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Investigating the function of Regulator G-Protein Signaling 4 (RGS4) protein in Central Nervous System under Chronic Inflammatory Pain

MSc thesis

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Ευχαριστίες

Ένα μεγάλο ευχαριστώ στην επιβλέπουσα καθηγήτρια μου κ.Βενετία Ζαχαρίου που με δέχτηκε στο εργαστήριό της και για την καθοδήγησή και την στήριξη της τον τελευταίο ένα χρόνο που βρίσκομαι στο εργαστήριο. Επίσης, θα ήθελα να ευχαριστήσω τα μέλη της τριμελούς επιτροπής κ. Π.Ποϊράζη, Χ.Δελιδάκη και Α.Ηλιόπουλο για την βοήθειά τους στο πλαίσιο των προφορικών εξετάσεων.

Τέλος, ευχαριστώ θερμά τα υπόλοιπα μέλη του εργαστηρίου Σέβη, Βίκυ, Alexander και Valeria για την άψογη συνεργασία και το ευχάριστο κλίμα στο εργαστήριο.

Περίληψη

Περίπου 10-15% του πληθυσμού παγκοσμίως πάσχει από χρόνιο πόνο που προκαλείται από εμμένουσα φλεγμονή. Η έλλειψη γνώσης σχετικά με τους μοριακούς μηχανισμούς που οδηγούν στην ανάπτυξη και διατήρηση του χρόνιου πόνου είναι ένας από τους λόγους όπου οι σημερινές θεραπευτικές προσεγγίσεις είναι ανεπαρκείς και εμφανίζουν σημαντικές παρενέργειες. Η έρευνά μας εστιάζεται στον χρόνιο φλεγμονώδη πόνο. Μεγάλη εξέλιξη έχει επιτευχθεί σχετικά με την κατανόηση των μοριακών και κυτταρικών μηχανισμών που μεταφέρουν την αισθητήρια πληροφορία στην σπονδυλική στήλη, ενώ οι αντίστοιχοι μοριακοί μηχανισμοί στον εγκέφαλο δεν έχουν διελευκανθεί. Τα σηματοδοτικά μονοπάτια των G-πρωτεϊνών υποδοχείς (GPCR) έχει δειχθεί ότι εμπλέκονται στον πόνο. Σημαντικοί κεντρικοί ρυθμιστές αυτών των μονοπατιών είναι οι πρωτεΐνες ρυθμιστές της σηματοδότησης μέσω των G-πρωτεϊνών (RGS). Προηγούμενες μελέτες του εργαστηρίου μας έχουν θεσπίσει ότι η RGS4 πρωτεΐνη είναι θετικός ρυθμιστής για την αναλγησία όταν χορηγούνται τρικυκλικά αντικαταθλιπτικά, φάρμακα που ευρέως συνταγογραφούνται για την θεραπεία του νευροπαθητικού πόνου. Στην παρούσα μελέτη χρησιμοποιώντας ως μοντέλο οργανισμό τον ποντικό (*Mus Musculus)* σε συμπεριφορικά παραδείγματα πόνου υποδεικνύουμε τον λειτουργικό ρόλο της RGS4 πρωτεΐνης στον χρόνιο φλεγμονώδη πόνο. Ενδιαφέρον είναι ότι ανακαλύψαμε ότι η RGS4 πρωτεΐνη παρουσιάζει τροπικότητα (modality) ως προς την απόκριση στα διάφορα ερεθίσματα σε καταστάσεις χρόνιου πόνου. Επιπλέον, η ρύθμιση των mRNA επιπέδων της RGS4 σε διάφορες περιοχές του κεντρικού νευρικού συστήματος που σχετίζονται με τον πόνο υποστηρίζουν τον φαινότυπο που παρατηρήθηκε. Συγκεκριμένα, εντοπίσαμε την περιοχή λειτουργικότητας της RGS4 πρωτεΐνης στον εγκέφαλο σε συνθήκες χρόνιου φλεγμονώδη πόνου. Η μελέτη μας παρέχει καινοτόμες πληροφορίες για τον ρόλο της RGS4 πρωτεΐνης στα σηματοδοτικά μονοπάτια στον εγκέφαλο σε καταστάσεις χρόνιου φλεγμονώδη πόνου και τα δεδομένα αυτά θα συμβάλλουν στην προσπάθεια εύρεσης νέων φαρμακευτικών στόχων για την αντιμετώπιση του χρόνιου πόνου.

Abstract

Around 10-15% of the population worldwide experience chronic pain induced by persistent inflammation. Part of the reason that the current therapeutic strategies are insufficient or produce major side effects, has to do with the incomplete understanding of molecular events involved in development and maintenance of chronic pain states. Our research focus on inflammatory pain. Increased progress has been made in understanding the molecular and cellular mechanisms that drive the sensory input to the spinal cord, whereas studies on signaling pathway adaptations in the brain centers are lacking. G-Protein Coupled Receptor (GPCR) signaling pathways are highly implicated in pain hypersensitivity and Regulator of G-protein signaling (RGS) proteins play a central modulatory action on these GPCR-mediated cascades. Previous studies from our laboratory have established that RGS4 is a positive modulator of analgesic responses to tricyclic antidepressant drugs, a class of drugs widely prescribed for the treatment of neuropathic pain conditions. Here, by using *Mus Musculus* model organism and well-established pain behavioral assays we report a functional role of RGS4 under chronic inflammatory pain states. Interestingly, we reveal that RGS4 act in a modality specificity manner. The regulation of RGS4 mRNA levels in various pain related CNS regions, further, support our observed phenotype under persistent inflammatory pain. Using advanced gene targeting approaches, we identified the critical brain region where RGS4 acts to modulate persistent inflammatory pain-like states. Our study provides novel insight on the role of RGS4 in intracellular adaptions in brain regions underling chronic inflammatory pain, pointing to novel targeted pharmacological interventions for the alleviation of chronic pain conditions.

Introduction

Chapter 1. Pain

1.1. Definition

Pain has been defined *as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage"* (Merskey & Bogduk, 1994). A revised definition identifies pain as "a somatic perception that contains a bodily sensation with qualities like those reported during tissue-damaging stimulation, an experienced threat associated with this sensation, and a feeling of unpleasantness or other negative emotions based on this experienced threat" (Price DD., 1999).

1.2. Classification

The *International Association for the Study of Pain* (IASP) classified pain according to specific characteristics:

- region of the body involved (e.g. abdomen, lower limbs)
- system whose dysfunction may be causing the pain (e.g., nervous, gastrointestinal)
- duration and pattern of occurrence
- intensity and time since onset
- etiology

Conventionally, *acute pain* is limited to pain of less than 30 days duration, whereas *chronic pain* persists for more than 6 months. There is an important functional distinction between acute and chronic pain. Acute pain has a protective role by limiting the use of injured or diseased body parts and that kind of pain departs when the limiting condition is resolved. Chronic pain, on the other hand, has little protective significance and persists beyond the expected period of healing (Turk and Okifuji, 2001). However, according to Woolf J. Clifford (2010), pain is essentially divided into two broad categories: adaptive and maladaptive. **Adaptive pain** contributes to survival by protecting the organism from injury (nociceptive pain) or promoting healing when injury has occurred (inflammatory pain*).* Nociceptive pain is the sensing of noxious stimuli via nociceptors and may occur with or without damage to the nervous system. Inflammatory pain is characterized by pain hypersensitivity and typically decreases as the tissue damage and inflammatory response is resolved.

On the other hand, maladaptive pain is the expression of a pathological situation of the nervous system (Woolf CJ., 2004). In particular that disease state is caused either by lesions to the peripheral or central nervous system (peripheral or central neuropathic pain, respectively) or in conditions in which there is no such damage or inflammation (dysfunctional pain) (Fig.1).

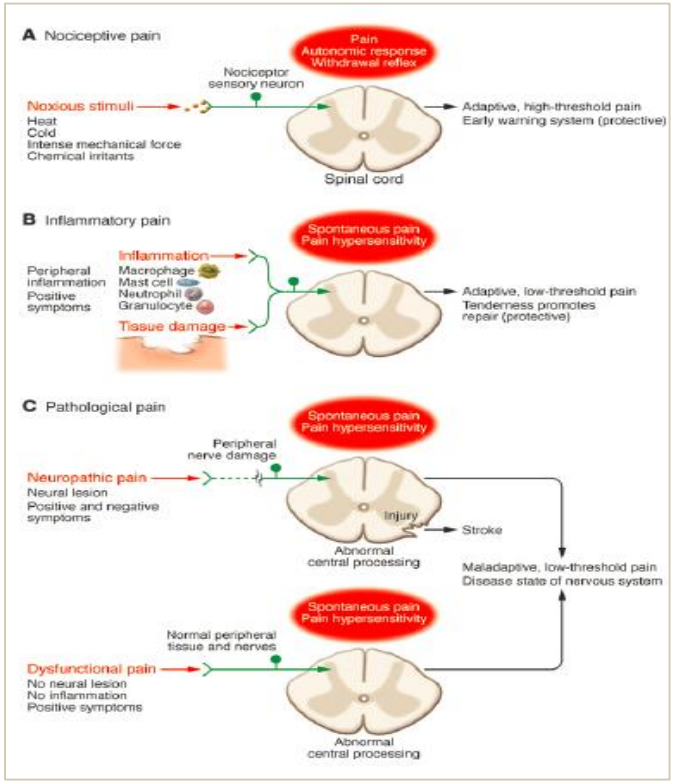

Figure 1: Pain classification
*Pain can be broadly divided into three classes. (**A**) Nociceptive pain (**B**) Inflammatory pain (**C**) Pathological pain either neuropathic or dysfunctional pain. (Adapted by Woolf CJ, 2010)*

1.3. Components of pain response

The generation of somatic pain in response to tissue injury involves four basic elements; transduction, transmission, transformation, perception (as shown in Fig.2). The pain signal is initiated by the detection of the noxious stimulus (mechanical, thermal, chemical stimuli) through specialized free nerve endings. That stimulus is converted into an action potential (AP), this process is collectively referred as *transduction*. The free nerve endings transmit the APs to the central nervous system from the periphery, and this process is called *transmission*. However, that signal can be modulated at synaptic sites of the spinal cord, or at other levels of central nervous system (through ascending and descending pathways), and that modulation is referred as *transformation*. The *perception* is the output component of pain which incorporates both sensory and emotional responses to the painful experience (Bourinet et al., 2014).

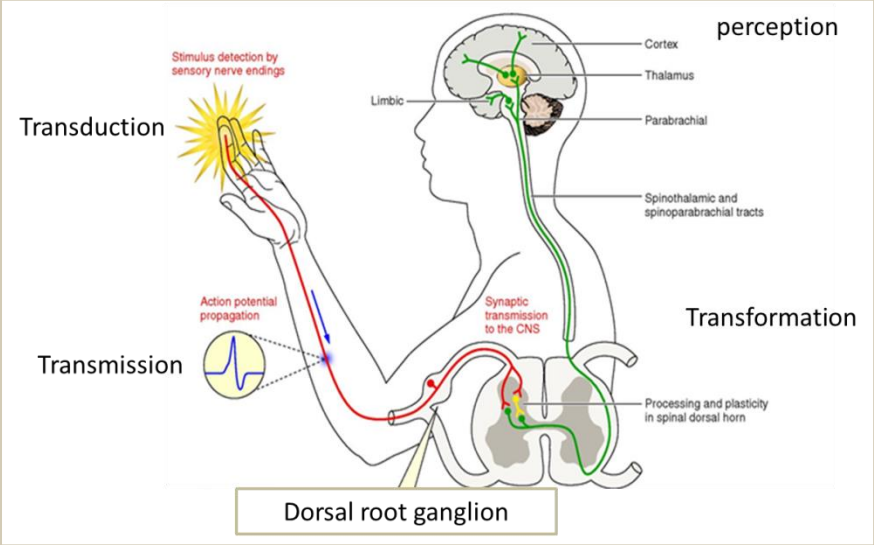

Figure 2: Components of pain response

Ascending pathways (green lines) and descending pathways (do not depicted here) arise from brain regions back to the dorsal horn of the spinal cord.

(Modified picture adapted by Bourinet et al., 2014).

1.4. Anatomy of Pain Pathway

1.4.1. Peripheral pain pathway

Nerve fibers that transmit signals from the periphery to the central nervous system (CNS) are known as **afferent neurons** whereas those that carry information to the periphery are known as **efferent neurons** (Albright et al., 2000). Cutaneous (skin), deep somatic tissues (tenders, joints, and ligaments) and muscles are innervated by **primary afferent neurons**, which translate the pain, itch, temperature or touch sensations into an AP. In particular, the free nerve endings of primary afferent neuron, defined as **nociceptors**, are stimulated by intense thermal, mechanical or chemical stimuli (Basbaum and Jessell, 2000, Bajwa et al., 2003). The cell bodies of nociceptors are located in the dorsal root ganglia (DRG) next to the spinal cord. They have both a peripheral and central axonal branch that innervates their target organ and the spinal cord, respectively. There are two major classes of nociceptors, based on their myelination level;

- Medium diameter myelinated afferents (Aδ) that mediate acute first-localized or fast pain. These fibers differ considerably from the larger diameter and rapidly conducting Aβ fibers that respond to innocuous mechanical stimulation (i.e. light touch).
- Small diameter unmyelinated C fibers that convey poorly localized, second or slow pain (not all C-fibers are nociceptors).

Aδ and C fibers are further subdivided according to neuroanatomical, molecular and electrophysiological studies. These functionally and molecularly heterogeneous classes of nociceptors associate with specific function in the detection of distinct pain modalities (Basbaum et al., 2009).

1.4.2. Central Pain Pathway

The grey matter of the spinal cord is organized into 10 laminae and laminae I- VI making up the dorsal horn (Rexed, 1952). The **dorsal horn** is the first site in the central nervous system where incoming nociceptive information is processed and modulated (Bajwa et al., 2003). Nociceptive fibers project to different levels at the dorsal horn structure (Fig.4):

- Aδ nociceptors project to lamina I and lamina V (deeper lamina)
- Aβ nociceptors project to deep laminae (III,IV, V)
- C nociceptors project more superficially to laminae I and II

Spinal cord neurons -within lamina I- are generally responsive to noxious stimuli via Aδ and C fibers and these spinal cord neurons are called ***nociceptive-specific neurons (NS)***, whereas neurons in laminae III and IV are primarily responsive to innocuous stimulation via Aβ fibers. Neurons in lamina V receive convergent non-noxious and noxious input via direct (monosynaptic) Aδ and Aβ inputs and indirect (polysynaptic) C fiber inputs. These convergent neurons have larger receptive fields and are called ***wide dynamic range (WDR) neurons*** since they respond to a broad range of stimulus intensities (Basbaum et al., 2009). The NS and WDR neurons are also known as ***second order neurons*** since they receive sensory information from the primary afferent neurons and send axons to the brain (Fig.4). In addition, non-neuronal cell types in the synaptic cleft can further influence the pain transmission.

1.4.2.1. Ascending pathway

Ascending pathways refer to the spinal neuronal tracts that transmit sensory information to higher brain centers from the somatic structures. ***The anterolateral system (ALS)*** is an ascending pathway that transmits pain, temperature and crude touch from the periphery to brain by a sequence of three neurons and interneurons. The neuron sequence comprises of (Fig.3):

- **First order neuron** or primary afferent neurons (nociceptors). It has been reported that the first order neuron may synapse with an **interneuron** that resides entirely within the dorsal horn, and whose axon synapses with the second order neuron.
- **Second order neurons** whose cell body is located within the dorsal horn and whose axon usually decussates and ascends i) in the direct pathway of the ALS (spinothalamic tract) to synapse to contralateral thalamus and sending some collaterals to the reticular formation, ii) in the indirect pathway of the ALS (spinoreticular tract) to synapse in the reticular formation.
- A **third order neuron** whose cell body is located in the thalamus, and whose axon ascends ipsilaterally to terminate in the somatosensory cortex (Patestas and Gartner, 2006).

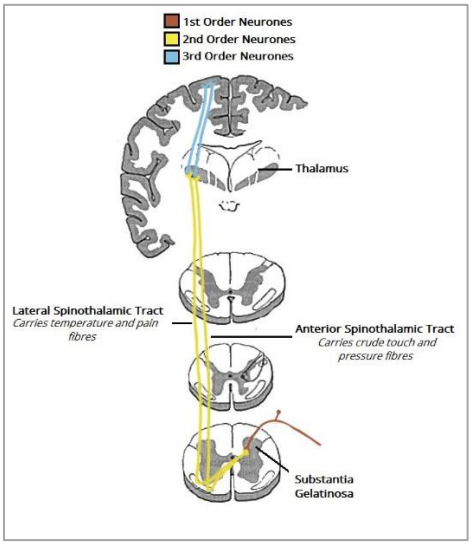

Figure 3: The spinothalamic tract
Neuron sequence in anterior and lateral STT.
(Adapted by teachMeAnatomy website)

The spinothalamic tract is the major pathway responsible for mediating pain sensation in humans and in rats. The STT fibers receive harmful input through Aδ and C-fibers and cross the midline at the segmental level of the spinal cord and ascend in two separate tracts, the anterior (ventral) STT and the lateral STT (Fig.3). The anterior STT transmits the spatial information related to crude touch and firm pressure, while the lateral STT transmits information related to temperature and pain. These tracts originate in different regions of the dorsal horn and show distinct projection sites at the brain. Both STT tracts terminate in

various nuclei of the thalamus. However, the lateral tract sends collateral branches to midbrain regions such as the periaqueductal gray, parabrachial area and reticular formation, whereas the anterior has collateral fibers to various brainstem nuclei. Concerning the anterior STT, the axon cells from the thalamic nuclei terminate to various cortical regions such as Anterior Cingulate Area (ACC), Broadman area, somatosensory cortex and insula. Interestingly, STT projections to the central lateral nucleus of the thalamus play a role in motivational-affective responses to pain, including emotional reactions (suffering, anxiety, depression), and the projection to lateral thalamus (the ventrobasal complex) is involved in sensory-discriminative aspects of pain (Patestas and Gartner, 2006).

1.4.2.2. Descending pathway

It has been shown that the nociceptive signal can be modulated via ***descending influences*** arise from multiple supraspinal structures including the hypothalamus, the amygdala and the rostral anterior cingulate cortex (rACC), ending up to the midbrain periaqueductal gray region (PAG) and with outputs from the PAG to the medulla (Fig.4). There are at least three separate descending systems; two of them originate from PAG and connect to dorsal horn neurons through at least one interneuron in the rostral ventromedial medulla (RVM) and in the dorsolateral pontine tegmentum (DLTP) nuclei and the third system is the noradrenalin (NA) serotonin system (Møller AR, 2006, Ossipov et al., 2010).

The PAG/RVM system is a well-studied descending pathway and provides a neuronal context for positive and negative pain modulation. Glutamatergic input arise from the dorsal horn and leading to firing of enkephalin-interneurons (INs) in the PAG. These INs inhibit GABA interneurons and thus glutamatergic connections towards the raphe nucleus (part of brainstem) are allowed to happen. A same disinhibition of GABA INs are performed in raphe nucleus, leading to the release of serotonin in the dorsal horn, activation of inhibitory INs and as a result attenuate the pain response. The RVM receives inputs, in addition to the PAG, from the thalamus, the parabrachial region and the noradrenergic locus coeruleus. RVM contains three types of cells: on-cells, off-cells and neutral cells. Neutral cells produce serotonin. Off-cells are thought to exert descending inhibition of nociception, whereas on-cells seem to increase their activity in response to noxious stimuli and facilitate nociceptive mechanisms at the spinal dorsal horn. Of note, that endogenous pain inhibitory system arise from PAG is opioid-mediated both in humans and animals. Studies performed in animal models of neuropathic or inflammatory pain indicate that an imbalance between the inhibitory and facilitatory descending pain modulatory systems may underlie pathological pain states (Ossipov et al., 2014). Interestingly, an imaging study with humans showed that

activation of this region is specifically related to development and maintenance of central sensitization (Lee et al., 2008).

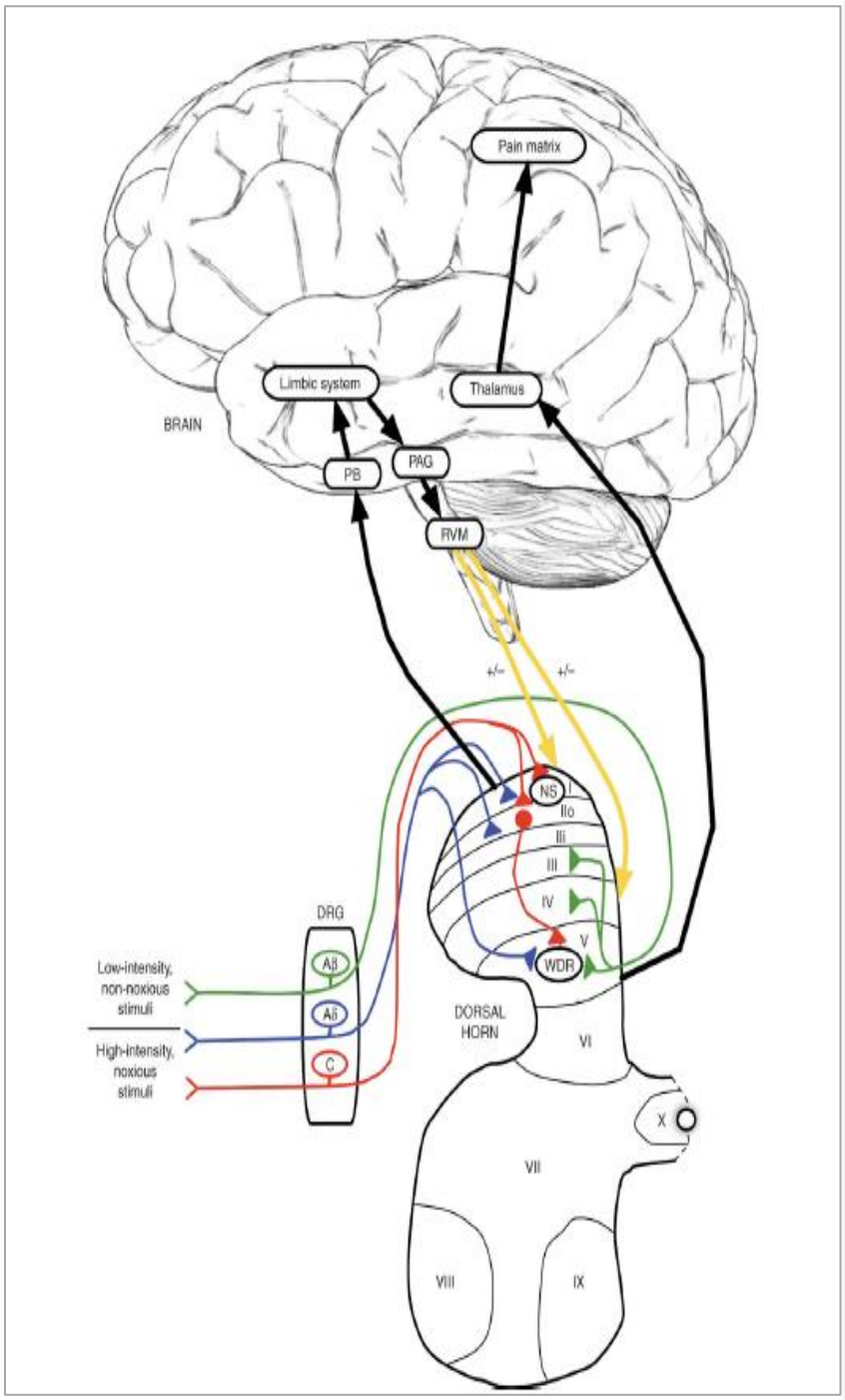

Figure 4: Pain pathways from periphery

Primary afferent transmit impulses from the periphery, through the DRG and into the dorsal horn of the spinal cord. NS cells are mainly found in the superficial dorsal horn, whereas most WDR neurons are located deeper. Projection neurons from lamina I innervate areas such as the parabrachial area (PB) and PAG and such pathways are affected by limbic areas. From here descending pathways (yellow arrows) from brainstem nuclei such as RVM are activated and modulate spinal processing. Lamina V neurons mainly project to the thalamus (STT), and from here the various cortical regions forming the 'pain matrix' (primary and secondary Somatosensory, insular, anterior cingulate, and prefrontal cortices) are activated.

(Adapted by Melllo and Dickenson, 2008)

Chapter 2. Inflammatory Pain

Inflammatory pain results from the activation and sensitization of nociceptors by inflammatory mediators (Vardeh et al., 2016). In addition, the reciprocal cross talk between Immune and Nervous system permits amplification of maladaptive feedforward inflammatory loops that contribute to the development of pain (Talbot et al., 2016). Inflammatory pain can last a short time, take for instance the post-operative pain, or it can be a chronic state such as rheumatoid arthritis, osteoarthritis, diabetes, cancer pain, inflammation of peripheral and cranial nerves. Inflammation can affect visceral organs, while that kind of inflammation differs from previously referred. Inflammation makes tissue become more sensitive to painful stimulation (named as hyperalgesia) and innocuous stimulations may produce a sensation of pain (called as allodynia). Notably, inflamed tissue may generate pain without external stimulation (spontaneous pain) (Møller AR, 2011).

2.1. Peripheral Mechanisms of Inflammatory Pain

Peripheral sensitization constitutes a decreased threshold and increased responsiveness of nociceptors as a result of post-translational changes in and altered trafficking of transducer receptors and ion channels (Fig.5). This is caused by local inflammatory mediators, and leading to the development of pain hypersensitivity at the site of the inflamed tissue (zone of ***primary hyperalgesia***) (Costigan et al., 2009, Woolf, 2011). Some key mediators are produced locally or by the immune cells that reside within or infiltrate in the site of injury (*Farquhar-Smith and Kerr,* 2005).

2.1.1. Inflammatory mediators produced locally

Protons. Protons are produced in inflamed tissue and in common with serotonin (5-HT) can act directly on primary afferent neurons. Ion permeability is increased by these and exposure of C- and Aδ-fibers to pH of 6 or less can activate acid-sensing ion channels (ASICs) with subsequent generation of action potential (*Farquhar-Smith and Kerr,* 2005). Also, extracellular protons and lipids function as positive allosteric modulator of Transient Receptor Channels (TRP) (Bausbaum et al., 2009). TRP channels are directly responsible for transduction, whereas others act indirectly such as NaV1:8 (Gold and Gebhart, 2010).

Kinins. Kinins are peptides cleaved from circulating proteins that are activated at the site of injury. Bradykinin (BK) is one of the main kinins found in raised concentrations in inflamed tissue. It activates nociceptors through G-protein-coupled BK K1/K2 receptor- Protein kinase C (PKC) signaling. BK acts

synergistically with other algogenic substances (including prostaglandins (PG) and nerve growth factor (NGF)) and can stimulate the release of other pro-inflammatory cytokines.

ATP. Adenosine triphosphate (ATP) acts upon Purinergic2X (P2X) receptors, which are selectively expressed on primary afferents. That activation contributes to the hyperalgesia and pain.

NGF. Nerve Growth Factor (NGF) is released locally from a number of cells (including fibroblasts) and performs a central role in the inflammation cascade. It has been associated with heat and mechanical hyperalgesia both in human inflammatory pain states and animal models (*Farquhar-Smith and Kerr,* 2005).

2.1.2 Inflammatory Mediators Released & Produced by Immune Cells

Various immune cell types can be recruited and contribute to abnormal pain sensitivity including mast cells, activated macrophages either resident or recruited from the blood, neutrophils and T- and B- cells. Indicatively secreted products are the following;

Products of COX and LOX metabolism. PGs are lipids compounds that are secreted by the enzymatic activity of cyclo-oxygenase (COX) and lipo-oxygenase (LOX) on arachidonic acid (AA) and perform a number of pro-inflammatory tasks through binding to a series of prostanoid receptors on nociceptors. (*Farquhar-Smith and Kerr,* 2005).

Cytokines. Cytokines are small regulatory proteins and are clustered into several classes such as interleukins (IL), tumor necrosis factors (TNF), interferons, and chemokines. Abundant evidence indicates that TNF-a, IL-6, IL-1beta, and IL-17 induce thermal and mechanical hyperalgesia (Schaible, 2014). Sensitization of nociceptors can be achieved either by direct phosphorylation of ion channels or by activation intracellular pathways leading to transcriptional up-regulation of certain neurotransmitters (e.g. GABA,CGRP,SP) and receptors, such as TRPV1, IL-6R, IL-1R etc (Ji et al., 2014).

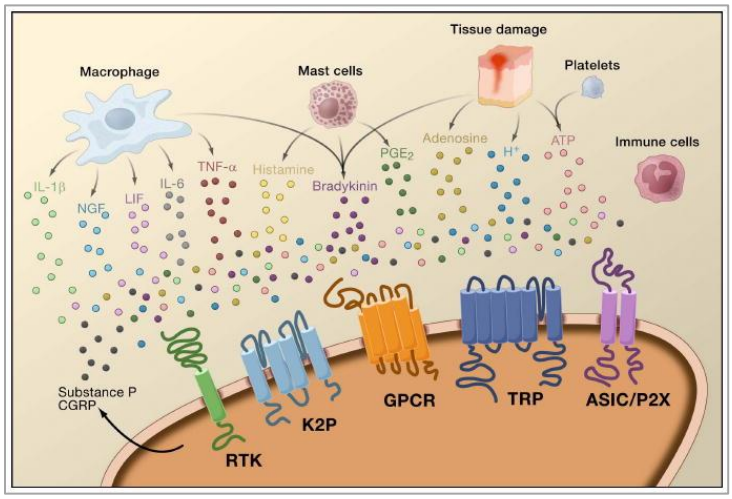

Figure 5: Peripheral Mediators of inflammation

Inflammatory mediators by activated nociceptors or nonneural cells that reside within or infiltrate into the injured area. This "inflammatory soup" of signaling molecules includes a variety of factors act directly on the nociceptor by binding to GPCR, TRP channels, ASIC, two-pore potassium channels (K2P), and receptor tyrosine kinases (RTK), as depicted on the peripheral nociceptor terminal. (Adapted by Meyer et al., 2008)

2.2. Plasticity in neural substrates of pain

Neural plasticity is a key element of chronic pain and can be occurred on molecular, synaptic, cellular and network levels. Disease-induced plasticity can be both functional and structural. Molecules may become functional sensitized in an activity-dependent manner via transcriptional and post-transcriptional modification. Apart from altering their function (for example, a drop in the activation threshold of an ion channel), these modifications may change their localization (for example, endocytosis or trafficking). At the synaptic level, a silent synapse can be transform to a potentiated synapse and evokes amplified excitatory postsynaptic potentials in spite of unchanged neurotransmitter availability. As shown in fig.6, this transformation usually involves the insertion of glutamatergic AMPA receptors (AMPARs) into postsynaptic membranes, driven by activation of glutamatergic NMDA receptors (NMDARs). Concerning the presynaptic mechanisms (Fig.6), activation of kinase cascades will increase the neurotransmitter release in synapses with a low release probability under physiological conditions. Furthermore, mediators released by microglia further promote the previously analyzed plasticity events (Kuner, 2010, Luo et al., 2014). Long-term potentiation of nociceptive transmission has been reported in the spinal dorsal horn and anterior cingulate cortex (Ikeda et al., 2003, Toyoda et al., 2009). Interestingly, a different type of potentiation, namely Wind-up have been discovered in spinal cord, which is a short-term increase in electrical response in the dorsal horn neurons and it is caused by repeated stimulation of group C fibers (Melzack & Wall, 1965).

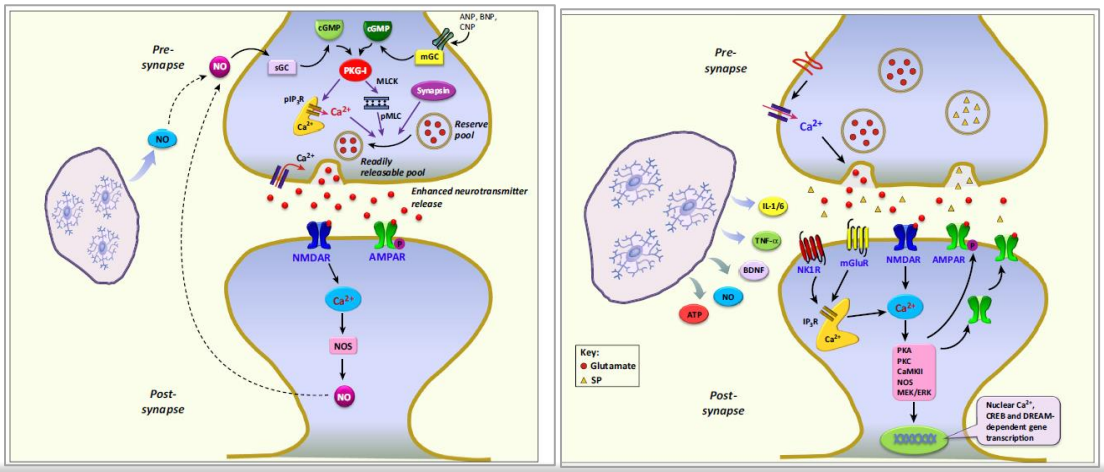

Figure 6: (left) Pre-synaptic & (right) Postsynaptic mechanisms of synaptic plasticity.

(L) : Synaptic potentiation between C-fibers and spino-PAG projection neurons involves activation of cyclic GMP-dependent kinase I (PKG-I) in presynaptic terminals, which in turn phosphorylates IP3R and myosin light chain kinase (MLCK) to yield an increase in the probability of neurotransmitter release. Nitric Oxide (NO) transported retrogradely across the synapses and may contribute to cGMP synthesis. (R): synaptic potentiation involves Ca2+ influx via NMDARs and insertion of AMPPARs. Mediators are released by microglia and augment potentiation. *(Adapted by Luo et al., 2014)*

Intriguingly, individuals with chronic pain develop pain hypersensitivity outside the initial injury (called as ***secondary hyperalgesia***). This reflect plasticity changes in neurons at the spinal and supraspinal regions. In particular, it has been proposed the mechanism of ***central sensitization*** which can be broadly described as the increased responsiveness of nociceptive neurons in the CNS to their normal or subthreshold afferent input (IASP), leading to enhanced processing of nociceptive (pain) messages (Latremoliere & Woolf 2009, Woolf 2011). In addition to post- and pre-synaptic mechanisms and neuro-glia interactions, the degeneration of inhibitory spinal neurons (Scholz & Woolf, 2007, Gangadharan & Kuner, 2013) further facilitate the establishment of a pathological low-threshold pain hypersensitivity (fig.7). Interestingly, recent reports provide evidence that the blocking of spinal GABA-ergic and glycinergic interneurons results to the development of hyperalgesia and mechanical allodynia (Zeilhofer et al., 2012, Guo & Hu, 2014, Foster et al., 2015). Endogenously released cannabinoids, opioids and adenosine also perform inhibitory actions at the spinal cord level (Gangadharan & Kuner, 2013).

Importantly, structural plasticity adds complexity contributing to the long-term nature of chronic pain. Notably, expansion of the peripheral receptive fields has been observed and that allows hyperalgesia to spread to uninjured regions. More examples including an increase or a decrease in the density of synaptic spines, degeneration or sprouting of axons leading to aberrant connectivity, cells atrophy such as loss of inhibitory interneurons and proliferation of astrocytes and microglia, which influence nociceptive processing by releasing modulatory substances (Kuner, 2010, Kuner & Flor, 2017).

Recent publications dissect the specific spinal cord circuitry underlying central sensitization (Maier et al.,2010, Duan et al., 2015) proving that different microcircuits encode mechanical allodynia depending on the injury (Peris et al., 2015).

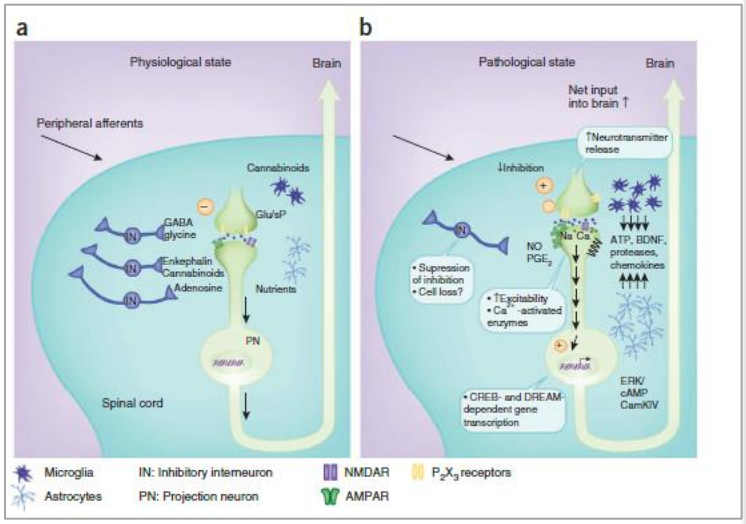

Figure 7: Spinal mechanisms of physiological pain and disease-induced pain hypersensitivity

mediators & cell-cell interactions in the spinal cord in physiological (a) and disease states (b). Putative changes in pathological states include suppression of inhibition, potentiation of presynaptic release and postsynaptic excitability, increases in synapse-to-nucleus communication and gene transcription, release of neuromodulators from activated microglia and astrocytes. (Adapted by Kuner, 2010)

2.3. Signaling pathways that mediate disease-induced pain hypersensitivity

It is published that diverse molecules modulate spinal pain processing by activating cell surface receptors in discrete spatial and temporal patterns (Woolf & Salter, 2000, Ji et al., 2003, Sandkuhler et al., 2009). Main receptors including (Fig.8):

i. Ligand-gated ion channels, which regulate neuronal excitability at a scale of microseconds to seconds, such as NMDA and AMPA-type glutamate receptors and ATP-gated P2X3-type ion channels. Increase of sodium and calcium activates NO and COX-2 synthase and their products function as retrograde messengers.

ii. G protein–coupled receptors (GPCRs) are activated by diverse neurotransmitters and neuromodulators, including glutamate, adenosine, ATP, cannabinoids, opioids and prostaglandins, and modulate pain processing over seconds to minutes. A variety of receptors are included such as metabotropic glutamate receptors (1/5), beta 1,2- adrenergic, protease activated receptors, neurokinin 1 receptor etc. G_q, leads to activation of PLC_B,PKC, calcium release and mediates facilitatory effects of Substance P and glutamate, whereas G_s induces production of cAMP and subsequent activation of PKA and CREB in cell nucleus (Wettschureck & Offermanns 2005).

iii. Receptor tyrosine kinases (RTKs) are activated in sensory pathways by several growth factors (Pezet & McMahon, 2006, Milenkovic et al. 2007) and act over temporal scales of minutes to hours. Prominent targets related to RTK activation including phosphorylation of ion channels, ERK1/2 activation and CREB activation.

In general, signaling transducers activated by each of these three kinds of receptor increase excitability and can directly or indirectly modulate gene transcription, which allows long-term modulation of pain.

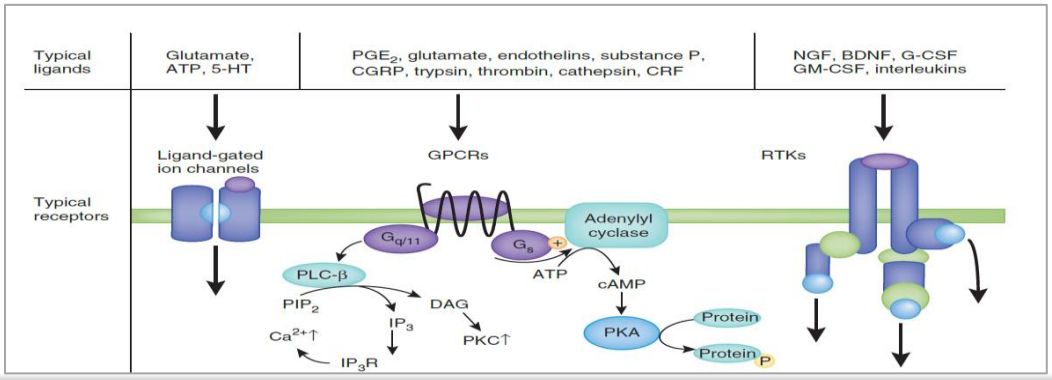

Figure 8: Overview of typical signaling pathways that mediate disease-induced pain hypersensitivity. *Activation of above signaling mediators induces changes in the expression or function of target proteins, thereby leading to characteristic functional changes over diverse timescales. (Adapted by Kuner, 2010)*

Notably, there is limited literature on the signaling pathways in supraspinal structures associated with acute or chronic pain. A few reports indicate a critical role of mGluR1/mGluR5-PLCβ pathway (Miyata et al., 2003, Cheong et al., 2008) and Homer/glutamate receptor (Obara et al., 2013) in the thalamus. In ACC synaptic mechanisms of potentiation such as GluR-A–containing AMPARs, activation of ERK1 and ERK2 and the calcium-stimulated adenylyl cyclase-1 have been reported (Cao et al., 2009, Jeon et al., 2010). In nociceptive amygdala (CeA) enhanced transmission is mediated by G protein signaling through mGluR1 and mGluR5 and corticotrophin-releasing factor receptors in rats with arthritic pain (Neugebauer et al., 2003, Fu & Neugebauer, 2008).

2.4. Animal Models for Pain Studies

Animal models of inflammatory pain have been used to study the mechanisms of tissue injury -induced persistent pain. A variety of inflammatory agents or irritants, such as complete Freund's adjuvant, carrageenan, formalin, capsaicin, bee venom, zymosan, mustard oil, acidic saline, and lipopolysaccharide, have been used to produce tissue injury and hyperalgesia in cutaneous/subcutaneous tissues, joints, and muscles. Nevertheless, these models do not simulate every aspect of chronic pain, they do model key features of human inflammatory pain. Studies in animals provide knowledge about human pain conditions and lead to improved pain management for patients. Most widely used models are CFA, Formalin, Carrageenan and Capsaicin Model (Zhang and Ren, 2011). A comparison between Inflammatory Models is depicted at the table 1. Here, we will use the CFA which is described in more detail below.

CFA Model

Injection of complete Freund's adjuvant (CFA, composed of inactivated and dried Mycobacterium and adjuvant) into the footpad produces localized inflammation and persistent pain. After a CFA injection into the footpad, cutaneous inflammation appears in minutes to hours and peaks within 5–8 h. CFA produces dose-dependent inflammatory responses. Around 30–200 mg of Mycobacterium butyricum suspended in oil/saline (1:1) yield significant edema and thermal hyperalgesia in the injected hind paw (Zhang et al., 1999) (Fig.9). The edema peaks around 24 h after the injection. The hyperalgesia and allodynia peak around 5 h after injection and persist for approximately 1–2 weeks. CFA-induced hyperalgesia and allodynia in rats are consistent with those seen in humans receiving inadvertent injections of CFA. Rats with CFA-induced inflammation exhibit minimal reductions in weight and show normal grooming behavior. Exploratory motor behavior is normal, and no significant alterations occur in an open field

locomotion test. The physiological and biochemical effects of CFA are restricted to the affected limb and there are no signs of immune response or systemic disease.

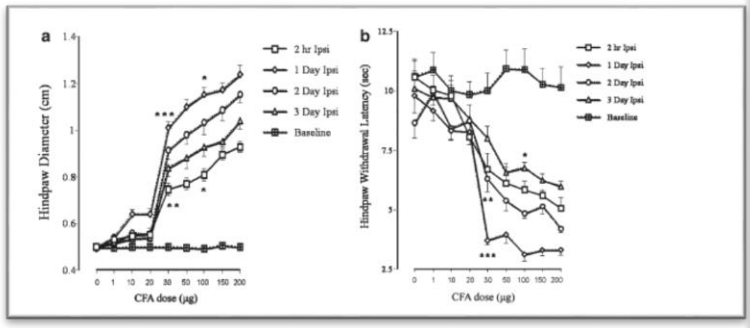

Figure 9: Inflammation and hyperalgesia produced by intraplantar injection of complete Freund's adjuvant in rats

(a) Edema of the rat hind paw after injection of different doses of CFA, determined by measuring the dorsal-ventral thickness of the injected hindpaw. (b) Changes in hind paw withdrawal latency to a noxious thermal stimulus at different time points (2 h to 3 days) after injection of different doses of CFA into the hindpaw. (Zhang et al., 1999)

Chemical	Hyperalgesia	Allodynia	Time of onset	Duration
CFA	Yes	Yes	2-6 h	1-2 weeks
Carrageenan	Yes	Yes	1 h	24 h
Formalin I	Not Applicable	Not Applicable	<1 min	5-10 min
Formalin II	Not Applicable	Not Applicable	10 min	1 h
Capsaicin	Yes	Yes	1 min	<1h

Table 1: Comparison of cutaneous/subcutaneous inflammatory pain models

(Zhang and Ren, 2011)

2.5. Assessment of Pain in Animal Models

While we cannot ask an animal directly about the ongoing nature of its pain experience, a wide range of nociceptive tests has been developed to assess nociception in laboratory animals (Le Bars et al., 2001). Rats and mice are by far the most common species used in pain studies.

2.5.1. Tests Based on Thermal Stimuli

The Tail-Flick, Hargreaves, Hot Plate test and test using cold stimuli are used in order to evaluate responses to thermal stimulation. In the present study, we apply the following two tests;

The Paw Withdrawal Test Using Radiant Heat (Hargreaves test) radiant heat was applied to a paw that had already been inflamed by a subcutaneous injection of carrageenan or CFA. One advantage is that the animal moves freely on a glass surface. Animal is tested in three to four sequential trials at approximately 5-min intervals to avoid sensitization of the response (Randall and Selitto, 1957, Hargreaves et al., 1988).

Tests Using Cold Stimuli place the animal on a cold surface or a cold plate (-5° -25° C) cooled by cold water circulating under it. The time taken for the first brisk lift or stamp of the ipsilateral hind paw to occur is recorded. Alternatively, the total number of jumps, flicking and licking of the ipsilateral hind paw are recorded (Chao Ma and Yuguang Huang, 2016).

2.5.2. Tests Based on Mechanical Stimuli

Researchers use Randall and Selitto, Pricking Pain, Von Frey and Q tip or Cotton Swab Test for the behavioral assessment in a mechanical stimulation context. Here, we apply the Von Frey Test, which is analyzed below.

Von Frey Test. Von Frey monofilaments are short nylon calibrated filaments, inserted into a holder that allows the investigator to exert a defined pressure on a punctiform area of the rodent paw. The animal is repeatedly stimulated with increasingly stronger filaments to determine the threshold where a paw withdrawal response is reliably elicited. Stimuli are always presented in a consecutive fashion, either ascending or descending. In the absence of a paw withdrawal response to the initially selected hair, a stronger stimulus is applied. **Electronic Von Frey hair** devices are, also, available (Chao Ma and Yuguang Huang, 2016).

Chapter 3. RGS Proteins

3.1. Heterotrimeric G proteins & G-protein Coupled Receptors

G proteins compromise a diverse family of proteins and participate in several cellular functions. A wide expression of these proteins in brain and peripheral tissues has been reported. Mammalian G proteins can be subdivided into heterotrimeric G proteins and small G proteins. **Heterotrimeric proteins** are involved in transmembrane signaling in the nervous system and are made up of three subunits α, β, and γ. Notably, multiple types of heterotrimeric G protein exist in nervous system and 20 different Ga subunits exist which are subdivided into four main categories based on their sequence homology (Neubig & Siderovski, 2002):

- G_s family stimulates adenyl cyclase (AC), which catalyzes the synthesis of cAMP.
- G_i family (G_0,G_{gust},G_z) can inhibit the AC and voltage-gated Ca^{2+} channels, albeit activate a certain type of K^+ channel, MAP-kinase pathway or activate phosphodiesterase
- G_q family ($G_q\alpha$,$G_{11}\alpha$, $G_{14}\alpha$, $G_{16}\alpha$) is implicated in activation of phospholipase C_β (PLC_β) and inhibition of GIRK channels
- G_{12} family ($G_{11\text{-}16}$) activates Rho-GEFs (Guanine Exchange Factors)

Their molecular weight varies between 38- 52 kDa. These distinct types of alpha subunits share common β and γ subunits; 5 β subunits (Mr 35-36 kDa) and 11 γ subunits (Mr 6-9 kDa) are known till now. More than 35 heterotrimeric G protein subunits had been identified in brain and peripheral tissues (Zachariou et al., 2012). These heterotrimeric proteins interact to a variety of cell-surface proteins that span the membrane seven times, such as neurotransmitter, hormone, cytokine and chemokines receptors, and so these structures termed as G protein-coupled receptors (GPCRs). Expression profiling had shown that single tissues and brain regions express an unexpectedly large number of GPCRs with a unique combination in order to sub serve their diverse functions (Vassilatis et al., 2003).

The functional cycle of heterotrimeric proteins, as shown in fig.10, involves their dissociation and re-association in response to extracellular signals (Zachariou et al., 2012).

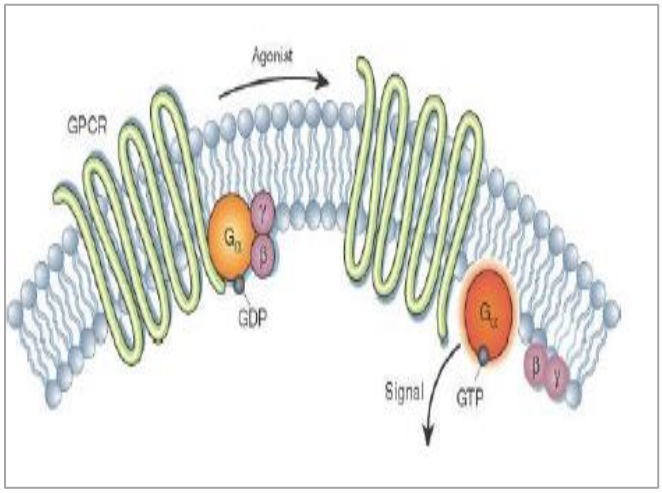

Figure 10: Activation of the G alpha subunit of a G-protein-coupled receptors

In unstimulated cells, the state of G alpha is defined by its interaction with GDP, Gβγ and a GPCR. Upon receptor stimulation by a ligand called an agonist, the state of the receptor changes. G alpha dissociates from the receptor and Gβγ, and GTP is exchanged for the bound GDP, which leads to G alpha activation. G alpha and Gβγ then go on to activate other molecules in the cell. (Adapted by Li, J. et al., 2002).

3.2. Regulator G-protein Signaling (RGS)

GPCRs represent the largest family of membrane proteins in human genome and are the targets of several prescribed drugs including neurological and neuropsychiatric disorders (Terzi et al. 2009). Growing evidence, further, indicates that GPCR cascades are highly implicated in pain hypersensitivity (Geppetti, 2015, Ghanemi, 2015, Veldhuis, 2015). The GPCR signal transduction pathway is controlled by the heterotrimeric G proteins and particularly by the lifetime of the Galpha-GTP form. The intrinsic GTPase activity of the Galpha subunit that leads to its inactivation is too slow to account for the rapid shut-off of GPCR signaling. That timing paradox was explained by the discovery of the regulator of G-protein signaling (RGS) proteins or also termed as GTPase-activating proteins (GAPs) (DiBello et al., 1998). Their functional domain, named RGS, consists of 125 conserved amino acids. RGS proteins bind to the GTP-bound Ga subunit and markedly accelerate its rate of GTP hydrolysis (Fig.11) and so on modulate the duration and the amplitude of signal, leading to the termination of the signaling event.

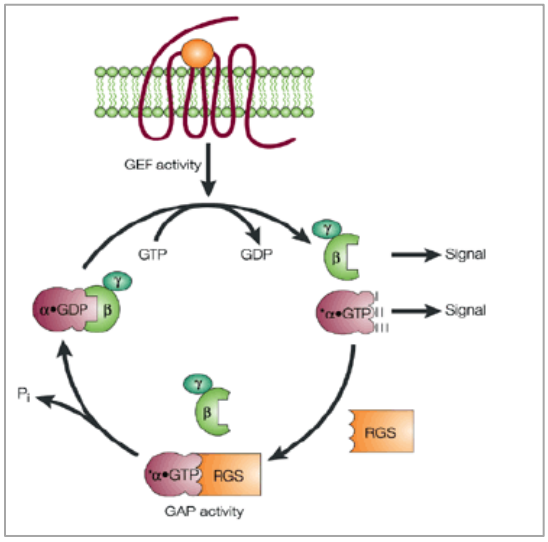

Figure 11: RGS proteins as Gα GTPase-accelerating proteins (GAPs)

*The binding of ligands to GPCRs enhances the receptor guanine-nucleotide-exchange (GEF) activity, leading to the loading of GTP by the Gα subunit, conformational changes in the Gα switch regions, producing the active * alpha conformation, dissociation of the Gα-Gβγ complex, and resultant effector interactions (denoted by 'signal' in Figure). (Adapted by Neubig & Siderovski, 2002)*

Moreover, the RGS proteins have specificity for discrete G alpha subfamilies and modulate selectively particular GPCR actions. Each tissue expresses a distinct repertoire of RGS protein and these proteins can be differentially up- or downregulated by physiological signals or pathological situations (Neubig & Siderovski, 2002). In human, RGS proteins comprise a structurally and functionally diverse superfamily with 37 gene products, which are divided into 10 subfamilies (Fig.12).

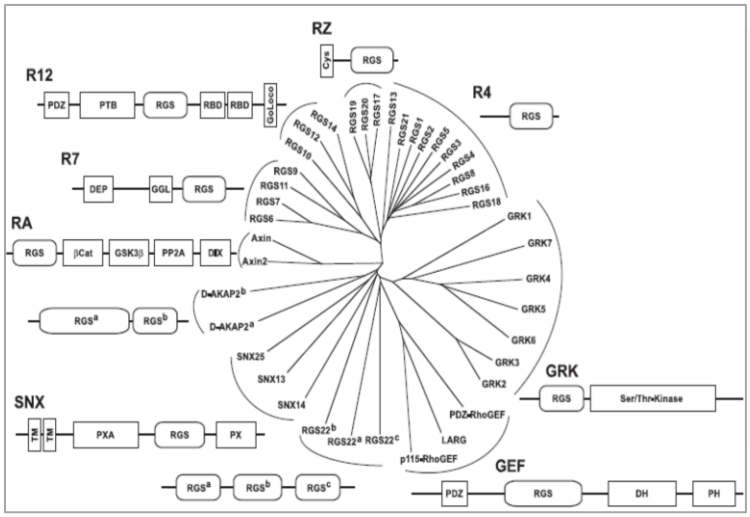

Figure 12: Subfamily categorizations of the 37 RGS domain-containing proteins identified in humans

The categorization based on sequence similarities and domain architectures (Adapted by Kimple et al., 2011).

Several members of the RGS family are expressed in the brain and similar expression pattern observed between rodents and human (Gold et al., 1997, Grafstein-Dunn et al., 2001, Larminie et al., 2004). These molecules regulate essential physiological processes such as vision (Chen et al., 2000, Nishiguchi, 2004),

locomotion (Rahnan et al., 2003) and working memory (Buckholtz et al., 2007), whereas their dysfunction is associated with several neuropathological conditions (Terzi et al., 2009).

3.3. Regulator G-protein Signaling 4 (RGS4)

RGS4 belongs to R4 family of RGS protein and has a molecular weight around 24 KDa. It consists of a single RGS domain with modest flanking amino and carboxy terminal regions. *In vitro* studies have revealed a direct interaction between RGS4 and the 3rd intracellular loop of several GPCRs, including muscarinic and opioid (Bernstein et al., 2004, Georgoussi et al., 2006). Rgs4 also acts as a GAP on both Gai/o and Gaq subunits (Huang et al., 1997). In situ hybridization data indicate that RGS4 is expressed throughout the brain (Gold et al., 1997). Specifically, this molecule is abundant in cortex, thalamus, striatum, Locus Coreleus, the pyramidal cells of the Hippocampus, and in the spinal cord (Gold et al., 1997, Larminie et al., 2004). Nevertheless, the RGS4 mRNA levels are the highest RGS measured levels within the brain (Larminie et al., 2004), the RGS4 basal protein levels are typically low, because this protein contains an N-terminal cysteine-rich sequence which promotes its rapid proteolytic degradation. Five different RGS4 isoforms are found in human brain, which are highly abundant in the cortex and totally absent from cerebellum. Regarding the mouse brain, only 3 splice variants are expressed, with differences only at their 3' UTR (Ding et al., 2009).

Polymorphisms in RGS4 and decreased protein expression in the dorsolateral prefrontal cortex are implicated in schizophrenia. RGS4 is widely distributed at the synapses in the pyramidal cortical neurons (Paspalas et al., 2009) and modulates G protein signaling, specifically 5-HT1A serotonin receptor signaling, both pre- and post-synaptically in the prefrontal cortex. A recent report indicates that RGS4 can limit the crosstalk between the a2 adrenergic Receptors (R) and GABA-B Rs, aiding the inactivation of the G proteins and preventing interference between the two receptors' neuromodulatory functions despite their close proximity (Lur and Higley, 2015). In Parkinson disease decreased striatal dopamine upregulates RGS4 expression specifically in cholinergic interneurons where RGS4 diminishes signaling through presynaptic M4 muscarinic acetylcholine auto receptors (Ding et al., 2006). Furthermore, RGS4 regulates dopaminergic control of striatal LTD via modulation of G-protein signaling postsynaptically, in a particular type of GABA-ergic inhibitory neurons (indirect pathway medium spiny neurons/ D2-type MSNs) within the striatum (Gerber et al., 2016). Moreover, RGS4 blocks mGluR5-mediated retrograde opioid release from neuroendocrine cells in the hypothalamus, increasing GABA release onto these neurons, while glucocorticoid receptor activation suppresses RGS4 expression and permits mGluR5 signaling, leading to the establishment of synaptic plasticity in this area. In the hippocampus, RGS4 has been shown to inhibit

signaling through group I metabotropic glutamate receptors (mGluR1 and 5), blocking mGluR5-mediated inhibition of the after-hyperpolarization current in CA1 neurons (Saugstad et al., 1998).

Studies indicate that RGS4 is implicated in pain modulation. Drugs of abuse have been shown to affect RGS4 mRNA levels in Locus Coeruleus (LC) and nucleus accumbens (NAc). Acute morphine or amphetamine administration decreased RGS4 expression in the LC, whereas the same treatment upregulated RGS4 in the NAc. Interestingly, in the LC, repeated morphine administration leads to increased RGS4 transcription and RGS4 protein acts as a negative regulator of morphine reward and promotes analgesia by opioids (such as methadone and fentanyl) in the NAc (Han et al., 2010). Notably, in a model of neuropathic pain it was proved that only RGS4 is upregulated in rat spinal cord during the development of hyperalgesia (Garnier et al., 2003). Interestingly, RGS4 is a positive modulator of analgesic response to antidepressant drugs in a neuropathic model as well (Stratinaki et al., 2013). Another study demonstrates that RGS4 mutations are a risk factor for fibromyalgia (Smith et al., 2012). Recently, it was published that appliance of intrathecal RGS4 Inhibitor reduces the nociceptive responses and enhances opioid-mediated analgesic effects in the mouse formalin test (Seo-Yeon Yoon et al., 2015). However, the exact molecular role of Rgs4 to chronic pain behaviors and particular in brain is unknown.

Aim

Based on previous findings (Stratinaki et al. 2013) that RGS4 is a positive modulator of analgesic response to antidepressant drugs in a neuropathic pain model, we aim to investigate the exact functional role of RGS4 in brain under chronic inflammatory pain states.

Materials & Methods

Animal studies

For all behavioral assays, we used 2- to 3-mo-old male and female RGS4KO (*Rgs4*-Knock-out) mice and their RGS4WT (*Rgs4*-Wild-type) littermates (RGS4KO mice were generated by Han et al., 2010). RGS4WT and KO mice were bred in house from homozygous RGS4 breeders. Mice were group-housed (maximum five per cage) on a 12-h light/dark cycle, provided with food and water ad libitum. Animal handling was in accordance to the animal care and use committee of Icahn School of Medicine at Mount Sinai. Mice were genotyped with polymerase chain reaction (PCR), using DNA extracted by ear of each mice. For viral infections, we used 2- to 3-mo-old male floxed RGS4 mice. The mutant mice used were on DBA background, which explains differences in some of the baseline responses. For qPCR assays, adult C57BL/6 male mice were used (The Jackson Laboratory).

The Complete Freud's Adjuvant (CFA) model for inflammatory pain

Complete Freud's Adjuvant (CFA) (Sigma, Alidrich) was diluted 1:1 with saline (0.9% NaCl) to a final concentration of 1 mg/ml until it was emulsified. 25-30µl of the emulsion were injected to the plantar surface of the left paw of each mouse. All assays of inflammatory pain took place at least 24h after the injection.

Stereotaxic Surgery and Viral-Mediated Gene Transfer

Conditional deletion of *Rgs4* gene was achieved via application of viruses (bilaterally injection) AAV2-CMV-Cre-GFP and AAV2-CMV-GFP (as control) into nucleus accumbens (NAc) and ventral posteromedial thalamic nuclei (VPm) of floxed RGS4 mice. AAV serotype 2 shows tropism for neurons and a high expression peak at 14 days post infection (slow onset action). Stereotaxic coordinates for vector injections in NAc were as follows: anteriorposterior+1.6 mm, lateral ± 1.5 mm, and dorsoventral −4.4 mm at an angle of 10° from the midline (relative to Bregma) (Stratinaki et al., 2013). Regarding the VPm nucleus: anteriorposterior -1.7 mm, lateral 2.2 mm, dorsoventral -3.5 mm at an angle of 10° from the midline. For all stereotaxic surgeries, mice were anesthetized with avertine and experiments were performed 2 weeks later.

Behavioral Assays

Von Frey Test for Mechanical Allodynia. Von Frey filaments of varying degrees (0.07-2 g) were used (IITC). Each filament was applied five times in a row against the lateral area of the paw. Hindpaw withdrawal or

licking induced by the filament was defined as positive pain response. A positive response in three of five repetitive stimuli was defined as the pain threshold. Mice were habituated to the Von Frey apparatus for 1 hour every day for 7 days, and baseline were measured before the CFA injection.

Hargreaves test for Thermal hyperalgesia. A heat lamp is applied on the inflamed hinpaw and the experimenter records the time (sec) until a reaction is observed. Hind paw withdrawal and licking defined as positive reaction to the stimulus. As a cut-off is defined 20 sec and the intensity of heat lamp (AI) is at 30%. Mice were placed on glass apparatus (IITC) 20-30 min before the measurements. Male mice were habituated to the apparatus for 30 min for 2 days and baseline were measured before the CFA injection.

Cold plate for cold allodynia. Mice were placed on a cold surface (at 0 ◦C using IITC hot/cold – plate) and the nocifensive behavior will be assessed by the measurement of total number of jumps and hindpaw flicking/licking for a period of 5 minutes (Colburn et al., 2007).

RNA extraction and Real-time quantitative polymerase chain reaction (RT-PCR or qPCR) assay

Brain tissue from adult male C57BL/6 mice (8 weeks old) pain free mice (naïve) and mice were injected with CFA was dissected, and RNA was extracted by using trizol, Isopropanol and ethanol. qPCR (data expressed as log2 fold change) was performed by using SYBR green on an Applied Biosystems 7500 system. Reactions were run in triplicate and analyzed by using the ΔΔCt method and GADPH gene expression as normalization control.

Generation of conditional RGS4 knock-out mice in Transient Receptor Potential Vallinoid type-1 (TRPV1)-expressing cells

A TrpV1-Cre line were used, in which the expression of Cre recombinase is driven by the TRPV1 promoter. TrpV1 Cre mice (C57BL/6 background), obtained from The Jackson Laboratory, were crossed to floxed RGS4 (female) mice (DBA background) in order to generate floxed RGS4/TrpV1Cre$^{+/+}$. After 3 progenies, floxedRGS4/TrpV1Cre$^{+/+}$ and their WT controls (RGS4WT/ TrpV1Cre$^{+/+}$) were obtained.

Statistical analysis

Two way ANOVA or unpaired two-tailed Student t tests were utilized to examine significant effects of CFA over genotype for all behavioral experiments and qPCR assays, respectively. Significant post-hoc effects were revealed by the Bonferroni post-hoc test, and effects were considered to be significant at $p<0.05$ (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

Results

Rgs4 mRNA levels are highly regulated under chronic inflammatory pain

Our first set of studies examined *Rgs4* mRNA levels in several regions involved in nociceptive processing at a late time point after the induction of inflammatory pain (day 17 post CFA). For these experiments, we used two groups of adult male C57BL/6 mice (8 weeks old) pain free mice (naïve) and mice were injected with CFA. Quantitative PCR (qPCR) analysis reveals that *Rgs4* mRNA levels are highly upregulated in the thalamus (Naïve=1.04±0.13, 17 days post CFA=1.46±0.08; *P<0.05) and the PeriAqueductal Gray (PAG) (Naïve=1.0±0.02, 17 days post CFA= 1.28±0.03; ***P<0.001), while they do not change in the raphe nucleus in CFA-treated mice (Fig.1b). Importantly, the regulation on mRNA levels is not observed at early time points (day 4 post CFA, Fig.1a) neither at the thalamus (Naïve=1.02±0.9, 4 days CFA=0.92±0.11) nor at the PAG (Naïve=1.05±0.17, 4 days post CFA=0.93±0.1). Interestingly, an opposite regulation on *Rgs4* mRNA levels at the ipsilateral (named according to the injected-paw, here is the left side of spinal cord) spinal cord was quantified (Naïve=1.07±0.16, 17 days post CFA=0.54±0.08; *P<0.05). The late time point regulation of *Rgs4* mRNA levels suggests a role of RGS4 in signal transduction adaptions under persistent pain.

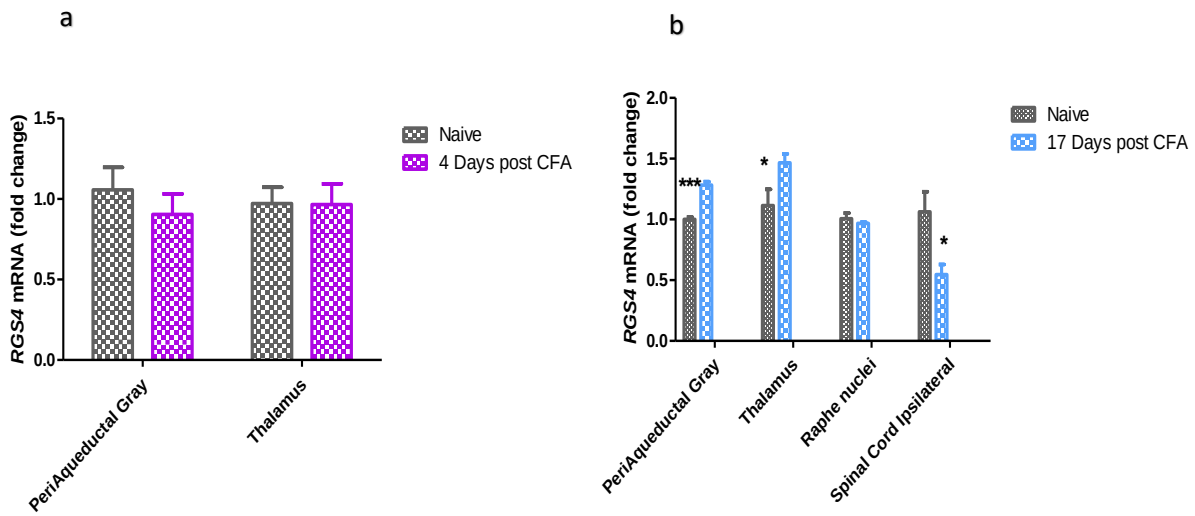

Fig.1: ***Rgs4* mRNA levels are regulated in CFA-induced persistent inflammatory pain model**. qPCR analysis of tissue from brain regions and spinal cord reveals that *Rgs4* mRNA levels are highly upregulated in PAG and thalamus, whereas they are down-regulated in ipsilateral spinal cord side on 17 days post CFA (b). In contrast, *Rgs4* mRNA levels do not change on day 4 post CFA (a). *P<0.05;**P<0.01;***P<0.001 (t test, unpaired two-tailed); mice per group (n) 6 -7. Data are presented as mean ± SEM.

RGS4 modulates mechanical allodynia associated with chronic inflammatory pain states

Based on previous findings we hypothesized that RGS4 plays a functional role in sensitized behaviors after the induction of peripheral inflammation. To test this hypothesis, we first used genetically modified mice to examine whether *Rgs4* deletion affects the behavioral responses under persistent inflammatory pain. Constitutive knockout mice of *Rgs4* injected with CFA show a gradual recovery from mechanical allodynia started from day 9 post CFA, in contrast to their wildtype controls. Interestingly, on day 17 post CFA RGS4KO mice recover from mechanical allodynia, whereas RGS4WT are still in deep pain (Fig. 2a). Independent groups were monitored in mechanical allodynia for 33 days post CFA and RGS4WT group started to recover after 33 days post CFA injection (data not shown). Notably, both genotypes show increased thermal hyperalgesia in response to hind paw CFA injection, and RGS4WT and RGS4KO groups recover in a similar way from thermal hyperalgesia. Collectively the data suggest a modality selective role of RGS4 in modulation of central sensitization (Fig.2b).

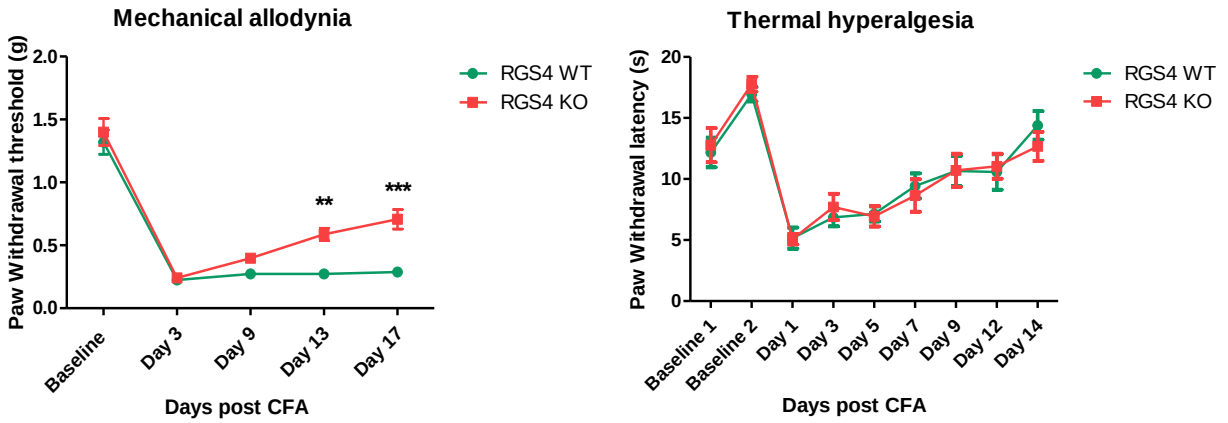

Fig.2: **RGS4KO male mice recover from mechanical allodynia, while they do not recover from thermal hyperalgesia.** (a) On day 17 post CFA RGS4KO mice recover more rapidly from mechanical allodynia (b) Both WT and KO male mice are under deep thermal hyperalgesia and have similar recovery profile from hyperalgesia. (*P<0.01, **P<0.001, ***P<0.0001), 2-way ANOVA followed by Bonferroni post-tests; (a) n=14-15 per group; (b) n=11 per group). Data are presented as mean ± SEM.

RGS4 do not modulate mechanical allodynia in a sex-dependent manner

In order to figure out if RGS4 acts in a sex-specific manner under pathological pain, we monitor female constitutive RGS4KO and their WT littermates in mechanical allodynia and thermal hyperalgesia tests. In case of Von Frey measurements, RGS4KO mice recover from mechanical allodynia on day 8 post CFA, in contrast to their WT controls. Intriguingly, the recovery profile is different from male RGS4KO mice (Fig.2a) as female RGS4KO mice do not show a gradual recovery instead they recover more rapidly and retain the same level of mechanical recovery till day 17 (Fig.3a). Similarly to male group, RGS4KO and RGS4WT female mice show increased thermal hyperalgesia and no genotype differences were noticed regarding the recovery from hyperalgesia (Fig.3b).

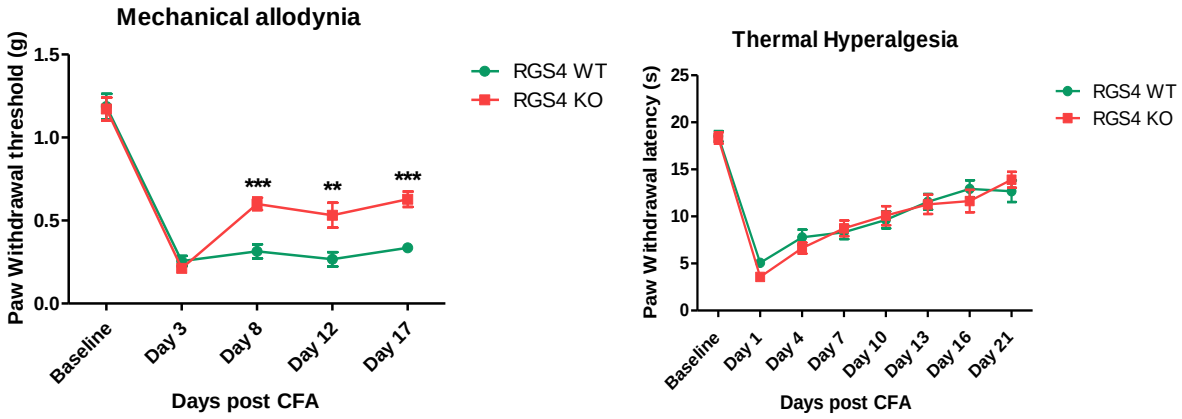

Fig.3: **RGS4KO female mice recover from mechanical allodynia, while they do not recover from thermal hyperalgesia.** (a) On day 8 post CFA RGS4KO mice recover rapidly from mechanical allodynia, a small drop at withdrawal threshold was noticed on day 12 post CFA, albeit statistical significance was stable till day 17 post CFA. (b) Both WT and KO female mice have a similar slow recovery profile from thermal hyperalgesia (*P<0.01, **P<0.001, ***P<0.0001), 2-way ANOVA followed by Bonferroni post-tests; (a , b) n=14-15 per group). Data are presented as mean ± SEM.

RGS4 modulates responses to cold allodynia

Our behavioral studies so far suggest that RGS4 acts in a modality specificity manner since RGS4KO mice only recover from mechanical allodynia (Fig.2 & 3). Cold plate test were applied in order to investigate whether in addition to mechanical allodynia, RGS4 modulates responses to cold allodynia. Interestingly, female RGS4KO mice show attenuated response to cold plate test on day 9 post CFA in contrast to their RGS4WT littermates (Fig.4). This finding further supports a selective role of RGS4 in particular neuronal pathways contributing to allodynia.

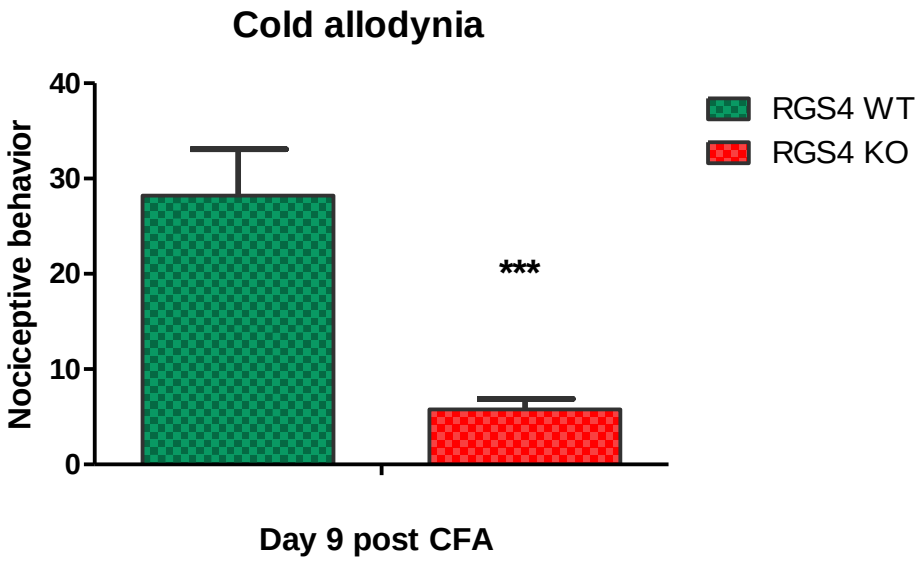

Fig.4: **Constitutive RGS4KO female mice attenuate response to cold (0° C) on day 9 post CFA**. Nociceptive behavior represents the total number of jumps and hindpaw flicking/licking for a period of 5 min. ***P<0.001 (t test, unpaired two-tailed); n=14-15 per group. Data are presented as mean ± SEM.

RGS4 actions in ventral posteromedial thalamic nucleus (VPm) modulate mechanical allodynia

Based on our qPCR results (Fig.1) we hypothesize that the thalamus and the PAG are two of the key brain regions involved in the phenotype observed on the RGS4KO mice. We hypothesize that ventral posterior thalamic region plays a key role, since lesions of Ventroposterior nucleus can result to persistent neuropathic pain (Kim et al., 2007, Klit et al., 2009,Hong et al., 2010) and individuals with neuropathic pain have reduced thalamic volume (Pattany et al., 2002, Apkarian et al.,2004, Gustin et al., 2011). We target the ventral posteromedial thalamic nucleus (VPm), which is the major thalamic relay nucleus, where the dorsal horn neurons terminate, and thalamic neurons then send projections to the somatosensory cortex (Graig et al., 1994). As shown in Fig.5, this VPm specific knockdown of RGS4 results in the same mechanical recovery phenotype to that observed in constitutive KO mice (Fig.2a), suggesting that RGS4 actions in VPm mediate this behavior.

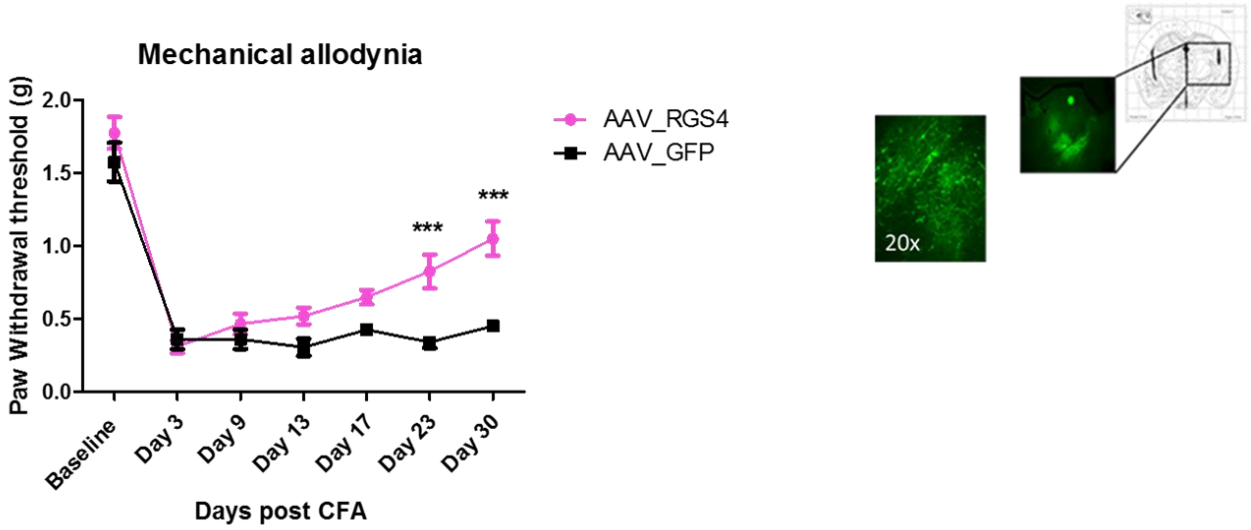

Fig.5: ***Rgs4* Knockdown in VPm leads to recovery from mechanical allodynia in a CFA model.** Mice with selective KD of *Rgs4* (AAV_RGS4) show a gradual recovery from mechanical allodynia started on day 9 post CFA. On day 23 and 30, AAV_RGS4 group has completely recover, whereas AAV_GFP group shows increased allodynia. Right side: viral injection site (VPm nucleus) at Paxinos and Watson mouse brain atlas (***P<0.001, 2-way ANOVA followed by Bonferroni post-tests); n=8 per group. Data are presented as mean ± SEM.

RGS4 actions in nucleus accumbens (NAc) do not modulate mechanical allodynia

Previous work from our lab demonstrates that RGS4 in the NAc act as a positive modulator of the antiallodynic-like actions of several monoamine-directed antidepressant drugs which are usually prescribed for neuropathic pain. We next investigated the tactile responses of the selective knockdown of *Rgs4* from NAc under chronic inflammatory states. As shown in Fig.6, loss of RGS4 in NAc does not affect the recovery from mechanical allodynia. The serotype (AAV2 Cre) that used leads to 67±11 % reduction on *Rgs4* levels in neurons (Stratinaki et al., 2011).

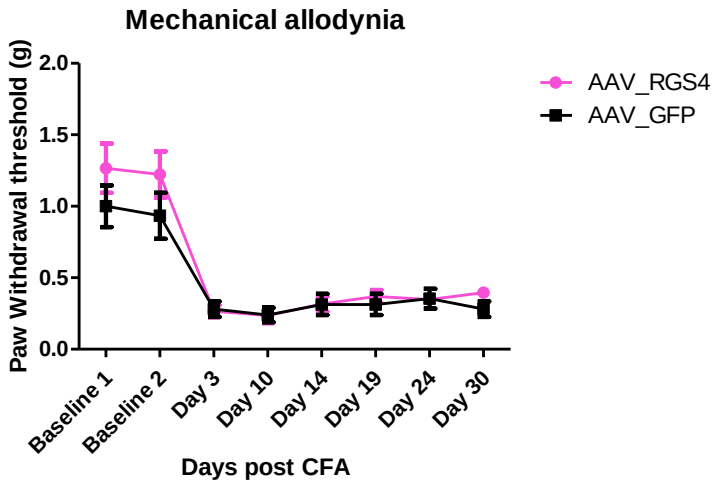

Fig.6**: *Rgs4* Knockdown in NAc does not affect recovery from mechanical allodynia in a CFA model**. Both mice with selective KD of *Rgs4* (AAV_RGS4) and control group (AAV_GFP) show still maximal allodynia a month after the onset of peripheral inflammation. (2-way ANOVA followed by Bonferroni post-tests); n=9 per group. Data are presented as mean ± SEM.

Collectively, these data set of experiments provide evidence about the exact brain area associated with RGS4 actions under inflammatory pain states.

RGS4 actions in a somatosensory neural network expressing Vallinoid type-1 receptors modulate in part the mechanical allodynia in response to peripheral inflammation.

Since there is tremendous potential for plasticity at network-level processing of nociceptive inputs (Woolf & Salter 2000, Kuner 2010), we also, investigate the functional role of RGS4 in a nociceptive network. Transient Receptor Potential (TRP) channels expression differentiates somatosensory neuron subtypes involved in nociception. We used the Cre recombinase approach in order to ablate the *Rgs4* gene in specific subsets of somatosensory neurons and we performed mechanical allodynia studies under

inflammatory pain-induced sensitized behaviors. We focus on TRPV1 non-selective cation channel, which appears to respond to a wide range of stimuli such as heat, botanical compounds, pro-inflammatory agents and notably intracellular signaling pathways engaging to inflammation (Venkatachalam & Montell, 2007, Le Pichon & Chesler, 2014). TRPV1 channels are highly expressed in C nociceptive fibers that detect the noxious stimuli in periphery, albeit reveal a highly restricted brain expression pattern in brain such as pain related regions; PAG and raphe nuclei (Cavanaugh et al., 2011). Interestingly, it was published that TRPV1 channels in the spinal GABAergic interneurons mediate the mechanical allodynia in neuropathic pain, whereas TRPV1 channels in C nociceptive neurons do not implicated in mechanical allodynia (Kim et al., 2012). Cre recombinase is driven by the TRPV1 promoter, thus targeting *Rgs4* in neurons expressing TRPV1 channels will determine whether RGS4 plays a crucial downstream role in the TRPV1-neuronal network in the development of mechanical allodynia under chronic inflammatory pain. As shown in Fig.7 floxedRGS4/TrpV1Cre$^{+/+}$ male mice started gradually to recover from mechanical allodynia on day 9 till day 17 post CFA, whereas the control group (RGS4WT/ TrpV1Cre$^{+/+}$) shows maximal allodynia. Interestingly, from day 24 post CFA and on, control group started rapidly to recover from mechanical allodynia. These findings indicate that RGS4 action in TrpV1-expressing neurons has a mild effect on male mice (Fig.7) compare to the one observed when we knocked-down *Rgs4* in the thalamus (fig.5), suggesting that while RGS4 actions in TRPV1 positive nociceptive neurons affect the recovery from mechanical allodynia, RGS4 actions in the thalamic neurons have a more potent role in modulating inflammatory pain-induced sensitized behaviors.

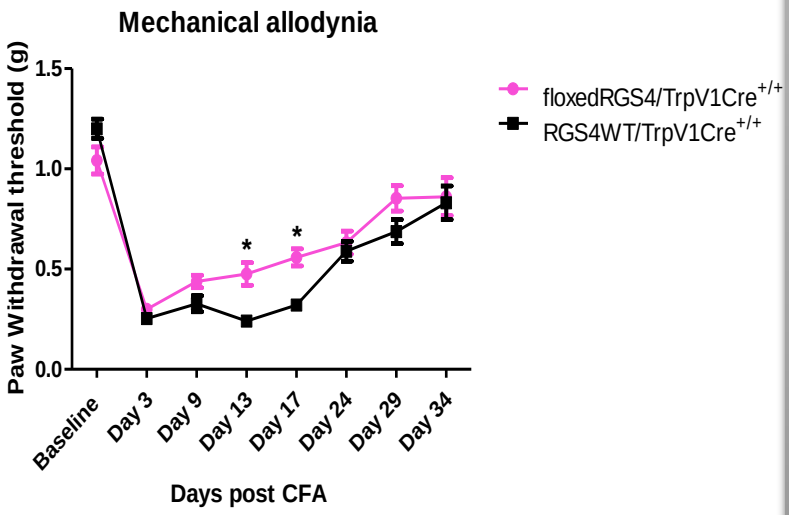

Fig.7: ***Rgs4* Knock out in neurons that express TrpV1 channels leads to partial recovery from mechanical allodynia in a CFA model.** On day 13 and day 19 post CFA, mice lacking *Rgs4* in TrpV1-expressing neurons recover from mechanical allodynia, while control group (RGS4WT/ TrpV1Cre+/+) started to recover rapidly from day 24 post CFA.

(*p<0.05; 2-way ANOVA followed by Bonferroni post-test); n=18-19 per group. Data are presented as mean ± SEM.

Discussion

Reports on pain studies indicate that approximately 1.5 billion people worldwide suffer from chronic pain disorders (Global Analysis, 2011). Part of the reason that the current therapeutic strategies are insufficient or produce major side effects, has to do with the incomplete understanding of molecular events involved in development and maintenance of chronic pain states (Schaible, 2015). The data presented here demonstrate a functional role of RGS4 protein in chronic inflammatory pain states. We show that RGS4 modulates the development and maintenance of both mechanical and cold allodynia in the CFA inflammatory model. Our work provides evidence that, RGS4 actions in the thalamus (VPm nucleus) and in a subset of nociceptive neurons (neurons expressing TRPV1 channels) contribute to mechanical allodynia observed after the induction of peripheral inflammation. The present study is the first study that reveals a direct role of a RGS member, the RGS4 protein, in intracellular mechanisms of pain and signal transduction adaptations in regions outside of the spinal cord.

We focused on the investigation of RGS4 because this molecule is a potent modulator of GPCR activity in the nociceptive pathway under pain states (Garnier et al., 2003, Yoon et al., 2015). In addition previous work from our lab, used a neuropathic pain model to demonstrate that RGS4 is a positive modulator of analgesic responses to tricyclic antidepressant drugs, a class of drugs widely prescribed for the treatment of neuropathic pain conditions (Stratinaki et al., 2013). Here, studies on constitutive knock-out male and female mice reveal a unique role of RGS4 in inflammatory pain-induced sensitized behaviors. Mice lacking the *Rgs4* gene recover from mechanical allodynia, albeit do not recover from thermal hyperalgesia. Our behavioral findings also show that RGS4 does not act in a sex-dependent manner. Notably, rapid recovery from mechanical allodynia has been noticed, in addition to the inflammatory model, under neuropathic pain states (data not shown). These findings strictly indicate a functional role of RGS4 under a wide range of chronic pain disorders and encourage efforts towards to the global drug development by targeting RGS4 activity.

Another novel piece of information extracted from the present study, concerns the role of RGS4 in cold allodynia. Our behavioral studies so far reveal that RGS4 acts in a modality-specificity manner, since constitutive KO mice attenuate responses to mechanical and cold allodynia. Future work should elucidate any sex-specific RGS4 action in cold allodynia. Nevertheless, these data suggest a crucial and unique role of an intracellular signal transduction molecule; RGS4 protein in pain fibers that contribute to allodynia.

Moreover, this interpretation indicates that RGS4 participates in central sensitization by acting downstream in intracellular signaling pathways that are activated in specific pain fibers (fibers contribute to allodynia).

The wide distribution of RGS4 in the central nervous system, the absence of antagonists that can selectively target RGS4 and can permeate the blood brain barrier are the main reasons that little is known about its action. To generate targeted interventions in RGS4 activity, we are using genetically modified animal models and particularly models for conditional knock-down of *Rgs4*, in addition to constitutive knockout that show potential developmental deficits associated with global deletion of *Rgs4* gene. Interestingly, the conditional knockdown approach revealed that RGS4 in ventral posteromedial thalamic nucleus (VPm) is a positive modulator of mechanical allodynia observed under chronic inflammatory states. In contrast, at the level of NAc RGS4 actions do not affect mechanical allodynia *de novo*, whereas RGS4 in this brain region acts as a positive modulator of the antidepressant-like and antiallodynic-like actions of several monoamine-directed antidepressant drugs (Stratinaki et al., 2013).

Research over the past years has provided knowledge on the molecular and cellular events on the spinal cord that result in the development and maintenance of persistent pain (Basbaum et al., 2009, Kuner, 2010, Gangadharan & Kuner, 2013, Luo et al., 2014). However, there are very few studies on the intracellular mechanisms involved in pain processing on supraspinal sites. The thalamus has a key role in gating, filtering and processing sensory information to the cerebral cortex. Two reports indicate a key role of metabotropic GlutamateR1/mGluR5-PLCβ complexes in thalamus under chronic inflammatory and visceral pain (Miyata et al.2003, Cheng et al, 2008). Thus, it is urgent to further understand the signal transduction events in the thalamus under chronic pain states. Our study shed light on the intracellular signaling adaptations are taking place in VPm thalamic nucleus under persistent inflammatory pain and reveals the critical role of RGS4, an intracellular modulator, in the development and maintenance of mechanical allodynia. This finding is consistent with the known role of VPm as it is part of a major relay station of nociceptive input in the brain (Graig et al., 1994). In future studies, we aim to identify, whether RGS4 actions in VPm mediate cold allodynia. Molecular studies on the signal transduction pathways affected by RGS4 in chronic pain are needed in order to figure out the mechanism via which RGS4 leads to mechanical allodynia. Based on published reports, we suggest that either mTOR (Jiménez-Díaz et al., 2008, Price et al., 2009, Melemedjian et al., 2010, Ferrari et al., 2013) or WNT pathway (Zhang et al., 20113, González-Fernández et al., 2014) components or their target genes are regulated by RGS4 leading to mechanical allodynia. Interestingly, deletion of a major negative downstream effector of the mTOR

pathway (the eukaryotic initiation factor 4E-binding protein 1 (4E-BP)), leads to mechanical but not to thermal pain hypersensitivity (Khoutorsky et al., 2015). This phenotype is the same one we observed on RGS4 constitutive KO. Another important clue is that β-catenin regulates the expression of genes (eg. GABA receptors, T- type channels) that are critical for neuronal excitation and the function of thalamocortical relay neurons (Wisniewska et al., 2012, Wisniewska et al., 2013). The function of these cells is very important since all sensory information enters the neocortex by way of the thalamus.

The Thalamic Reticular Nucleus (TRN) is another important structure that modulates the transfer of information from the thalamus to the cortex. This nucleus consists of a thin sheet of neurons, exclusively GABergic, which surrounds the VPm. Gustin et al. (2011) proposed the following central events which result in the development and/or maintenance of chronic neuropathic pain. An initial loss of neurons in the VP results in a loss of excitatory inputs to the TRN which in turn results in altered inhibitory GABA input in VP thalamus. This disturbed thalamocortical activity may result in the constant perception of intense pain. Furthermore, GABAergic inhibition via the TRN is differentially regulated by metabotropic glutamate receptors (mGluRs). Indeed, a recent report adds that mGluR-mediated Ca2+-signalling in the TRN to the state-dependent modulators of the thalamocortical system (Neyer et al., 2016). As mGluRs are regulated by the RGS4 (Saugstad et al., 1998, Salt et al., 2013) we speculate that RGS4 may act at that level by regulating the mGluR-mediated signaling.

Importantly, we developed genetic tools to examine the network-specific actions of RGS4 and demonstrated that RGS4 is a partial modulator of mechanical allodynia in TRPV1-neuronal network. This network-specific approach inspires for the development of novel pain therapies by coupling key nociceptive molecules (TRPV1) that specify neuronal types with key intracellular signaling molecules (RGS4) that modulate signaling events leading to particular pain symptoms (mechanical allodynia). Nevertheless, several studies in the pain field have attributed specific noxious stimulation to specific sensory cell types, these genetic studies create a clearer picture of how specific modalities are encoded by the somatosensory system revealing the central mechanisms of that integration (Pichon & Chesler 2014). Towards to this, targeting intracellular molecules (RGS4) in cell types helps to unravel the downstream signaling pathways that result to pain hypersensitivity in neural circuits. Our data suggest that in TRPV1-neuronal network RGS4 is activated downstream and in part mediates the mechanical allodynia in chronic inflammatory pain. We conclude that while RGS4 actions in TRPV1 positive nociceptive neurons affect the recovery from mechanical allodynia, RGS4 actions in the thalamic neurons have a more

potent role in modulating central sensitization, and prevention of RGS4 activity in the VPm thalamic nucleus leads to substantial alleviation of mechanical allodynia.

Our work highlights the need to figure out the critical molecular brain mechanisms underlying chronic pain states. Overall these data suggest that RGS4 actions in both thalamus and TRPV1 positive neurons promote sensitized behaviors that develop after peripheral inflammation. Therefore, blockade of RGS4 activity, will alleviate allodynia via actions in several RGS4 controlled-networks.

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