess et al., 1989; Jeftinija, 1994a). On the other hand, PLA\(_2\) mediates the synthesis of arachidonic acid, which is converted into pro-inflammatory agent PGE2. PGE2 sensitise nociceptors by binding to EP2 receptor and by activating PKA mediated intracellular pathways (Wang et al., 2006). In addition, Bradykinin sensitises other proteins involved in nociception. Indeed, TrpV1 is tonically inhibited by PIP2. Activation of PLC\(_{\beta}\) degrades PIP2 which results in TrpV1 sensitisation (Chuang et al., 2001). Activation of PKC also results in phosphorylation and enhancements of currents mediated by TrpV1 (Premkumar and Ahern, 2000) and Na\(_v\)1.8 (Gold et al., 1998; Khasar et al., 1999; Okuse, 2007).

ATP binds to P2X3 receptor, a member of the P2X protein family. P2X is a group of nucleotide gated channels comprising seven proteins (P2X1-7); each protein is thought to comprise two transmembrane segments, with a long extracellular domain, and to form homo-hetero-oligomers (North, 2002). P2X3 is the only member of the P2X family which is exclusively expressed in DRG neurons. It has been found that P2X3 is mostly expressed in IB4 positive, non peptidergic nociceptors, and to a lesser extent in IB4 negative nociceptors (Ruan et al., 2004; Vulchanova et al., 1998). ATP is released by necrotic/apoptotic cells after trauma, during metabolic imbalances and inflammatory states (Cook and McCleskey, 2002). ATP is a pronociceptive compound and elicits a sensation of pain if applied to the skin (Bleehen and Keele, 1977). It excites nociceptors by binding to P2X3; upon binding a conformational change opens the channel and allows an influx of Calcium ions into the cells, which depolarize the neurons and trigger action potentials (Bouvier et al., 1991; Gu and MacDermott, 1997). Similarly to the other chemical transducers, P2X3 is the target of intracellular pathways and contributes to peripheral sensitisation. For example, Bradykinin and Substance P increase P2X3 mediated currents and this is has been hypothesised to occur through a PKC dependent pathway (Paukert et al., 2001). Also, intrathecal delivery of NGF and GDNF to primary afferents has been shown to up-regulate P2X3 levels in nociceptors (Ramer et al., 2001).

The common characteristic of the aforesaid receptors is that they are expressed in nociceptors, which also express Na\(_v\)1.8 (Djouhri et al., 2003a). Therefore the chemical
stimulation of DRG neurons with capsaicin, ATP and Bradykinin provides a tool to stimulate this class of neurons and investigate Na\textsubscript{\textit{v}}1.8 mediated action potentials.

### 3.1.5 Aims.

The studies presented in this chapter were aimed at disrupting lipid rafts and interfering with the association between them and Na\textsubscript{\textit{v}}1.8. The methods employed to test the effective disruption were both biochemical and optical. To assess the biological meaning of the association of Na\textsubscript{\textit{v}}1.8 with rafts we aimed to develop functional assays (mechano- and chemical stimulation of DRG neurons) and utilised them to investigate Na\textsubscript{\textit{v}}1.8 function.
3.2 Materials and Methods

3.2.1 Sterol complexes preparation.

7-ketocholesterol (7KC) and cholesterol were complexed with Methyl-β-cyclodextrin (MBCD) (Sigma) as previously described in literature (Klein et al., 1995; Rentero et al., 2008). Sterols (Sigma) were dissolved in 96% Ethanol (EtOH) to a final concentration of 15 mg/ml. Methyl-β-cyclodextrin (MBCD) was dissolved in sterile water to a final concentration of 50 mg/ml. 400 μl of 50 mg/ml MBCD was heated to 80°C and 4x10 μl of 15 mg/ml sterols added every 5’. This preparation led to stock sterols solutions (3.4 mM 7KC, 3.5 mM cholesterol). The compounds were prepared fresh on the day of the experiment.

3.2.2 Lipid raft disruption.

DRG neurons were washed three times with pre-warmed DMEM and incubated with 10 mM MBCD or 50 μM 7KC, diluted in DMEM for 30’ at 37°C. Control cells were either incubated in DMEM only or with 50 μM cholesterol diluted in DMEM for 30’ at 37°C. After treatment, cells were washed three times with pre-warmed DMEM and processed for subsequent applications. For live cell imaging experiments, cells were firstly loaded with the fluorescent dye (see section 3.2.4) and then rafts were depleted.

3.2.3 Di-4-ANEPPDHQ staining and visualisation.

Di-4-ANEPPDHQ (Invitrogen) was dissolved in 96% EtOH to a final concentration of 0.2 mM and stored at 4°C in the dark. DRG neurons were treated with 7KC, cholesterol and MBCD as described above. After the different treatments cells were washed three times with Normal solution. Normal solution composition was: 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 2 mM MgCl₂, 10 mM D-Glucose, 10 mM HEPES, pH 7.4. Solution was sterilised by filtering it through a 0.20 μm filter. All compounds were cell culture grade from Sigma. After washes the cells were incubated with 4 μM di-4-ANEPPDHQ diluted in Normal solution for 10’ at 37°C in the dark. Cells were imaged under a Leica SP5 inverted confocal microscope with a 20x air objective. The dye was excited with a 476 nm Argon laser line. Emission spectra were constructed by

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performing a $\lambda$ scan. $\lambda$ scan records a series of individual images within a user-defined wavelength range; each image will be detected at a specific emission wavelength. This can be used to measure the emission spectrum a fluorescent molecule in the sample. In more details, the fluorescence intensity was recorded across the visible spectrum in the range 510-690 nm with 19 readings (each reading detected the fluorescence intensity in a window of 9.5 nm). To build the emission spectra at the end of the scan the fluorescence intensities were automatically plotted against the respective range of the spectrum they were recorded from.

### 3.2.4 Fluo-4 AM loading and imaging.

Membrane permeable calcium fluorescent indicator Fluo-4 AM (Molecular probes; Invitrogen) was reconstituted in dimethyl sulfoxide to a final concentration of 4 mM and stored at -20°C. DRG neurons were washed three times with Normal solution and incubated with 4 $\mu$M Fluo-4 AM diluted in Normal solution for 30’ at R.T. in the dark. After three washes in Normal solution cells were left 30’ at R.T. for the de-esterification step. Following three washes neurons were imaged on a Leica SP5 inverted confocal microscope equipped with a temperature controlled chamber. The microscope is suited for live cell imaging and fast recording, thanks to the presence of a Leica TCS Resonant Scanning head. The cells were imaged at 37°C in Normal solution. Fluo-4 was excited with a 488 nm Argon laser line set at 15% of the laser’s maximum power. Emitted fluorescence was detected in the range 500-570 nm. Gain and offset values of the photomultipliers were set by using the “Smart gain/offset” tool in combination with the “Saturation Pseudocolor” mode. To allow fast recording (approximately one frame every 130 msec) the following settings were selected: Scan-mode = xyt; Visible shutter = 1; Frame accumulation/average = 1; Line average = 2; Resolution = 8 bits; CCD camera frequency = 8000 Hz; Image dimension = 512 x 512 pixels. Files were recorded as TIFF images. Post acquisition processing was performed with LAS AF Lite software. Pictures are presented either in green hue or in pseudocolor (GlowDark look-up-table).
3.2.5 Viability assay.

DRG neurons were treated with 7KC, cholesterol and MBCD as described above. After the treatments DRG neurons were washed with DMEM and incubated with 1 μg/ml Propidium Iodide diluted in DMEM for 15’ at R.T. After washes cells were fixed with 4% PFA for 10’ at R.T. and nuclei counterstained with Hoechst 33342 (see section 2.2.6).

3.2.6 Mechano-stimulation.

DRG neurons were prepared as described in Chapter 2 (see section 2.2.3). Five thousand neurons were plated on 35 mm borosilicate glass-bottom dishes (MatTek) coated with poly-L-lysine (Sigma) and Laminin (Invitrogen). Two days after plating cells were loaded with Fluo 4-AM and treated with different compounds (7KC, cholesterol, MBCD). Mechano-stimulation was performed under a Leica SPS inverted confocal microscope equipped with micro-manipulator Inject Man N1 2 (Eppendorf). The motorised head of the micro-manipulator was set at 45° against the main surface of the culture wells. Mechano-stimulation of the neurons was achieved by using a fine glass tip with an outer diameter 1.0 µm (Femtotip; Eppendorf). The glass tip was moved to the focal plane of DRG neurons by using the “coarse mode” control and mechano-stimulation performed by using the analogic “fine mode” control (0-5 µm/sec). When axonal stimulation was needed fields of view comprising the axon and the cell body it arises from were chosen. Cells were imaged with a 63x water objective and Calcium dynamics were recorded by visualising Fluo-4 fluorescence over the course of mechano-stimulation by recording 9 frames per second (111 msec between every frame). Different electronic magnification values were used between different neurons. Every frame has been acquired with 2 lines average and pinhole aperture was set at the maximum value (5.4 Airy Units, minimal confocality). All the experiments were performed in Normal solution in a controlled temperature incubator set at 37°C. Quantification of the fluorescence intensities was performed by using Leica SP lite software.
3.2.7 Campenot set-up and chemical stimulation.

Campenot chambers were custom designed and fabricated with Teflon™ (Tyler Research Corporation). Teflon™ is an inert substance which is routinely used to set-up compartmentalised cultures (Campenot and Martin, 2001). The chambers (Fig. 3.1) were cleaned with a mild detergent, rinsed, soaked in 70% EtOH for 5’, washed in distilled water and autoclaved before every use. Dividers were stored in sterile conditions till the day of use. The chambers were set-up the day before plating the neurons. 35 mm plastic dishes (BD Falcon) were scratched with a pin-rake (Tyler Research Corporation) in the middle portion. The length between each incision measured 300 µm. The scratched region was coated with 0.1 mg/ml poly-L-lysine for 2 hours, washed three times with distilled water and left to dry. The middle portion of the scratched region was overlaid with 30 µl of 1% Methylcellulose (Sigma), 10 µg/ml Laminin (Invitrogen), 50 ng/ml NGF (Peprotech) diluted in DMEM with antibiotics, in order to wet the region where axons will cross under the divider. The bottom surface of the Teflon™ dividers was greased with autoclaved high-vacuum grease (Dow Corning) by using a syringe (Tyler Research Corporation) fitted with a blunt 23G needle. Particular care was taken in laying the grease with a continuous single movement. The greased side of the dividers was gently moved on top of the scratched region of the plastic dishes. The dividers were sealed to the plastic dishes by turning upside-down the dish-chamber complex and by applying gentle pressure to the plastic dishes with a fine forceps. These procedures were performed under a stereo-microscope in sterile conditions. The assembled Campenot chambers were left 2 hours at 37°C to equilibrate. 200 µl of DMEM was applied to the side chambers and left over-night at 37°C to test for leakage. The following day the leaky chambers were discarded. Chambers were washed three times with pre-warmed DMEM, and coated with 10 µg/ml Laminin (Invitrogen). DRG neurons were prepared as described in Chapter 2 (section 2.2.3) and 50,000 neurons were plated in the smallest chamber (soma chamber; A in Fig. 3.1) in DMEM without NGF and supplemented with 10% FBS, antibiotics, 10 µM Aphidicolin. Axonal outgrowth was promoted by NGF diluted in 10% FBS DMEM with antibiotics and 10 µM Aphidicolin. The day of plating 50 ng/ml NGF was added to the middle chamber (B in Fig. 3.1), and NGF was maintained in this chamber until the axons grew in proximity of the wall between the middle chamber and the side chamber (C in Fig. 3.1), furthest from the soma chamber. When axons started growing beneath this wall, the furthest chamber from the soma was supplemented with 50 ng/ml of NGF and the middle chamber was shifted to 25 ng/ml. Axon outgrowth was checked daily and
media changed every two days. The soma chamber was always left without NGF. Media was changed every two days. Experiments were carried out 14 days after plating, when extensive neurite outgrowth in the furthest chamber was obtained.

![Diagram of the Campenot chamber](image)

Figure 3.1) Specifications of the Campenot chamber used for the compartmentalised DRG culture. A=soma chamber, where DRG neurons were plated, B=middle chamber, C=furthest chamber from soma chamber (side chamber).

The day of the experiment DRG neurons were loaded with Fluo 4-AM. If lipid raft disruption was needed, this was carried out only in the middle chamber (B in Fig. 3.1). For imaging purposes the chambers were filled with 150 µl of Normal solution and axon endings in chamber C where chemically stimulated with 10 µM Capsaicin (Fluka), 10 µM Bradykinin (Sigma) and 300 µM ATP (Sigma) diluted in Normal solution. Cell bodies were visualised with a 10x air objective on a Leica SP5 confocal microscope equipped with a heated chamber maintained at 37°C. Fluo-4 fluorescence intensity was recorded with the same settings used for the mehano-stimulation assay, with the difference that the pinhole aperture was set at 7.4 Airy Units (minimal confocality).
3.2.8 Statistical analysis.

Data were computed and analysed with OriginPro 8.5 software (Originlab Corporation). For multiple comparisons between groups one-way ANOVA (di-4-ANEPPDHQ calculated maximum emission of the spectra; viability assay) was used. Fisher’s exact test was used for categorical data (Mechanostimulation assays). Non parametrical Mann-Whitney test was used for comparison between individual groups (Speed of conduction; chemical stimulation in the Campenot set-up). Data are expressed as mean ± SEM unless stated otherwise. P value < 0.05 was considered statistically significant. Power analysis of the collected data was computed and shown when applicable.
3.3 Results

Investigation of the disruption of the association between Na\textsubscript{v}1.8 and lipid rafts.

We have demonstrated in the previous chapter that Na\textsubscript{v}1.8 associates with lipid rafts *in vitro*. We extended this finding in order to understand if the association between Na\textsubscript{v}1.8 and lipid rafts has got a functional meaning. The next section will focus on the results about lipid raft disruption and on the consequences of this process on Na\textsubscript{v}1.8-raft association. The second section will describe the effort we made to develop assays to investigate Na\textsubscript{v}1.8 role in action potential conduction and how this role is affected by disrupting Na\textsubscript{v}1.8-raft association.

3.3.1 Lipid raft disruption: biochemical evidence.

We used different agents to disrupt lipid rafts *in vitro*. The agents of choice were 7-ketocholesterol (7KC) at a concentration of 50 µM and Methyl-β-cyclodextrin (MBCD) at a concentration of 10 mM. Control cells were left untreated (CTR). We have investigated the effect of the different compounds on lipid rafts stability, by detergent extraction and gradient ultracentrifugation. Lipid raft stability is dependent on cholesterol, which allows the tight packing of the saturated fatty acid chains (Simons and Vaz, 2004). A common method to disrupt lipid raft is to deplete cholesterol from the cell membrane by using MBCD. We also used 7KC to interfere with the lipid phase of lipid rafts, in an attempt to interfere with their stability. The hypothesis was that, by interfering with cholesterol content and with the physical lipid phase of rafts, the stability of these micro-domains would be affected and this, in turn, would impair their ability to be resistant to non-ionic detergent at 4°C. We treated 15000 DRG neurons after 2DIV with the different compounds for 30’ at 37°C and extracted lipid rafts. We used Flotillin-1 as the protein marker of lipid rafts in the different conditions. We found that 10 mM MBCD, and 50 µM 7KC negatively affected raft stability. In fact, upon detergent extraction, Flotillin-1 profiles between 7KC, MBCD and CTR were different. In CTR condition (Fig. 3.2 A, B) the majority of Flotillin-1 is retrieved from the top fractions (lane 2 and 3). In 7KC...
treated neurons Flotillin-1 displayed a reduced amount on the top fractions (lane 2 7KC vs. lane 2 CTR) and a tailing effect towards the bottom fractions. In MBCD treated samples, lipid raft disruption was even more evident, with Flotillin-1 being recovered only from the bottom fractions (lanes 7, 8 and 9) (Fig. 3.2 A, B).

**Figure 3.2** Lipid raft disruption in cultured DRG neurons. A) DRG neurons have been treated with 7KC and MBCD to disrupt lipid raft. CTR (as in Fig. 2.8 of Chapter 2, section 2.3.3) is shown here to ease the comparison between the different conditions. Fractions from a density gradient were analysed by western blotting for Flotillin-1 to test the effect of MBCD and 7KC on lipid rafts stability. Different fractions are labelled from 1 to 9, with 1 being the top fraction and 9 the bottom fraction. M is the protein ladder. B) Shows the bands intensity plotted against the fraction number.
We next probed these samples for Na\textsubscript{v}1.8, to ascertain its partitioning upon raft depletion. Remarkably we found that, compared to CTR sample, all Na\textsubscript{v}1.8 is recovered from the bottom, non raft, fractions. In fact, the lipid raft associated pool (lane 2 in CTR) is absent in both 7KC and MBCD treated samples (Fig. 3.3A, B). This result indicates that treatments with MBCD and 7KC led to the dissociation of Na\textsubscript{v}1.8 from lipid rafts.

![Figure 3.3](image)

**Figure 3.3) 7KC and MBCD impair Na\textsubscript{v}1.8-raft association.** A) DRG neurons have been treated with 7KC and MBCD to disrupt lipid raft. CTR (as in Fig. 2.9 of Chapter 2, section 2.3.3) is shown here to ease the comparison between the different conditions. Fractions from a density gradient were analysed by western blotting for Na\textsubscript{v}1.8 to test the effect of MBCD and 7KC on its association with lipid rafts. Different fractions are labelled from 1 to 9, with 1 being the top fraction and 9 the bottom fraction. M is the protein ladder. B) Shows the bands intensity plotted against the fraction number.
3.3.2 Lipid raft disruption: imaging evidence.

We also employed imaging techniques to monitor 7KC and MBCD mediated lipid rafts disruption. We exploited the remarkable feature of fluorescent dye di-4-ANEPPDHQ to act as a sensor for the membrane lipid phase (Jin et al., 2005). It displays a blue-shift of the emission spectrum in the liquid ordered phase (raft-like) compared to liquid disordered phase (non raft) (Jin et al., 2006). We treated cells with 50 µM 7KC and 10mM MBCD to disrupt lipid rafts; Control cells were either treated with 50 µM cholesterol (CHOL) or left untreated (CTR). To determine the effect of the compounds on the lipid phase we constructed the emission spectra of di-4-ANEPPDHQ by performing a λ scan. Since MBCD and 7KC, in different ways, disrupt lipid rafts we hypothesised that the emission of di-4-ANEPPDHQ would be red-shifted compared to controls samples, because of a reduced liquid ordered phase (reflecting a decreased amount of liquid ordered lipid raft microdomains). Fig. 3.4 A shows representative live neurons treated with the different compounds, and stained with di-4-ANEPPDHQ. The dye, as expected, clearly shows a membrane localisation. Cell morphology (highlighted both by the fluorescent dye and DIC imaging – in the insets) is not altered by the different treatments, as cell bodies and axons do not show structural abnormalities. The results of λ scans are summarised in Fig. 3.4 B. The normalised spectra are constructed by reading fluorescence intensity from 510 nm to 690 nm (each data point is presented as the mean fluorescence intensity of at least eleven neurons ± SEM). CTR and CHOL treated samples show completely overlapping spectra, suggesting that CHOL treatmeant does not alter the phase of the membrane. On the contrary, 7KC and MBCD both determine a red-shift of the spectra, compared to CTR and CHOL treated samples (Calculated emission maxima, presented as mean emission maxima ± SEM: CTR= 590 ± 0 nm; CHOL= 590 ± 0 nm; 7KC= 596 ± 1.8 nm *·.; MBCD= 596 ± 1.6 nm *·,#; *p < 0.01 vs CTR, #p < 0.01 vs CHOL. One-way ANOVA, followed by Tukey’s post-hoc tests. Calculated power=0.99). Also, MBCD treatment determines a less steep shoulder of the spectra at wavelengths higher than 600 nm, compared to the other treatments. This result clearly indicates that 7KC and MBCD, alter the lipid phase of the neurons, shifting it to a less ordered phase, consistent with raft disruption.
Figure 3.4) Lipid raft disruption detected by using lipid phase probe di-4-ANEPPDHQ. A) DRG neurons have been treated with 7KC and MBCD to disrupt lipid raft. CHOL and CTR were treated with cholesterol and left untreated, respectively. The images show representative live
neurons stained with di-4-ANEPPDHQ after the treatments. B) The graph shows the normalised spectra of di-4-ANEPPDHQ emitted fluorescence in the different conditions.

3.3.3 Lipid raft disruption and Na\textsubscript{v}1.8 sub-cellular localisation.

We sought to investigate if lipid raft disruption had an effect on Na\textsubscript{v}1.8 sub-cellular localisation. In particular, we focused on the clustered appearance of Na\textsubscript{v}1.8 along the neurites of cultured DRG neurons, which we discussed in the Chapter 2. After 2DIV we treated neurons with 50 µM 7KC, 10 mM MBCD, and 50 µM CHOL for 30’ at 37°C. CTR cells were left untreated. After the treatments cells were washed, fixed and processed for immunofluorescence. We found that the different treatments did not induce macroscopic alteration in Na\textsubscript{v}1.8 distribution. In fact, regardless of the treatments, Na\textsubscript{v}1.8 clusters were evident along the axons (Fig. 3.5, arrows), suggesting that raft integrity is not needed for the stability of the clusters.
Figure 3.5) Lipid raft disruption does not alter Na\textsubscript{v}1.8 clustered distribution along the axons. Images show immunofluoresce for Na\textsubscript{v}1.8 after lipid raft disruption. Arrows indicate clusters along the axons of neurons. Cell bodies are identified by nuclear stain Hoechst. Scale bars are 20 µm.

### 3.3.4 Lipid raft disruption and GM1 sub-cellular localisation.

We previously reported that GM1, when detected with biotinylated CTB and fluorescent Streptavidin, appears to be present in puncta on the cell surface (Chapter 2, section 2.3.6). Given its role as a lipid raft marker we also investigated GM1 localisation on neurons upon lipid raft disruption. We treated cells with 50 µM 7KC, 10 mM MBCD, and 50 µM CHOL for 30’ at 37°C. CTR cells were left untreated. After the treatments cells were washed and probed for GM1. We have found that, similarly to what we found for Na\textsubscript{v}1.8, lipid raft disruption did not abolish the punctuated appearance of GM1 on the neuronal surface (Fig. 3.6). Thus raft depletion does not alter the punctuated distribution of GM1 along the axons.
Figure 3.6) Lipid raft disruption does not alter GM1 clustered distribution on the cell surface. Images show GM1 localised on DRG neurons after lipid raft disruption. The framed areas are magnified to highlight GM1 puncta along the axons. Scale bars are 20 µm.

3.3.5 Lipid raft disruption and Flotillin-1-Dronpa sub-cellular localisation.

We also investigated the localisation of raft protein marker Flotillin-1 after lipid raft disruption. We have previously reported that, in our hands, the construct Flotillin-1-Dronpa when expressed in DRG neurons tends to be evenly distributed with puncta of higher fluorescence along the axons (Chapter 2, section 2.3.10). We have delivered the construct to DRG neurons and after 2DIV we treated the cells with 50 µM 7KC, 10 mM MBCD, and 50 µM CHOL for 30’ at
37°C. CTR cells were left untreated. After the treatments cells were washed and fixed. We have not found obvious changes in Flotillin-1-Dronpa localisation after the different treatments. Indeed, in control condition, similarly to what we have described before, Flotillin-1-Dronpa was evenly distributed with brighter puncta along the axons. 7KC, MBCD and CHOL treated cells were still characterised by the appearance of brighter puncta of the construct along the neurites (Fig. 3.7).

Figure 3.7) Lipid raft disruption does not alter Flotillin-1-Dronpa ability to cluster. Images show Flotillin-1-Dronpa after lipid raft disruption. Cell bodies are identified by nuclear stain Hoechst. Framed area are magnified to show the brighter puncta of the construct. Scale bars are 20 µm.

3.3.6 Viability assay.

To determine if the agents we used to disrupt lipid rafts cause cell death (apoptosis and/or necrosis) we performed a viability assay. We treated the cells for 30’ at 37°C with the different compounds, washed them and incubated them for 30’ at 37°C. After this incubation period we incubated the neurons with Propidium Iodide (PI) to identify dead cells. We counterstained the cells with nuclear dye Hoechst and counted the dead cells upon the different treatments (Fig. 3.8 A). We found that the different treatments did not induce cell death at this time point,
compared to CTR cells (% of PI positive nuclei ± SEM; CTR= 4.6 % ± 0.5, CHOL= 4.8 % ± 1.1, 7KC= 3.9 % ± 0.5, MBCD= 5.3 % ± 0.7; No statistically significant difference between the different treatments and CTR; n=3; p > 0.1, One-way ANOVA, followed by Tukey’s post-hoc tests. Calculated power=0.11) (Fig. 3.8 B).
In the previous sections we have demonstrated that 7KC and MBCD interfere with the lipid phase of DRG neurons, and they disrupt the association of Na\textsubscript{\textit{v}}1.8 with lipid rafts. Importantly, under these conditions the totality of Na\textsubscript{\textit{v}}1.8 is present in the soluble, non raft, fraction of the membrane. At the same time, the clustered appearance of Na\textsubscript{\textit{v}}1.8, Flotillin-1-Dronpa and GM1 (protein and lipid marker of lipid rafts, respectively) was not impaired by raft disruption treatments. To ascertain if Na\textsubscript{\textit{v}}1.8 raft association is functionally relevant we have developed two assays to determine the potential importance of such association. Since a comprehensive amount of literature demonstrated that Na\textsubscript{\textit{v}}1.8 plays a key role in action potential generation (Akopian \textit{et al.}, 1999b; Renganathan \textit{et al.}, 2001) and propagation in nociceptors (Brock \textit{et al.}, 1998; Djouhri \textit{et al.}, 2003a; Jeftinija, 1994a, b; Quasthoff \textit{et al.}, 1995; Steffens \textit{et al.}, 2001) we have studied the functional meaning of Na\textsubscript{\textit{v}}1.8-raft association in action potential propagation.
in DRG neurons. The next sections will describe how we developed assays to generate action potential in DRG neurons, by mechanical and chemical means, and how action potential propagation is affected in neurons where Na\textsubscript{1.8} does not associate with lipid rafts.

3.3.7 Mechano-stimulation based assay *in vitro*.

We developed an assay based on mechano-stimulation to study action potential propagation. DRG sensory neurons are mechano-sensitive *in vivo* (Kandel *et al.*, 2000; Tsunozaki and Bautista, 2009) and retain the property to trigger action potential, in response to mechanical stimuli, *in vitro* (Cheng *et al.*, 2010; Cho *et al.*, 2002; Drew *et al.*, 2007; Hu and Lewin, 2006; McCarter *et al.*, 1999). We exploited this ability to study action potential propagation in cultured DRG neurons. We plated DRG neurons and after 2DIV we loaded them with Fluo-4, a calcium indicator (Gee *et al.*, 2000; Paredes *et al.*, 2008). We mechanically probed the neurites of the cells in order to evoke action potentials and we recorded subsequent changes in Fluo-4 signals. We found that when the glass probe contacts the neurite of a responsive neuron an increase of Fluo-4 fluorescence can be detected. In particular, the increase of fluorescence is propagated from the point of contact in an antidromic and orthodromic fashion towards the end of the neurite and the cell body, respectively. When the wave of fluorescence reaches the cell body, this responds with a sharp increase of fluorescence (*Fig. 3.9*). Also, repetitive stimulation evokes multiple responses. *Fig. 3.10* clearly shows how multiple mechanic stimuli, along the axon of a neuron, determine related increases of fluorescence at the level of the cell body. In this neuron the neurite was stimulated for the first time at 29.2 \( \mu \text{m} \) from the cell body and the second and third stimuli were delivered at 10.8 \( \mu \text{m} \) from the soma. For this particular neuron we found that the second and third stimulation evoked greater responses than the first one.
Figure 3.9) Mechanostimulation of DRG neurons *in vitro*. The figure shows a representative neuron loaded with Fluo-4 responding to a mechanical stimulus. A) Shows the Fluo-4 fluorescence and DiC image of a DRG neuron. The glass probe is visible in the DiC image, at the moment of contact with neurite (arrow) which projects from the cell body. B) Shows the Fluo-4 fluorescence in pseudo-color, associated with different time points during the recording. C) The graph shows the recorded fluorescence intensity of different region of interests (ROIs), visible in A. The arrow indicates the time point when the cell has been stimulated; cardinal numbers refer to the time points which the images in B are associated to. Scale bar is 10 µm.
Figure 3.10) Multiple mechanostimulation of DRG neurons *in vitro*. The figure shows a representative neuron loaded with Fluo-4 responding to multiple mechanical stimulus. A) Shows the Fluo-4 fluorescence and DiC image of a DRG neuron. The glass probe is visible in the DiC image, at the moments of contact with neurite (arrows) which projects from the cell body. B) Shows the Fluo-4 fluorescence in pseudo-color, associated with different time points during the recording. C) The graph shows the recorded fluorescence intensity of the ROI, visible in A. The arrows indicate the time point when the cell has been stimulated; cardinal numbers refer to the time points which the images in B are associated to. Scale bar is 10 µm.
3.3.8 Mechano-stimulation in vitro in the absence of extracellular Calcium or Sodium ions.

Axon stimulation in vitro evokes cell depolarisation (Cheng et al., 2010; Hu and Lewin, 2006; Lin et al., 2009). To understand the nature of cell depolarisation associated with the increase of Fluo-4 we have carried out the mechano-stimulation in Calcium or Sodium free conditions. We investigated soma responsiveness upon mechanical stimulation of the neurite in medium devoid of Calcium and in the presence of 2mM EGTA. When calcium was omitted from the medium we did not detect any Fluo-4 fluorescence increase at the level of the cell bodies (n=8) (Fig. 3.11 A, B). This result shows that extracellular calcium is needed to evoke Fluo-4 changes in fluorescence, and rules out involvement of intracellular calcium stores.

**Figure 3.11** Mechanostimulation in vitro in Calcium free condition. The figure shows a representative neuron loaded with Fluo-4 not responding to a mechanical stimulus. A) Shows
the Fluo-4 fluorescence and DiC image of a DRG neuron. The glass probe is visible in the DiC image, at the moment of contact with neurite (arrow) which projects from the cell body. B) The graph shows the recorded fluorescence intensity of the ROI visible in A. The arrow indicates the time point when the cell has been stimulated. Scale bar is 10 µm.

We repeated the experiment in the presence of Calcium and absence of Sodium. In this condition, we replaced extracellular NaCl with an equimolar amount of Choline chloride to maintain the right osmolarity (Fitzgerald et al., 1999) and presence of positive charges in the medium. Importantly, in this condition we did not detect any soma response upon mechanic stimulation of the axons (n=15) (Fig. 3.12 A, B). This result indicates that sodium ions are necessary for the propagation of the depolarisation from the axon towards the cell body and that calcium ions alone are not sufficient for the propagation of the depolarisation. Occasionally we detected, after the axonal stimulation, an increase at the level of the cell body characterised by a very slow onset (20 secs) (Fig. 3.12 C, D).

**Figure 3.12** Mechanostimulation in vitro in Sodium free condition. A) Shows the Fluo-4 fluorescence and DiC image of a DRG neuron not responding to a mechanical stimulus. The glass probe is visible in the DiC image, at the moments of contact with neurite (arrow) which projects from the cell body. B) The graph shows the recorded fluorescence intensity of the ROI visible in A. The arrow indicates the time point when the cell has been stimulated. C) Shows
the Fluo-4 fluorescence and DiC image of a DRG neuron responding to a mechanical stimulus with a slow onset of fluorescence increase. The glass probe is visible in the DiC image, at the moments of contact with neurite (arrow) which projects from the cell body. D) The graph shows the recorded fluorescence intensity of the ROI visible in C. The arrow indicates the time point when the cell has been stimulated (Neuron stimulated at 11.2 sec and maximum fluorescence intensity was recorded at 32.7 secs). Scale bar are 10 µm.

3.3.9 Axonal Mechano-stimulation: effect of Lidocaine, Tetrodotoxin and lipid raft disruption.

We used the mechano-stimulation based assay to study action potential propagation in different conditions. In fact, we tested the effect of lipid raft disruption, a scenario where we demonstrated Na\textsubscript{v}1.8 shifts to the soluble fraction, on action potential propagation. We also further characterised the nature of the propagating depolarisation from the axon to the cell body, by performing the experiment in the presence of Lidocaine or TTX. For this series of experiments we probed axons originating from small diameter neurons (Diameter < 25 µm), most likely to be nociceptive, at a mean distance of 19.8 ± 7.6 µm (mean distance ± SD; n=188). We probed the cell three times and for our analysis we defined a neuron as “responsive” if the axonal stimulation evoked a soma response after one of these three stimulations. In case of multiple responses only the first stimulation was included in the analysis, to minimise the effect of sensitisation/desensitisation. Also, the increase of fluorescence at the level of the cell body to be classified as “response” had to be higher than 10% (threshold set arbitrarily), compared to baseline fluorescence, and to reach the maximum level within 20 seconds. In case the neurite was displaced during the stimulation and the cell was responsive, the time point considered as “contact” was the time point at which the neurite showed the maximum displacement. Cells that showed swelling or rupture of the axons were discarded from the analysis. Given this criteria we found that in the presence of Lidocaine (used concentrations: 10 mM (Gu and MacDermott, 1997) and 500 µM (Chevrier et al., 2004) the percentage of responsive cells dropped significantly compared to control cells (% of responsive cells; CTR=50.9%, Lidocaine 10 mM=12.5% *, Lidocaine 500 µM=0% *; *= p<0.05 vs CTR. Fisher's exact test. Table 3.1). These data clearly indicates that voltage gated sodium channels are needed for the propagation of the evoked depolarisation towards the cell body.
and for the response of this one. Also, the presence of 250 nM TTX did not affect the cell responsiveness, suggesting that TTX-r VGSC channels are sufficient to propagate and depolarise the cell soma (% of responsive cells; CTR=50.9%, TTX 250 nM=50%; Table 3.1). We also determined the effect of lipid raft disruption on action potential propagation. We performed the mechano-stimulation in Normal solution (see materials and methods for its composition; section 3.2.3) after lipid raft disruption. We treated the cells with 50 µM 7KC and 10 mM MBCD to disrupt lipid rafts, CHOL cells were treated with 50 µM cholesterol and CTR cells left untreated. Under this conditions we found that cholesterol treated cells were as responsive as control cells. Remarkably, upon lipid raft disruption and Na\textsubscript{v}1.8 shift to the soluble fraction of the cell membrane, we found a significant decrease in the number of soma able to respond to the mechanical stimulation of the axon (% of responsive cells; CTR=50.9%, CHOL 50 µM=54.2%, 7KC 50 µM=27.8% *, MBCD 10 mM=27.8% *; *= p<0.05 vs CTR. Fisher’s exact test. Table 3.1).
Table 3.1. Effect of different treatments on cell responsiveness, upon axonal stimulation.
Table summarises the results of axonal mecho-stimulation of DRG neurons in vitro. Percentages of neuronal cell bodies responsive to mechanical stimulation of the axon, in the different conditions, are listed. *= p<0.05 vs CTR. Fisher’s exact test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total number of screened neurons</th>
<th>Responsive neurons</th>
<th>% Responsive of responsive neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR</td>
<td>55</td>
<td>28</td>
<td>50.9</td>
</tr>
<tr>
<td>Lidocaine 10 mM</td>
<td>16</td>
<td>2</td>
<td>12.5 *</td>
</tr>
<tr>
<td>Lidocaine 500 µM</td>
<td>13</td>
<td>0</td>
<td>0 *</td>
</tr>
<tr>
<td>TTX 250 nM</td>
<td>8</td>
<td>4</td>
<td>50.0</td>
</tr>
<tr>
<td>CHOL 50 µM</td>
<td>24</td>
<td>13</td>
<td>54.2</td>
</tr>
<tr>
<td>7KC 50 µM</td>
<td>36</td>
<td>10</td>
<td>27.8 *</td>
</tr>
<tr>
<td>MBCD 10 mM</td>
<td>36</td>
<td>10</td>
<td>27.8 *</td>
</tr>
</tbody>
</table>

The mecho-stimulation based assay allowed us to calculate the time between the mechanostimulation and the soma response. Since also the time at which the cell soma starts responding is known, we calculated the mean speed of conduction of the depolarisation, between the point of stimulation on the axon and the cell body. We found that, compared to control condition, the mean speed of conduction upon lipid raft disruption was significantly lower. Cholesterol treatments had no effect (Mean speed (in µm/sec) ± SEM: CTR = 20.8 ± 2.1, n=28; CHOL = 19.1 ± 3.4, n=13; 7KC = 12.3 ± 2.2 *, n=10; MBCD= 12.4 ± 2.6 *, n=10; *= p<0.05 vs CTR., Mann-Whitney U Test. Calculated power value for the paired couples are: CTR vs. CHOL = 0.15, CTR vs. 7KC = 0.99, CTR vs. MBCD = 0.99 ) (Fig. 3.13).
Figure 3.13) Lipid raft disruption determines a reduction in the speed of conduction. The graph shows quantification of the speed of conduction in the different treatments. Values are presented as percentage ± SEM. *= p < 0.05 vs. CTR, Mann-Whitney U test.
3.3.10 Soma Mechano-stimulation: effect of lipid raft disruption.

We also directly probed the cell body of DRG neurons in the different conditions to test 1) the mechano-sensitivity of the soma and 2) the effect of lipid raft disruption on mechano-transduction. We found that cell bodies of DRG neurons were mechano-sensitive, and upon stimulation an increase of fluorescence was evoked. Fig.3.14 shows a representative response of a DRG cell body responding to mechano-stimulation.

**Figure 3.14** Mechanical stimulation of the cell soma of DRG neurons *in vitro*. The figure shows a representative neuron loaded with Fluo-4 responding to a mechanical stimulus delivered to the soma. A) Shows the Fluo-4 fluorescence and DiC image of a DRG neuron. The glass probe is visible in the DiC image, at the moment of contact with the soma (arrow) B)
Shows the Fluo-4 fluorescence in pseudo-color, associated with different time points during the recording. C) The graph shows the recorded fluorescence intensity of different region of interests (ROIs), visible in A. Note that increase of fluorescence was evident also along the axon (ROI 2). The arrow indicates the time point when the cell has been stimulated; cardinal numbers refer to the time points which the images in B are associated to. Scale bar is 10 µm.

Differently from what we found upon axonal stimulation, when we disrupted lipid rafts the cell response was not altered following this method of stimulation (Table 3.2).

Table 3.2. Effect of different treatments on cell responsiveness, upon soma mechanical stimulation. Table summarises the results of soma mecho-stimulation of DRG neurons in vitro. Percentages of neuronal cell bodies responsive to mechanical stimulation of the soma, in the different conditions, are listed. p > 0.5 vs. CTR. Fisher’s exact test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Responsive</th>
<th>% Responsive Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR</td>
<td>33</td>
<td>20</td>
<td>60.6</td>
</tr>
<tr>
<td>CHOL 50 µM</td>
<td>18</td>
<td>11</td>
<td>61.1</td>
</tr>
<tr>
<td>7KC 50 µM</td>
<td>33</td>
<td>21</td>
<td>63.6</td>
</tr>
<tr>
<td>MBCD 10mM</td>
<td>38</td>
<td>26</td>
<td>68.4</td>
</tr>
</tbody>
</table>
3.3.11 Chemical-stimulation based assay in vitro: DRG neuron culture in Campenot chambers.

We further investigated action potential propagation along the axons of DRG neurons in control and raft depleted conditions by exploiting the properties of Campenot chambers, which allow a compartmentalisation of different parts of the neurons.

We first developed a culture system where DRG neurons can be functionally segregated in different compartments. We used a Campenot chamber (for details of the dimensions see materials and methods; section 3.2.7) with three separate chambers. The protocol we used to set the culture chamber is known to segregate the different compartments (Campenot, 1977). Nevertheless, we wanted to confirm that in our hands the technique is efficient. We set a chamber and imaged the side chamber when a fluorescent dye was present in the middle chamber. The images in Fig. 3.15 show that no leakage occurred between the chambers.

**Figure 3.15** No leakage occurs between the chambers. We filled the middle chamber with 1% Texas Red in normal solution, and kept the side chambers filled with normal solution. After 5’ and 30’ we monitored the side chamber and no leak of Texas Red from the middle chamber was detected.
Once DRG neurons were plated, neurite outgrowth was promoted by creating a density gradient of NGF across the chambers (see section 3.2.7). At the day of the experiments, DRG cell bodies (Fig. 3.16, arrow heads) were confined in the Soma chamber and projected axons, through the Mid-chamber, to the furthest chamber (Axon chamber) (Fig. 3.16)

![Diagram of the Campenot chamber setup](image)

**Figure 3.16** DRG neurons cultured in Campenot chamber. The composite image shows an immunofluorescence for neuronal marker III β-Tubulin (βTub). Cell bodies (arrow heads) are restricted in the Soma chamber. Bundles of axons projects through the Mid-chamber to furthest Axon chamber. Note that axons beneath the walls are not visible because, during immunostaining, the walls prevented antibody binding. Scale bar is 100 µm.

### 3.3.12 Chemical stimulation of DRG neurons in Campenot chambers.

We tested the excitability of axons to chemical stimulation. We also ascertained if the axonal stimulation is strong enough to depolarise the axons to an extent that is sufficient to evoke depolarisations that travel towards the cell bodies. We loaded the cells (all compartments) with Fluo-4 and stimulated only the axons by applying a chemical mix to the Axon chamber (furthest chamber from Soma chamber, see also Fig 3.16). We used a cocktail composed of Capsaicin 10 µM, ATP 300 µM and Bradykinin 10 µM (CAB). These chemicals were used because DRG neurons have been shown to be excited by these agents and to respond by
triggering action potentials (see Introduction; section 3.1.4). When axons were subjected to CAB application they displayed a rapid increase of Fluo-4 fluorescence (stimulation and visualisation of the same chamber: Axon chamber; see Fig. 3.16), clearly showing that the axons in the Axon chamber can be efficiently excited with these chemicals (Fig. 3.17).

Figure 3.17) Chemical stimulation of DRG neurons: axonal stimulation and axonal recording. The figure shows axons in the Axon chamber loaded with Fluo-4 and responding to a chemical stimulus (CAB). A) Shows the Fluo-4 fluorescence and DiC image of a the axons. B) Shows the Fluo-4 fluorescence in pseudo-color, associated with different time points during the recording. C) The graph shows the recorded fluorescence intensity of different ROIs, visible in
A. The arrow indicates the time point when the cell has been stimulated; cardinal numbers refer to the time points which the images in B are associated to. Scale bar is 100 µm.

Most importantly, for the purpose of our experiment, we found that axonal stimulation carried out in the Axon chamber is able to elicit a Calcium influx that travels through the Mid-chamber and invades the cell bodies in the Soma chamber. In fact, CAB application only in the Axon chamber elicited a fluorescence increase at the level of the cell bodies in the Soma chamber. Application of vehicle did not elicit any response (Fig. 3.18).

Figure 3.18) Chemical stimulation of DRG neurons: axonal stimulation and soma recording. The figure shows the effect, at the level of the cell bodies, of axonal chemical stimulation. A) Shows the Fluo-4 fluorescence and DiC image of the cell bodies (Soma chamber, arrow heads) and axons projecting to the Axon chamber, through the Mid-chamber. B) The graph shows the recorded fluorescence intensity of different cell bodies visible in A (Soma chamber, arrow heads). Each data point is the mean fluorescence intensity ± SEM of different cell bodies (n=58). Images in pseudo-color represent the Soma chamber Fluo-4 fluorescence. The black arrow indicates the time point when Vehicle has been applied. Red arrow indicates when the
axons have been stimulated with CAB; cardinal numbers refer to the time points which the images in pseudo-color are associated to. Scale bar is 100 µm.

3.3.13 Chemical stimulation of DRG neurons in Campenot chambers: Sodium free condition and effect of TTX.

We have investigated the nature of the propagating depolarisation by substituting NaCl in the Mid-chamber only with equimolar Choline chloride. In this set-up, since the chambers do not allow mixing of the media, the Axon chamber, which is stimulated by CAB, and the cell bodies are in the presence of extracellular sodium. When CAB is applied to the axonal terminals, in the absence of sodium in the Mid-chamber, we found that no cell responded with an increase of fluorescence (0% responsive cells; experiment performed in triplicate). This data clearly shows that sodium ions are needed in the Mid-chamber for the propagation of the action potentials towards the cell bodies. Moreover, when Choline chloride is replaced with NaCl in the Mid-chamber, cell body responsiveness was restored and the CAB stimulation at the level of the axons determined an increase of fluorescence in the Soma chamber. In fact, the same cells which do not respond in the absence of sodium (Fig. 3.19 A, B) became responsive with the presence of sodium in the Mid-chamber, upon chemical stimulation.

A)

B)
Figure 3.19) Chemical stimulation of DRG neurons: Sodium free condition. The figure shows the effect, at the level of the cell bodies, of axonal chemical stimulation in the absence (Sodium free) and presence (Normal solution) of NaCl. A) Shows the Fluo-4 fluorescence and DiC image of the cell bodies (Soma chamber). C) The graphs show the recorded fluorescence intensity of different cell bodies visible in B. The black arrow indicates the time point when CAB has been applied. Scale bar is 100 µm.

We have also investigated the contribution of TTX-r currents in the propagation of action potential. For this purpose we have added 250 nM TTX to the Mid-chamber only. This concentration is known to completely block all TTX-s channels. Na\textsubscript{v}1.8, being TTX-r, is spared from blockage at this concentration (Akopian et al., 1996; Akopian et al., 1999b; Renganathan et al., 2001; Sivilotti et al., 1997). In this condition, the TTX-s channels in the axonal compartment (where stimulation is carried out) and in the soma compartment are not blocked. We have found that the blockage of TTX-s currents does not impair action potential propagation. In fact, compared to CTR condition, the same number of cells responded to chemical stimulation (mean percentage of responsive neurons in TTX treated samples, expressed as % of CTR ± SEM; CTR = 100% ± 35.9, TTX = 109% ± 12.1; n=3; p=1, Mann-Whitney test. Calculated power = 0.08) (Fig. 3.20). This result shows that the majority of sodium currents encoding the propagation of the action potential are indeed mediated by TTX-r channels.
Figure 3.20) TTX-r VGSC mediate action potential propagation from the axon terminals to the cell bodies. The graph shows the quantification, at the level of the cell bodies, of axonal chemical stimulation in the presence of TTX in the Mid-chamber.

3.3.14 Chemical stimulation of DRG neurons in Campenot chambers: lipid raft disruption.

Having demonstrated that action potential propagation is mostly mediated by TTX-r currents (e.g. Na\textsubscript{v}1.8) we investigated the effect of lipid raft disruption and Na\textsubscript{v}1.8 shift to the soluble fraction on the propagation of action potentials. For this purpose we have disrupted lipid rafts with 7KC or MBCD in the Mid-chamber only and applied CAB to the axonal terminals (Axon chamber) and recorded fluorescence intensity at the level of the cell body (Soma chamber). It is worth to stress the fact that axon terminals and cell bodies are unaffected from lipid raft disruption and the shift of Na\textsubscript{v}1.8 to the soluble fraction only occurs in the Mid-chamber. Control cells were either left untreated or treated with cholesterol. Upon CAB application we have quantified the number of cells responsive to the chemical stimulation. Cells that showed an increase of fluorescence were classified as responsive. In particular, the increase of fluorescence had to be higher than 3%, compared to baseline. This value was chosen as it represents at least three times the increase that occurred in few cells in CTR condition after vehicle application (1% increase in 1 out of 58 cells). Also, the increase of fluorescence had to show a transient profile to be classified as positive. Cells with fluorescence oscillation before
the chemical stimulation were not included in the analysis. Fig. 3.21 shows representative increases of fluorescence that occurred in cells bodies upon CAB stimulation of the axons.

Figure 3.21) Representative profiles of DRG neurons responding to axonal stimulation. The figure shows cell bodies in the Soma chamber loaded with Fluo-4 responding to a chemical stimulus (CAB). A) Shows the Fluo-4 fluorescence and DiC image of the cell bodies. B) Shows the Fluo-4 fluorescence in pseudo-color, associated with different time points during the recording. Arrow head indicates a cell body which responded to the chemical stimulation. C)
The graph shows the recorded fluorescence intensity of different ROIs, visible in A. The arrow indicates the time point when the axons have been stimulated; cardinal numbers refer to the time points which the images in B are associated to. Scale bar is 100 µm.

Importantly, the different treatments did not alter the morphology of axons in the Mid-chamber (Fig. 3.22).

<table>
<thead>
<tr>
<th>CTR</th>
<th>CHOL</th>
<th>7KC</th>
<th>MBCD</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Fluo-4" /></td>
<td><img src="image2" alt="Fluo-4" /></td>
<td><img src="image3" alt="Fluo-4" /></td>
<td><img src="image4" alt="Fluo-4" /></td>
</tr>
</tbody>
</table>

Figure 3.22) Treatments did not alter the morphology of neurites in the Mid-chamber. The figure shows neurites in the Mid-chamber loaded with Fluo-4, after the treatments. Scale bar is 100 µm.

In Table 3.3 we list the raw data relative to the chemical stimulation in Campenot chamber. We found variability across different experiments in terms of absolute number of responsive neurons, together with a consistent decrease in the percentage of neurons responding to chemical stimulation upon raft depletion. Because of this reason in Table 3.4 we show the percentage change of cells responding to the chemical stimulation compared to control cells, for ease of comparison between the different groups. We found that 7KC and MBCD treatments significantly decreased the percentage of cells responding to the chemical stimulation. On the other hand, cholesterol treatment did not cause any effect on action potential conduction.
Table 3.3. Campenot set-up raw data. Table lists the percentage and the absolute number of neurons responding to the axonal chemical stimulation (responsive neurons / total neurons per field of view).

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>CTR Treatment</th>
<th>CHOL Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.7% (17/82)</td>
<td>28.3% (19/67)</td>
</tr>
<tr>
<td>2</td>
<td>7.7% (8/104)</td>
<td>2.7% (3/109)</td>
</tr>
<tr>
<td>3</td>
<td>3.5% (3/86)</td>
<td>4.8% (5/105)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>CTR Treatment</th>
<th>7KC Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.9% (3/61)</td>
<td>0% (0/58)</td>
</tr>
<tr>
<td>2</td>
<td>5% (3/59)</td>
<td>2.1% (1/46)</td>
</tr>
<tr>
<td>3</td>
<td>2.5% (2/81)</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>1.9% (3/158)</td>
<td>0% (0/160)</td>
</tr>
<tr>
<td>5</td>
<td>3.5% (2/57)</td>
<td>2.2% (1/44)</td>
</tr>
<tr>
<td>6</td>
<td>10.5% (6/57)</td>
<td>2.2% (1/44)</td>
</tr>
<tr>
<td>7</td>
<td>20.7% (17/82)</td>
<td>14.1% (13/92)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>CTR Treatment</th>
<th>MBCD Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98.3% (57/58)</td>
<td>4.5% (3/66)</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>20% (20/100)</td>
</tr>
<tr>
<td>3</td>
<td>46.6% (14/30)</td>
<td>5.5% (4/72)</td>
</tr>
<tr>
<td>4</td>
<td>5.8% (1/17)</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>5.7% (3/52)</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>1.9% (3/158)</td>
<td>1.0% (2/184)</td>
</tr>
<tr>
<td>7</td>
<td>18% (6/33)</td>
<td>8.7% (5/57)</td>
</tr>
<tr>
<td>8</td>
<td>10.5% (6/57)</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3.4. Lipid raft disruption impairs action potential propagation in DRG neurons. Table shows the percentage of neurons responding to the axonal chemical stimulation after the different treatments. Data are presented as means of responsive neurons expressed as % of CTR ± SEM. *=p < 0.05 vs CTR, Mann-Whitney test. Calculated power value is shown in brackets for each paired couple.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CHOL Treatment</th>
<th>7KC Treatment</th>
<th>MBCD Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR</td>
<td>100 % ± 48.7</td>
<td>112 % ± 77.3 (0.05)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 % ± 7.7</td>
<td>34.5 % ± 12.8 * (0.99)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 % ± 27.1</td>
<td>34.4 % ± 10.8 * (0.89)</td>
<td></td>
</tr>
</tbody>
</table>
3.4 Discussion

Investigation of the disruption of the association between Na\textsubscript{v}1.8 and lipid rafts.

We have demonstrated in the previous chapter that Na\textsubscript{v}1.8 associates with lipid rafts \textit{in vivo} and \textit{in vitro}. We further explored this finding by investigating the functional meaning of this association. The strategy that we pursued was to disrupt lipid rafts and to test how Na\textsubscript{v}1.8 partitions. A functional assay was also needed to determine how Na\textsubscript{v}1.8 function changes after lipid rafts disruption, to unmask the potential physiological meaning of Na\textsubscript{v}1.8-raft association. The first section of the discussion will analyze the results regarding the disruption of rafts and the effect on Na\textsubscript{v}1.8 obtained by biochemical and imaging techniques. The second part of the discussion will focus on the development of functional assays and on the effect of lipid raft disruption, and Na\textsubscript{v}1.8 shift to the soluble portion of the membrane, on action potential propagation.

3.4.1 Lipid raft disruption: biochemical and imaging techniques.

We have disrupted lipid rafts by using two complementary approaches, which interfere with different aspects of lipid raft nature: MBCD treatment and 7KC delivery. MBCD is the most common agent used to disrupt lipid rafts (Simons and Toomre, 2000). Lipid rafts are highly enriched in cholesterol, and its presence is necessary for raft stability (Pike, 2003). MBCD is a cyclic oligosaccharide which binds to sterols. If added to the media it depletes cholesterol from the cell membrane and leads to raft disruption (Dart, 2010). On the other hand, 7KC represents a novel tool to disrupt lipid rafts. 7KC is an analogue of cholesterol which, if present on the membrane, impedes the tight packing of the saturated fatty acid chains. This physical alteration perturbs lipid raft stability by decreasing the lipid order (Owen and Gaus, 2010; Rentero \textit{et al.}, 2008) (for a detailed description of these compounds and mode of action see the introduction; section 3.1.2). We have first tested the effects of these compounds on raft
integrity *in vitro* by monitoring the flotation pattern of Flotillin-1, a protein raft marker. In fact, upon MBCD treatment or 7KC delivery two different scenarios can be predicted. In MBCD treated cells cholesterol is removed from the membrane and rafts, by lacking its constituent, are not present, or reduced, on the cell surface. In 7KC treated samples, on the other hand, cholesterol is still present in rafts, but the inclusion of 7KC decreases the lipid order. Hence, both treatments induce fundamental changes in rafts’ physical properties. Since lipid rafts are resistant to non-ionic detergents thanks to their physical properties any alteration of these should be reflected in their ability to resist non-ionic detergent lysis. These consequences can be monitored by analysing the floatation pattern of raft associated proteins. In fact, if rafts ability to resist lysis is affected they will be lysed with the rest of the membrane. Consequently raft proteins, since they do not associate with rafts anymore, lose their floating properties in a density gradient. In our conditions, both agents proved to impair raft stability. Indeed, compared to control condition, in 7KC and MBCD treated samples, Flotillin-1 is also recovered from the soluble fractions. This effect was more prominent for MBCD, where no Flotillin-1 was associated with floating fractions, compared to the tailing effect present in 7KC treated samples, where Flotillin-1 was distributed across the gradient. From this result we can conclude that both MBCD and 7KC impaired raft stability. These data confirmed two lipid rafts hallmarks in our system: they are dependent on cholesterol and on a lipid ordered phase. We also found that MBCD proved to be a stronger agent than 7KC. The effect could be attributed to the different modes of action of the compounds. It is possible that the effect of MBCD, which results in stripping of cholesterol from the membrane, has a more profound effect on raft stability, compared to the effect of 7KC, which impairs the ability of rafts to be detergent resistant by decreasing their lipid order and not by removing cholesterol from the cell surface. In summary, we have demonstrated that MBCD and 7KC disrupt lipid rafts.

We asked what the effect of these treatments would be on Na\textsubscript{v}1.8 buoyancy. Since we found that Na\textsubscript{v}1.8 associates with rafts *in vitro* we would expect an alteration of its floating pattern, upon raft disruption. We found that both MBCD and 7KC treatments had the same effect on Na\textsubscript{v}1.8. Na\textsubscript{v}1.8 was found to be associated only with the soluble portion of the membrane and the raft pool of Na\textsubscript{v}1.8 was completely absent after the treatments. This result demonstrates that the floating pool of Na\textsubscript{v}1.8 is indeed raft associated in DRG neurons and that the methods we employed to interfere with rafts integrity are useful tools to induce a shift of raft
associated Na$_{1.8}$ to the soluble portion (non-raft) of the membrane. This evidence also opens the way to the investigation of Na$_{1.8}$ properties when it is raft associated and when it is not.

We have further investigated the effect of MBCD and 7KC on the lipid phase of live cell. We have used a completely different approach: live cell imaging. Noticeably this method does not involve any detergent extraction and is carried out at physiological temperature. We have exploited the properties of the environmentally sensitive styryl dye di-4-ANEPPDHQ. Di-4-ANEPPDHQ is water soluble and shows minimal background fluorescence in water. When it binds the outer leaflet of the plasmamembrane it becomes fluorescent, and is characterised by a large Stoke shift. This dye has the interesting property to act as a lipid phase probe. It was first described to distinguish between liquid ordered and liquid disordered phase in model membranes. It displays a red-shift of the emission spectra in lipid disordered phase, compared to lipid ordered phase (Jin et al., 2005). It is worth to underline that this feature is dependent on the lipid phase itself and not on preferential binding of the dye to certain class of proteins. Indeed, it has been discovered that di-4-ANEPPDHQ emission and excitation spectra (in both lipid raft and soluble part of the membrane) are unaffected by the presence of peptides (Dinic et al., 2011). Also, di-4-ANEPPDHQ emission spectra is dependent on cholesterol content (Jin et al., 2006). It has been proposed that these properties rely on the effect of the solvent on the dye’s fluorescence emission. When the dye is buried into the plasmamembrane in a stiff environment (e.g. lipid raft) it shows a smaller Stoke shift, compared to a more soluble environment. A stiffer environment limits the dispersion of the energy from the excited dye to the surrounding lipids, hence more energy is retained by the dye for the emission phenomenon, which occurs at lower wavelengths (Jin et al., 2006). Importantly for the purpose of our discussion, this dye is suitable to image lipid phase in live cells. Di-4-ANEPPDHQ has been reported as a lipid raft probe in live HeLa cells, Neutrophils and T-cells (Jin et al., 2006; Owen and Gaus, 2010; Owen et al., 2006; Owen et al., 2007; Owen et al., 2010). We found that MBCD and 7KC impairs raft stability. We thought that this could correlate to an altered lipid phase. For this purpose we used di-4-ANEPPDHQ to stain live DRG neurons after lipid raft disruption. We found that both 7KC and MBCD determined a red shift of the dye’s spectra. Cholesterol treatment did not influence the lipid phase, as the related spectrum completely overlapped to the spectrum of untreated cells. This demonstrates that, in our model, the concentration of cholesterol we used was below the needed concentration to induce cholesterol-insertion dependent modification of the membrane, which is in accordance
with published findings (Rentero et al., 2008). When cholesterol homeostasis is studied, usually, much higher concentration are needed to detect physiological differences (Lundbaek et al., 2004). Our data perfectly match with the expected outcome of 7KC and MBCD treatments. In fact, regardless of the mode of action, both compounds interfere with lipid rafts; impairment of tight lipid packing (7KC action) and loss of rafts (MBCD action) action should correlate with a decrease of lipid order and we found that di-4-ANEPPDHQ emission spectra shifted to redder wavelengths compared to control cells upon both treatments. Hence, the dye’s spectrum shift is consistent with an increase of membrane disorder in DRG neurons and it confirms that 7KC and MBCD impairs lipid rafts in our model. Interestingly, we also found that the spectra of 7KC and MBCD treated cells was different at longer wavelengths (e.g. 7KC was narrower). This effect is consistent with the dye’s properties. Indeed, it has been reported that an increase of cholesterol concentration determine a narrowing of the emitted spectrum, or in other words the decrease of cholesterol results in a broader emission spectrum (Jin et al., 2006). The difference between 7KC and MBCD spectrum could be due to the fact that, even though both treatments increase lipid disorder and both spectra are red-shifted compared to control and cholesterol samples, in MBCD treated samples membrane cholesterol is depleted and this is mirrored in a less steep shoulder of the spectrum, compared to 7KC (where cholesterol content is unaffected).

In conclusion, we have used to two different methods to disrupt lipid rafts and two complementary techniques to confirm and assess the degree of disruption. The data combined from biochemistry and imaging approaches demonstrate that 7KC and MBCD determine a loss of lipid order on the membrane and lead to lipid rafts disruption. Importantly, in these conditions, Na\textsubscript{v}1.8 is present in the soluble part of the membrane and the cells completely devoid of raft associated Na\textsubscript{v}1.8.

By imaging techniques we have also investigated the sub-cellular localisation of raft markers and Na\textsubscript{v}1.8. We reported (see Chapter 2; Results section) that GM1, Flotillin-1-Dronpa and Na\textsubscript{v}1.8 display a characteristic clustered distribution along the axons of neurites. Since one of lipid rafts role is to act as platforms to stabilise protein complexes on the membrane (Dart, 2010; Hering et al., 2003; Zhu et al., 2006) we hypothesised that raft disruption would lead to a dispersion of the clusters into an even distribution. For example, it was reported that AchR clusters at the post-synaptic site in neuromuscular junctions are stabilised by cholesterol, and its depletion via MBCD lead to a disassembly of the clusters. In more details, it was found that
rafts allowed SRC-Family protein tyrosine Kinases (SKFs) to act in the stabilisation of AchR clusters by phosphorylating AchR and by maintaining their association with the cytoskeleton (Willmann et al., 2006). MBCD also disrupts the clustered distribution of AchR in the somatic spines of ciliary neurons. It was found that raft integrity was necessary to maintain the clusters at the cell surface (Bruses et al., 2001). We found that regardless of the treatments, GM1, Flotillin-1-Dronpa and Na\textsubscript{v}1.8 clusters were still present on the membrane. GM1 visualisation (as we discussed before, see discussion Chapter 2) depends on the patching mediated by CTB binding to GM1 and Streptavidin binding to CTB. Upon lipid raft disruption, changes at the molecular level may occur and alter GM1 distribution, which we cannot detect at a single molecule level because of the intrinsic limit of light microscopy. Hence, the clustering activity of CTB-Streptavidin method that we use to visualise raft components could mask the effect of raft disruption on GM1 sub-cellular localisation, by bridging molecules of GM1 that are not associated in rafts anymore. Distribution of Flotillin-1 is affected when rafts are disrupted, as we demonstrated by biochemistry techniques. Nevertheless, when we employed imaging method to detect its sub-cellular localisation we did not detect any gross change. For this purpose it would be interesting to employ microscopy techniques which overcome the limit of optical resolution, like FRET, PALM, SEM and single molecule tracking to understand what the local effect of raft disruption is at a molecular level. Na\textsubscript{v}1.8 clusters were also apparent after lipid raft disruption, suggesting that the clustered appearance may not be dependent on raft integrity. Our data suggests that Na\textsubscript{v}1.8 clusters represent a terminal organization of the channel (see data from ex vivo samples; section 2.3.5). It is known that sodium channels interact with membrane (e.g. VGSC \(\beta\) subunit, Contactin), and intracellular proteins (e.g. Ankyrin) that stabilise the channels on the membrane, by bridging it either directly or indirectly to the cytoskeleton (Cusdin et al., 2008; Lai and Jan, 2006; Poliak and Peles, 2003). Specifically for Na\textsubscript{v}1.8 it has been reported that Contactin regulates Na\textsubscript{v}1.8 expression and currents along the axons (Rush et al., 2005). Also, Na\textsubscript{v}1.8 has a conserved Ankyrin binding motif (VPIAEGESD) which may mediate a direct association of the channel, via its intracellular loop, with the cytoskeleton (Lemaillet et al., 2003). It could be hypothesised that lipid rafts role is not to maintain Na\textsubscript{v}1.8 clusters, and upon raft disruption Na\textsubscript{v}1.8 localisation is retained by its association with intracellular proteins. It is worth to highlight that our experimental set-up was aimed to analyse potentially short term effects of raft disruption on Na\textsubscript{v}1.8 localisation. It would be interesting to assess Na\textsubscript{v}1.8 localisation at later time points after lipid raft disruption.
We have demonstrated that 7KC and MBCD disrupt lipid rafts. We have tested if these treatments had an acute toxic effect on DRG neurons. For this purpose we used Propidium Iodide to stain DNA. Propidium Iodide is a fluorescence intercalating agent that increases its fluorescence 30 folds when it binds DNA. Since it is membrane impermeable it can only bind the nuclei of neurons with a compromised cell membrane. In our hands we found that lipid raft disruption did not induce any toxic effect resulting in membrane disruption. This data further supports that 7KC and MBCD short term effects on raft integrity and lipid phase of the cell membrane are specific and not due to pleiotropic toxic effects.

3.4.2 Lipid raft disruption: functional assays.

We demonstrated that Na\textsubscript{v}1.8 associates with lipid rafts in DRG neurons and that upon treatments with 7KC and MBCD it shifts to the soluble portion of the membrane. Na\textsubscript{v}1.8 mediates the majority of sodium influx during action potentials (Renganathan \textit{et al.}, 2001). It also encodes TTX-r currents which are able to propagate along unmyelinated fibres. In fact, classic studies pointed at Na\textsubscript{v}1.8 as a key player in action potential propagation in unmyelinated fibres. The first evidence that Na\textsubscript{v}1.8 supports action potential propagation in C-fibres came from studies performed in the early nineties. Jeftinija showed that in a spinal cord-dorsal root ganglion preparation, application of high concentration potassium to DRG (cell bodies, dorsal root and peripheral processes) evoked action potentials that propagated to the spinal cord where they triggered excitation of dorsal horn neurons. Inhibition of TTX-s channels blocked action potential propagation in large fibres, but it did not affect the propagation in unmyelinated nociceptors. This indicates that Na\textsubscript{v}1.8 is sufficient to mediate the propagation of action potentials in slowly conducting fibres (Jeftinija, 1994b). The same author later described a similar finding by using Bradykinin, a pro-inflammatory peptide, as the excitation agent. It was found that the stimulatory effect of Bradykinin (depolarisation and firing of action potentials) on DRG neurons was TTX resistant. In the presence of TTX, application of Bradykinin to DRG neurons still evoked excitatory action potentials in dorsal horn neurons. Bradykinin also potentiated the postsynaptic potentials recorded in the dorsal horn, induced by electrical stimulation of TTX-resistant afferent fibres. This finding further demonstrates that TTX-r currents are able to mediate propagation of action potentials from DRG neurons to the spinal cord (Jeftinija, 1994a). Furthermore, conduction of TTX-r action potentials has been
described in unmyelinated C-fibres from human sural nerve (Quasthoff et al., 1995). Na_\text{v}1.8 is expressed in the unmyelinated fibres in cornea, which originate from the trigeminal ganglia, where it encodes TTX-r currents (Black and Waxman, 2002). In these fibres TTX-r action potential propagation has also been shown. In fact ongoing electric activity, mechano, thermal and chemical stimuli evoke action potentials in the cornea, and these are conducted in the presence of TTX (Brock et al., 1998). Moreover TTX-r action potential propagation has been described to occur in C fibres along the dorsal roots (Steffens et al., 2001).

All these data collectively show that Na_\text{v}1.8, which mediates TTX-r currents, is sufficient to mediate in certain conditions action potential propagation. Given these evidence, in order to test if Na_\text{v}1.8-raft association is physiologically relevant, we decided to investigate action potential propagation in small diameter neurons (e.g. nociceptors) in control conditions and in neurons where, upon lipid raft disruption, Na_\text{v}1.8 is only associated with the soluble fraction of the membrane. We developed two assays to generate and propagate action potentials: direct mechano-stimulation and chemical stimulation of DRG neurons in vitro.

### 3.4.3 Mechano-stimulation based assay.

Our aim is to study the effect of lipid raft disruption on Na_\text{v}1.8 functions. We used in first instance, for this purpose, voltage sensitive dyes and a sodium sensitive dye, whose fluorescence is dependent on the membrane voltage and intracellular sodium concentration, respectively. Both methods proved to be hindered by technical limits (data not shown). For this reason we have investigated action potential propagation upon mechanical stimulation of the axons, by using Fluo-4, a calcium sensitive dye. Nociceptors, which express Na_\text{v}1.8 (Djouhri et al., 2003a), respond to high-threshold mechanic stimulation in vivo (Wall and Melzack, 2005) and maintain this ability when cultured. Nociceptors, following a direct mechanic stimulation of the cell bodies (Gschossmann et al., 2000; McCarter and Levine, 2006; McCarter et al., 1999) or the neurites (Hu and Lewin, 2006), encode inward currents and generate action potentials. Similarly to what we have reported, mechanical stimulation of the axons also evokes a propagating wave of Fluo-4 fluorescence from the point of stimulation (Sanchez et al., 2007). In agreement with data in literature we have found that a direct mechanical stimulation of the axons of DRG neurons in vitro evokes an increase of calcium at the level of
the cells body, showing that the focal stimulus on the periphery evokes an excitation event that is propagated to the cell body. In our system we have further investigated the nature of such excitation and correlated it to the function of Na\textsubscript{v}1.8. In agreement with published data (Gschossmann \textit{et al.}, 2000), we found that in the absence of extracellular calcium no cell body responded. This implies that the intracellular stores do not participate in the raise of calcium upon stimulation at the level of the axons and cell bodies. We also performed the experiment in sodium free conditions. We replaced extracellular sodium with Choline chloride. This compound is commonly used in electrophysiological experiments and allows maintaining positive charges and the right osmolarity in the extracellular media in the absence of sodium (Allard \textit{et al.}, 2006; Fitzgerald \textit{et al.}, 1999). Remarkably we found that in this condition no peak-like increase of fluorescence was detected at the level of the cell bodies, upon axonal stimulation. Occasionally we found that stimulation of the axons evoked a slow rise of fluorescence at the level of the cell bodies. Since calcium free conditions demonstrated that the intracellular stores are not involved in the propagation of the signal, the extracellular calcium could be the reason of this slow increase of fluorescence. In literature it has been reported that voltage gated calcium channels can underlie action potential (Quasthoff \textit{et al.}, 1995; Renganathan \textit{et al.}, 2001). It is possible that these channels are responsible for this slow component of the excitation which propagates to the cell bodies. For this reason we only focused on the fast, sodium dependent, component of the increase of fluorescence at the level of the cell bodies. The sodium free condition clearly shows that the event of stimulation evokes a sodium conductance which is responsible for the excitation of the cell bodies. This strongly points to an involvement of sodium channels in this process. Furthermore, we have correlated the increase of fluorescence of the cell bodies to voltage gated sodium channels and Na\textsubscript{v}1.8 activity. We used Lidocaine, a local anaesthetic, at concentrations which block voltage gated sodium channels (Chevrier \textit{et al.}, 2004; Gu and MacDermott, 1997), to investigate the role of this class of protein in action potential propagation. We found that the blockage of voltage gated sodium channels dramatically decreased the number of cell bodies responding to the axonal stimulation, demonstrating the these channels are involved in the propagation of the depolarisation to the soma from to the focal contact on the axons. Also, the pharmacological modulation of mechano-stimulation, together with the evidence that we were able to repetitively stimulate the same cells, show that we produced mechanically gated currents as opposed to artifacts (e.g. increase of ionic conductance due to membrane rapture). Na\textsubscript{v}1.8 is a TTX-r channel, hence we exploited this feature to analyse its role in action potential propagation.
propagation. In the presence of TTX, at a concentration which blocks all TTX-s channels (Akopian et al., 1999b; Goldin et al., 2000; Renganathan et al., 2001; Yu and Catterall, 2003), the axonal stimulation still evoked an increase of fluorescence at the level of the cell body. This shows that Na\textsubscript{v}1.8, in our condition, is sufficient to underlie action potential propagation in DRG neurons. Our combined data demonstrate that mechanical stimulation evokes a depolarisation at the level of the neurite which propagate away from the point of contact towards the cell body. The propagation of the excitation relies on the presence of sodium and more specifically on the activity of voltage gated sodium channels. Most importantly, Na\textsubscript{v}1.8 alone is sufficient to let the action potentials propagate.

A potential limitation of our set-up is that is not possible to directly correlate the mechano-stimulation with a stimulus that would produce nociception in vivo. In fact, unmyelinated fibres in adult animals are surrounded by other cell types/extracellular matrix which could be physically involved in the propagation of the force from the point of stimulus to the axons. A direct investigation of mechano-transducers in vivo has been hampered by technical difficulties; the small size and inaccessibility of unmyelinated mechano-sensitive endings have avoided a direct analysis of electric properties of such neurons, hence a direct correlation between pain/mechano sensations in animals and in vitro is not available at the moment. Nevertheless, mechano-stimulation in vitro has been proposed to potentially correlate to noxious mechano-sensation (Hu and Lewin, 2006). Drew et al. found that nociceptors (identified by capsaicin responsiveness) responded to higher level of pressure, compared to non-nociceptive neurons, which correlates with the finding that in vivo nociceptors have high threshold of mechanical activation (Drew et al., 2002). The same group later discovered that a conopeptide toxin analogue selectively blocked mechanically evoked currents in DRG neurons. These currents were slowly adapting and associated with small nociceptive sensory neurons (identified according to capsaicin sensitivity and expression of Peripherin). Interestingly, this peptide blocked pain behaviour in response to high intensity mechanical stimuli but did not affect the threshold to low intensity pressure, highlighting a correlation between the currents evoked in vitro and in vivo and supporting the view that the slowly adapting mechano-sensitive channels mediate the responses to noxious pressure (Drew et al., 2007). Additional evidence on the mechanostimulation of nociceptors in vitro has been provided by Di Castro et al. This group showed that signaling pathways involved in inflammatory pain conditions directly modulate the mechano-transduction apparatus of sensory neurons. PKC activation (which
induces mechanical hyperalgesia in vivo) and NGF (which is a pro-nociceptive agent) administration increased mechano activated currents. It was found that PKC mediates insertion of mechano-transducers into the membrane, while NGF increases their transcription rate (Di Castro et al., 2006). Moreover, ATP and UTP sensitise nociceptors through P2Y2 receptors and determine potentiation of mechanically activated currents in vitro via a G protein dependent pathway (Lechner and Lewin, 2009). In addition, it has been reported that DRG neurons express a channel (whose molecular identity is unknown) which is gated by a high pressure mechanical stimulus in vitro. This channel is only present in small diameter neurons and its current is modulated by pro-inflammatory agent PGE2 (Cho et al., 2002). Even if ultimately it cannot be ascertained if our mechanical stimulation in vitro mirrors nociception in vivo, it must be stressed that with our model we developed an assay to study action potential propagation, mediated by Na\textsubscript{v}1.8, in small diameter sensory neurons, which are likely to be nociceptive (Djouhri et al., 2003a).

We used this set-up to study Na\textsubscript{v}1.8 function in control samples, where the channel associates with lipid rafts and in samples where, upon raft disruption, it is associated only with the non-raft (soluble portion) of the membrane. Remarkably, in raft depleted neurons we have found a drastic reduction of cell bodies responding to propagating action potentials and the neurons which still responded showed slower action potential propagation. These data shows how the association between Na\textsubscript{v}1.8 and rafts correlates with proper action potential conduction and disruption of such association has a detrimental effect in action potential propagation. In fact less cell bodies, compared to control, respond to the propagating depolarisation. The speed of conduction is also lowered in raft depleted cells. There are several determinants of speed of conduction including membrane resistance (R\textsubscript{m}), fibre thickness, temperature, magnitude and kinetics of the active sodium currents (Hille, 2001). It would be interesting to assess the passive properties of the membrane to determine if these parameters are affected, and if they are, to which extent by raft depletion. On the other hand, it is known that rafts exert an influence on fundamental properties of proteins, and their disruption could lead to altered activity of the Na\textsubscript{v}1.8. Supporting the idea that impaired electrophysiological properties could alter speed of conduction, it is demonstrated that modification in the inactivation kinetics decrease the speed of conduction along the axons of unmyelinated fibre (De Col et al., 2008). In our experimental model we were interested in an acute perturbation of lipid rafts. It would be interesting also to gather information about the re-constitution of rafts upon their
depletion. It could be possible to deliver cholesterol after MBCD treatment to replenish its content, or allow the cells to recover after the lipid phase change and test if the raft depletion has a chronic or transient effect. It must be underlined that with the mechano-stimulation set-up we cannot exclude a priori that the mechano-transducer(s) itself is located in lipid rafts and that raft disruption alters mechano-transduction. Since the molecular identity of mechano-transducers is unknown we could not test if they partition into rafts by biochemical and imaging method. In addition, the lack of a valid positive control for mechano-stimulation does not rule out the possibility that the decrease in responsiveness of neurons upon raft depletion may be due to pleiotropic effects of the raft depleting agents, rather than a direct effect on the propagation of the depolarisation. Because of these reasons we exploited our functional assay and have investigated mechano-stimulation of the cell bodies directly, a method of stimulation which does not involve propagation of action potential. We found that raft disruption does not alter cell responsiveness. We can hypothesis that the increase of intracellular calcium can be originated by a direct flux from the extracellular media through mechano-transducers only (Gschossmann et al., 2000). Alternatively, even though several lines of evidence argue against this hypothesis (Gschossmann et al., 2000; Sharma et al., 1995), both mechano-transducers and calcium specific channels (e.g. voltage gated calcium channels), which are activated in response to the cell depolarisation, contribute to calcium influx. In our hands, direct stimulation of the cell bodies demonstrates that both mechano-transducers and/or other calcium channels potentially involved in calcium influx are not affected by raft disruption. This demonstrates that upon raft depletion the mechano-transduction mechanism and output measurement is not impaired. We cannot be sure if Na\textsubscript{v}1.8 participates in cell responsiveness upon direct soma stimulation. We can hypothesise a scenario where calcium influx is independent of voltage gated ion channels and one where they are involved. If the latter case is true we did not find any difference in cell response upon raft disruption, indicating that this method of stimulation is not affected from the association of VGSC and Na\textsubscript{v}1.8 with rafts. Interestingly, we have found that at the level of the cells bodies Na\textsubscript{v}1.8 and GM1 (a marker of rafts) did not co-localise (see chapter 2, Fig. 2.15; section 2.3.6). It is tempting to speculate that, in the hypothetical scenario where Na\textsubscript{v}1.8 does indirectly contribute to soma stimulation mediated calcium influx, raft disruption does not impair its activity since at the level of the cell body Na\textsubscript{v}1.8 is not associated with rafts in the first instance.
The data gathered from the mechano-stimulation based assay highlights that raft disruption and parallel Na₉.1.8 shift to the soluble portion, do not lead to pleiotropic effects and specifically accounts for impairment in action potential conduction. This data strongly points to a biological and physiological importance of Na₉.1.8-raft association.

3.4.4 Chemical stimulation based assay.

We explored action potential propagation also with Camenot chambers and chemical stimulation. The great advantage of this method is that it is possible to segregate different parts of DRG neurons in vitro. Camenot chambers are named after the author who first developed this method. This set-up was traditionally used in the investigation of the effects of trophic factors on different compartments of the neurons (e.g. soma vs. axons). By using these chambers it was demonstrated how NGF promotes axon outgrowth from the cell bodies to the adjacent chambers (Campenot, 1977). Compartmentalised chambers are also used in the study of protein trafficking (Cui et al., 2007; Ure and Campenot, 1997), axonal degeneration (Nikolaev et al., 2009) and viral infection (McGraw and Friedman, 2009). This method, albeit with differences, was used to study DRG-dorsal horn neurons connectivity. In this model, DRG cell bodies were forced to project axons in the adjacent chamber, where they formed functional synapses with dorsal horn neurons. It was shown that electric and chemical stimulation of the DRG neurons evoked action potential that propagated between the chambers. Also, as we have demonstrated, TTX-r action potentials propagation was described to occur in nociceptive neurons (Vikman et al., 2001). By using a three-chamber set-up we segregated the cell bodies from the conducting portion of the axons and the axonal terminals that we used to excite the cells. We don’t know the molecular fingerprint of the cells projecting to the further chambers. However, we exploited the activity of NGF to promote neurite outgrowth (Campenot, 1977; Petruska and Mendell, 2004; Sofroniew et al., 2001), therefore the cells projecting neurites are most likely peptidergic nociceptors expressing NGF receptor TrkA (Averill et al., 1995; Fang et al., 2005). It is well known that the chemicals we used to excite the axonal terminals are able to depolarise the neuronal membrane of nociceptors and to trigger action potentials. Capsaicin elicits a noxious sensation of burning pain when it binds to nociceptors in vivo. Specifically, it binds to TrpV1, a non-selective cation channel expressed by nociceptors, which is also gated by noxious temperature (Caterina et al.,
Once gated, TrpV1 depolarises the membrane due to the influx of cations, voltage gated sodium channels are engaged and action potentials generated. ATP also elicits pain sensation in vivo (Coutts et al., 1981). It binds to P2X3, a cation channel exclusively expressed in DRG neurons, and encodes a depolarizing current (Bouvier et al., 1991). Bradykinin is a nonapeptide with pro-inflammatory activity which binds to receptors B1, B2 and causes sensation of pain. Its application to nociceptors also evokes depolarising currents (Burgess et al., 1989; Jeftinija, 1994a; Wang et al., 2006) and mediates sensitisation of other pain transductors (Okuse, 2007; Premkumar and Ahern, 2000). Chemical stimulation excites DRG neurons in vitro and this activity correlates well with nociception in vivo. In fact, the chemicals described above have all been shown to bind to nociceptors in adult animals, excite them and elicit pain sensation (Wall and Melzack, 2005). We have used a cocktail of chemicals in order to maximise the responsiveness of DRG cell bodies. DRG neurons are a heterogeneous population, and small fibres can further be classified according to the expression of certain class of proteins (e.g. peptidergic vs. not peptidergic). Na\textsubscript{v}1.8 is expressed in virtually all nociceptive neurons (both peptidergic and non peptidergic) (Choi et al., 2007; Djouhri et al., 2003a). We used chemicals whose receptors are expressed in nociceptors (for details see Introduction; section 3.1.4). TrpV1 has been shown to be present in nociceptive cells, both peptidergic and non peptidergic, which encode TTX-r currents (Arbuckle and Docherty, 1995; Hwang et al., 2005; Wood and Docherty, 1997); P2X3 is present in non peptidergic nociceptors (and to a lesser extent in peptidergic fibres) (Ruan et al., 2004); Bradykinin receptors, B1 and B2 are also expressed in nociceptive neurons. B1 has been found in IB4 positive, TrpV1 expressing nociceptors if GDNF supplemented (Vellani et al., 2004) and B2 in TrkA expressing cells (Lee et al., 2002). We have demonstrated that chemical stimulation of the axonal terminals triggers calcium influx that propagates towards the cell bodies. This propagation is sodium dependent, which demonstrates that sodium channels are necessary. This finding also indirectly shows that calcium and calcium channels do not play a major role in action potential propagation. In the presence of TTX only in the middle chamber, the number of cell bodies responding to the chemical excitation was only slightly reduced (and not to a statistically significant level) compared to control cells. Importantly, this data confirms that Na\textsubscript{v}1.8 clearly mediates the majority of action potentials in the cells responding to the chemical stimulation. If the population of cells responding to the chemical stimulation was expressing mostly TTX-s channels, like non-nociceptors which do not express TTX-r channels (Djouhri et al., 2003a; Fang et al., 2002), TTX would have blocked most of the action potential conduction. The main
contributor to TTX-s currents in nociceptors is Na\textsubscript{v}1.7 (Djouhri \textit{et al.}, 2003b). In DRG neurons it is thought to boost ramp currents and enhance depolarisations which determine recruiting Na\textsubscript{v}1.8 (Cummins \textit{et al.}, 1998; Waxman, 2006b). Even though a comprehensive analysis of its sub-cellular distribution is still missing, reports showed that Na\textsubscript{v}1.7 is enriched at the axon terminals where it amplifies cell depolarisations to reach thresholds for action potential firing (Toledo-Aral \textit{et al.}, 1997; Waxman, 2006b). In our model, the finding the TTX does not impair action potential propagation supports that Na\textsubscript{v}1.7 is present at the axon terminals. On the other hand a report showed that a Na\textsubscript{v}1.7 blocker does stop action potential propagation in C fibres. However, it must be noted that the concentration of blocker used for the experiment was at least 100 times higher than the calculated IC\textsubscript{50} and at this concentration blockage of other channel isoforms could occur (Schmalhofer \textit{et al.}, 2008). It is also worth noting that Na\textsubscript{v}1.9, the other TTX-r channel expressed by nociceptors, is mainly expressed in peptidergic neurons which do not express TrkA (Fang \textit{et al.}, 2006). Since we used NGF to promote the axon outgrowth of TrkA positive neurons the expression of this channel in our system is likely to be minimal. Na\textsubscript{v}1.9 is also characterised by extra-slow kinetics and is thought to underlie a persistent current which contributes to set the resting membrane potential rather than to evoke transient currents and action potential generation (like Na\textsubscript{v}1.8) (Dib-Hajj \textit{et al.}, 2002; Priest \textit{et al.}, 2005; Waxman, 2006b). Moreover, in our set-up we used NGF but not GDNF as trophic factors and the presence of GDNF has been reported to be crucial for the expression of Na\textsubscript{v}1.9 \textit{in vitro} (Fjell \textit{et al.}, 1999a). In conclusion, the Campenot set-up allows us to investigate Na\textsubscript{v}1.8 mediated action potential conduction, and most importantly to interfere with the association between Na\textsubscript{v}1.8 and lipid rafts only in specific region of the cells. Because we sought to investigate action potential propagation we interfered with rafts stability only in the middle chamber, which contains the axonal compartment responsible of action potential propagation. Since Campenot chambers allow growth of axons beneath the septa, yet it stops media from different chambers to mix, when we disrupted lipid rafts in the middle chamber we only affected portion of the neurons present in this chamber. On the other hand, the axonal terminals, which responded to chemical stimulation, and cell bodies, which responded to the propagated action potentials, were unaffected. Thus, Campenot chambers set-up represented a valuable tool to interfere, from a spatial point of view, specifically with Na\textsubscript{v}1.8 raft association. Remarkably, when we disrupted the association of Na\textsubscript{v}1.8 with rafts in the middle chamber action potential propagation was negatively affected, as shown by less cell responsiveness compared to control.
To summarise the data presented so far we have found that Na\textsubscript{v}1.8 resides in lipid rafts along the axons of small diameter neurons, most likely nociceptors. The functional assays that we have described confirm the existing data showing that Na\textsubscript{v}1.8 mediates the majority of sodium influx during action potential (Renganathan et al., 2001) and is able to let propagate TTX-r currents (Jeftinija, 1994a, b; Quasthoff et al., 1995). They also strongly point to a functional meaning of the association between Na\textsubscript{v}1.8 and rafts. In fact our results show impaired action potential propagation in nociceptors upon raft disruption.
Chapter 4: General discussion and conclusions
4.1 Discussion

Meaning of the association between Na\textsubscript{v}1.8 and lipid rafts: possible explanations.

We have demonstrated that Na\textsubscript{v}1.8 associates with lipid rafts in small sensory neurons. Moreover, the disruption of lipid rafts leads to the dissociation of Na\textsubscript{v}1.8 from these micro-domains and this is correlated with impaired Na\textsubscript{v}1.8 mediated action potential propagation in nociceptors \textit{in vitro}. These findings strongly suggest that the association between Na\textsubscript{v}1.8 and rafts is of fundamental importance for the correct functioning of this channel and for its involvement in action potential propagation in nociceptors. In the next section we shall discuss the hypothesis to explain what the physiological base of such association might be.

4.1.1 Lipid rafts are involved in trafficking (exo-endocytosis) and clustering of Na\textsubscript{v}1.8.

We originally hypothesised that rafts on the membrane acted as platforms necessary for the maintenance of Na\textsubscript{v}1.8 clusters on the cell surface. Our experiments in raft depleted samples showed that clusters of Na\textsubscript{v}1.8 and raft markers (GM1, Flotillin-1-Dronpa) were still present. Hence, at least in the short period, lipid rafts are not necessary for the stability of Na\textsubscript{v}1.8 large clusters. Nevertheless, this finding does not rule out rafts’ potential involvement in Na\textsubscript{v}1.8 trafficking to the membrane and/or development of clusters. Lipid rafts have been shown to act as cues on the membrane where proteins, including ion channels are targeted to (Dart, 2010; Echarri \textit{et al.}, 2007; Martens \textit{et al.}, 2004; Pristera and Okuse, 2011). For example it has been found that rafts regulate Nicotinic AChR clustering at neuromuscular junctions, by facilitating the molecular interaction of Nicotinic AchR with Rapsyn and by regulating the Agrin/MuSK signaling (Zhu \textit{et al.}, 2006). It would be interesting to monitor Na\textsubscript{v}1.8 forward trafficking and cluster nucleation in control and raft depleted samples with live cell imaging (e.g. FRAP, FLIP with Na\textsubscript{v}1.8-Dronpa; Na\textsubscript{v}1.8-ACP) to assess the role of rafts in these processes.
It would be also interesting to clarify p11 role. It is not known if p11 is important for the trafficking of the channel to the membrane or for its stabilisation on it, but it is a necessary factor for Na_\text{v}1.8 presence on the membrane and, notably, it does associate with rafts. It may be proposed that Na_\text{v}1.8 targets rafts \textit{per se} or via the interaction with p11 and/or other proteins. A direct association of Na_\text{v}1.8 with rafts may be mediated by cholesterol binding or a fatty acid modification (palmitoylation, myristoylation). To date, a direct binding of Na_\text{v}1.8 to cholesterol has not been described and even though palmitoylation can occur to VGSC (Schmidt and Catterall, 1987) it has not been reported for Na_\text{v}1.8. Thus, Na_\text{v}1.8 could translocate to rafts \textit{per se} via still unknown mechanisms but at the moment evidence for this process are missing. On the other hand, it may be proposed that p11 binds to Na_\text{v}1.8 in the exocytosis pathway and promotes its insertion and/or stabilisation onto membrane lipid rafts. Given these considerations, raft disruption could determine defective Na_\text{v}1.8 trafficking/retention to the membrane, which would result in reduced sodium currents and action potential propagation failure in nociceptors. Insights on this process could come from biochemical raft preparation and imaging study in p11 KO mice, which features less Na_\text{v}1.8 on the membrane and reduced TTX-r currents, compared to wild-type animals (Foulkes \textit{et al.}, 2006).

VGSC beta subunits are known to bind the alpha sub-unit and to regulate its trafficking and electrophysiological properties (Isom, 2001). It has been found that beta subunits partition into rafts in mouse adult brain and cortical neurons \textit{in vitro} (Brackenbury \textit{et al.}, 2008; Wong \textit{et al.}, 2005). We did not investigate Na_\text{v}1.8 beta sub-units partition into rafts but their involvement in Na_\text{v}1.8 raft association could also be possible, both in terms of forward trafficking and of stabilisation on the neuronal membrane, similarly to what we have proposed for p11. The proteins we listed above are obvious interesting targets, because of their known role in Na_\text{v}1.8 trafficking and/or association with rafts. Na_\text{v}1.8 interacts with several proteins (Malik-Hall \textit{et al.}, 2003) whose function is not fully understood and it would be interesting to assess their roles in Na_\text{v}1.8 trafficking/raft association.

Complementary to forward trafficking, lipid rafts have also been shown to regulate endocytosis of membrane proteins. One potential explanation of action potential conduction failure in raft depleted samples could be that in the absence of rafts, Na_\text{v}1.8 is recruited into endocytotic pathways. This would lead to a reduction of TTX-r currents which may impair the conductivity along the axons. A similar mechanism has already been described for an ionic
channel in hippocampal neurons. In fact, lipid raft depletion leads to a reduction of AMPA receptor molecules on the membrane, both in basal and AMPA stimulated neurons, due to their recruitment into the endocytotic pathway (Hering et al., 2003).

Another consideration can be raised about raft biosynthesis and Na$_v$1.8 raft association. In fact, the exact mechanisms of raft synthesis are largely obscure (Munro, 2003). It could be possible that, as discussed above, rafts are important for Na$_v$1.8 forward trafficking and that Na$_v$1.8 is targeted to pre-existing lipid rafts; alternatively, lipid rafts could nucleate around Na$_v$1.8 clusters. Neuronal protein NAP-22 has been described to promote raft formation by cholesterol binding, in synthetic conditions (Epand et al., 2001).

### 4.1.2 Lipid rafts directly modulate the electrophysiological properties of Na$_v$1.8.

There is a constantly growing body of evidence which focuses on how lipid rafts directly alter the fundamental properties of membrane proteins. This implies that lipid rafts not only affect cell excitability by regulating ion channel trafficking but also by shaping the electrophysiological properties of the ion channels. Evidence supporting this is numerous and includes different classes of ion channels. Even though the exact mechanisms are unknown, it is thought that rafts exert a physical effect on the membrane proteins. Rafts are characterised by a liquid ordered phase, with different lateral pressure, viscosity and by-layer thickness, compared to non raft regions of the membrane. All these parameters can influence protein properties by modulating, for example, folding, kinetics of transition between different conformational states (e.g. the process open-closed-inactivated) and the stabilisation of allosteric states (e.g. open vs. closed vs. inactivated). Indeed, whenever a conformational change occurs (as it is for voltage gated channels when they experience voltage changes across the membrane) the protein will disturb the neighbouring lipids. Hence, the kinetics of state transitions or stabilisation into a certain conformation, do not only depends on the intrinsic properties of the channel but also on the interaction between the protein itself and the lipid environment (Andersen and Koepppe, 2007; Dart, 2010; Lee, 2004; Lundbaek et al., 2004; Phillips et al., 2009; Tillman and Cascio, 2003). Dramatic direct alteration of electrophysiological properties upon raft disruption has been described for ion channels. For example, lipid raft disruption, by cholesterol depletion, increases the activity of TrpM8 to
multiple stimulation, both when menthol and cooling stimuli are applied, suggesting that lipid rafts have an inhibitory role towards this channel. When the electrophysiological properties of the channel were examined it resulted that its threshold of activation, in MBCD treated cells, was lower than control cells (i.e. warmer stimuli were able to gate the channel) and the voltage dependence of activation shifted to a more negative value. Interestingly the trafficking of the channel is unaffected in treated cells, suggesting the lipid rafts directly modulate the gating properties of the channel rather than its amount on the membrane (Morenilla-Palao et al., 2009). \( K_{v2.1} \) is a voltage gated potassium channel mostly associated with non-caveolae type lipid rafts. It is expressed in the brain and localises preferentially to the dendritic compartment of neurons (Lim et al., 2000). The depletion of cholesterol from lipid rafts leads to altered buoyancy and electrophysiological properties. In fact, \( K_{v2.1} \) steady state inactivation curve is shifted towards more hyperpolarised values, while current density and activation kinetics are unchanged (Martens et al., 2000). Also, modulation of cholesterol content has been correlated to changes in the shape of action potentials and neuronal firing in hippocampal neurons (Guo et al., 2008). To date there are a few examples of an effect of membrane phase/lipids on sodium channel electrophysiological properties. \( Na_{v1.4} \) is a voltage gated sodium channel expressed in the skeletal muscle. It has been shown that, when expressed in heterologous system, cholesterol depletion determines a hyperpolarising shift in the voltage dependence of steady-state inactivation and a decrease of inactivation kinetics of \( Na_{v1.4} \) (Lundbaek et al., 2004). Interestingly, it has also been conjectured that membrane lateral lipid pressure influences \( Na_{v1.6} \) properties at the nodes of Ranvier. \( Na_{v1.6} \) transient current displays irreversible hyperpolarising shifts of steady-state inactivation and of activation upon membrane trauma, which leads to changes in later membrane pressure (Wang et al., 2009). All these data support a scenario where rafts depletion may change \( Na_{v1.8} \) intrinsic electrophysiological properties and this, in turn, affect action potential propagation, like we proved in our model. Indeed, changes in the voltage dependence of steady-state activation/inactivation, kinetics of channel opening/closing and conductivity may affect the overall \( Na_{v1.8} \) mediated sodium conductance and ultimately action potential propagation. We also found that both methods used to disrupt lipid rafts determined the same output. Since different tools were employed, it is possible that different mechanisms were involved. In fact, MBCD could lead to differences due to both depletion of cholesterol and parallel lipid phase change, while 7KC exerts its effect through lipid order change. This data suggests that a phase order change is sufficient to drive changes in membrane excitability. If this effect is mediated
solely by changes of Na\textsubscript{v}1.8 (e.g. trafficking as previously discussed or electrophysiological properties) or due to a combination of different factors is not yet known. In fact, with a direct effect of lipid rafts to Na\textsubscript{v}1.8 electrophysiological properties we can also consider indirect effects. We have not investigated, for example, if other sodium channels partition into rafts. We have demonstrated that TTX-s channels (e.g. Na\textsubscript{v}1.7 in nociceptors) do not substantially contribute to action potential propagation in our system. Nevertheless it may be hypothesised that Na\textsubscript{v}1.7 partitions in lipid rafts and has an indirect effect on Na\textsubscript{v}1.8 mediated action potential propagation. If rafts depletion altered Na\textsubscript{v}1.7 properties we could speculate that a defective boost of ramp currents (mediated by this channel) may impair Na\textsubscript{v}1.8 recruitment, due to its high threshold of activation (Rush \textit{et al.}, 2006). Also, Na\textsubscript{v}1.9, even though it is not involved in action potential generation, contributes to set the resting membrane potential. If raft disruption altered Na\textsubscript{v}1.9 gating properties a change in resting membrane potential could affect Na\textsubscript{v}1.8 availability to fire action potentials. These examples could be extended to other class of proteins which contribute to membrane excitability, like Sodium/Potassium ATPase and leaky potassium channels. For this purpose, a large scale proteomic profiling of the sciatic nerve/DRG neurons would be helpful to define the raft associated protein population. To unravel if raft depletion is indeed directly affecting Na\textsubscript{v}1.8 electrophysiological properties, electrophysiological techniques would be necessary. Both soma and axonal patching would reveal if and how rafts affect Na\textsubscript{v}1.8 properties.

4.1.3 Lipid rafts indirectly modulate the electrophysiological properties of Na\textsubscript{v}1.8 through signaling protein complexes on the membrane.

Lipid rafts modulate, by segregating or by facilitating the interaction of certain molecules, cell signaling (Golub \textit{et al.}, 2004; Simons and Toomre, 2000; Tsui-Pierchala \textit{et al.}, 2002). A potential way rafts could also influence action potential propagation is by indirectly influencing Na\textsubscript{v}1.8 properties. Na\textsubscript{v}1.8 currents are modulated by PKA and PKCe (Okuse, 2007). Interestingly, we have found that Na\textsubscript{v}1.8 co-localised with TrkA, the NGF receptors which is upstream of PKCe. Also, in neuronal cells PKC and TrkA have been reported to translocate into lipid rafts upon activation (Botto \textit{et al.}, 2007; Limpert \textit{et al.}, 2007). In our model, a plausible hypothesis could be that raft depletion alters the signaling between TrkA, PKCe and Na\textsubscript{v}1.8 resulting in modified properties of Na\textsubscript{v}1.8. Supporting the hypothesis that rafts influence ion channel properties by
regulating cell signaling, there is the evidence of \( Na_v1.5 \). \( Na_v1.5 \) is mainly expressed in the cardiac tissue, where it triggers action potentials and supports their propagation through the tissue. \( Na_v1.5 \) resides in Caveolae-type lipid rafts in cardio myocites. Caveolin-3, a marker of rafts, is specifically involved in the \( \beta \)-adrenergic stimulation-mediated increase in sodium currents through the activation of the alpha sub-unit of \( G_s \) protein (Yarbrough et al., 2002). It has also been proposed that the association between potassium channel \( K_v2.1 \) and rafts facilitates the interaction between \( K_v2.1 \) and signaling molecules known to be enriched in rafts such as kinases. The altered properties of \( K_v2.1 \) upon raft disruption may depend on misregulated signaling between kinases and their effectors (including \( K_v2.1 \)) (Martens et al., 2000).

In conclusion, we propose that the effect of lipid rafts on \( Na_v1.8 \), which translates in an influence on cell excitability and ultimately on action potential propagation, may be mediated through one or a combination of the mechanisms we have proposed above. In fact, the complex feed-back between intracellular signaling, gating properties, channel trafficking, association with other proteins and the known involvement of rafts in these processes suggest that multiple ways may occur to tune \( Na_v1.8 \) activity.
4.2 Conclusions

Since the concept of lipid rafts was proposed, a vast and constantly growing amount of data has been clearly showing how these domains influence protein properties and cell behaviour. Traditionally, studies on rafts have been conducted on immune cells. The interest has moved also into other cell types, and at the moment there is great excitement about the role of these micro-domains in regulating cell processes in neurons. Recent studies have highlighted how lipid rafts affect ion channels function in DRG neurons, and how this translates to modulation of cell excitability (Morenilla-Palao et al., 2009; Vacca et al., 2004). The cohort of ion channels expressed on the membrane of neuronal cells and their sub-cellular localisation defines the excitability and firing properties of each cells. The understanding of the detailed mechanisms shaping cell excitability in nociceptors is of obvious importance, given the role of these cells. In fact, they represent the first gate in the perception of painful stimuli in normal conditions and in chronic pain states, where sensitisation and discharge of abnormal ectopic stimuli contribute to hyperalgesia and allodynia (Wall and Melzack, 2005). The great majority of studies conducted so far in the molecular and cellular aspects of pain have unveiled the role of different classes of proteins in cell excitability. Nowadays it is becoming clear that also the lateral organisation of the membrane, with different rafts and domains, and the selective partition of classes of proteins between these domains represent a further level of tuning over protein function and a new field of research.

We focused our study on Na\textsubscript{v}1.8 and lipid rafts in nociceptors. Na\textsubscript{v}1.8 is one of the major determinants of nociceptor excitability. Na\textsubscript{v}1.8 has been found to play an undisputed role in nociception, pain pathways, inflammatory pain and in neuropathic conditions, even though in this latter case there is contrasting evidence. Our main finding was that Na\textsubscript{v}1.8 is associated with lipid rafts \textit{in vivo} along the sciatic nerve and it retains this association in cultured DRG neurons. Also, we have found a characteristic sub-cellular distribution of Na\textsubscript{v}1.8. It was found in clusters, both \textit{in vivo} and \textit{in vitro}. Since cell excitability and ultimately action potential propagation relies on the gating properties of ion channels and their distribution within the cell, this finding may shed light on the precise mechanisms of action potential propagation in unmyelinated nociceptors.
We have also investigated the role of the association between rafts and Na\textsubscript{v}1.8 in unmyelinated nociceptors, and this association proved to be of physiological importance: we have demonstrated that raft disruption results in altered cell excitability. In fact, both mechanical and chemical stimuli, with the latter known to trigger pain \textit{in vivo}, evoked action potentials which failed to propagate along the axons of raft depleted nociceptors. Notably, this directly correlates with the shift of Na\textsubscript{v}1.8 from lipid rafts to non-lipid raft compartments. This finding highlights the importance of rafts in shaping Na\textsubscript{v}1.8 mediated action potential propagation. The discovery of Na\textsubscript{v}1.8 associating with rafts represents a novel insight not only into the pain field but also in the lipid raft field. Indeed, we are the first group to date to show that a voltage gated sodium channel segregates into rafts in neuronal cells and that this correlates with an effect on cell excitability. We have conducted experiments \textit{in vitro}. The advantage of this system is that precise control of certain experimental variables is possible. On the other hand, it would be intriguing to further extend our findings in \textit{in vivo} models. It would be interesting to translate our findings to pain models to assess what the role of the association between rafts and Na\textsubscript{v}1.8 would be, in terms of action potential propagation, cell excitability and ultimately pain behaviours in adult animals.

We conclude that the effect of lipid rafts on cell excitability represents a novel aspect to be considered in the efforts aiming to understand the fundamental properties of nociceptors and neuronal cells in general. Studies which are being carried out to discover new and effective drugs to treat pain conditions would potentially benefit from further investigation into the influence of rafts on neuronal excitability. Indeed, the exact mechanisms of protein behaviour in its lipid environment could lead to insights which may open new perspectives in the race to find new targets for effective painkillers.
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