UNIVERSITY OF CRETE
DEPARTMENTS OF BIOLOGY AND MEDICINE,
INSTITUTE OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY- FORTH
JOINT GRADUATE PROGRAM:

"MOLECULAR BIOLOGY AND BIOMEDICINE"

2013

The Role of Regulator of G-protein Signaling 20 (RGS20) in Inflammatory Pain Antinociception, Opioid-induced Analgesia & Morphine Tolerance

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Ευχαριστίες

Κλείνοντας αυτόν τον κύκλο, θα ήθελα να ευχαριστήσω αρχικά την επιβλέπουσα καθηγήτριά μου κ. Βενετία Ζαχαρίου για την καθοδήγηση και την στήριξη που μου παρείχε αυτόν τον ένα χρόνο που βρίσκομαι στο εργαστήριο, καθώς και τα μέλη της τριμελούς επιτροπής, κ. Δ. Καρδάση και κ. Κ. Πετράτο, για την συμβολή τους στο πλαίσιο των προφορικών εξετάσεων του προγράμματος.

Επίσης, ένα μεγάλο ευχαριστώ οφείλω στη Βίκυ, την Άρτεμις, την Δήμητρα και τη Μάρω για την άψογη συνεργασία και το ευχάριστο κλίμα στο lab ☺

Και φυσικά δεν θα μπορούσα να μην ευχαριστήσω την οικογένειά μου..!!Χωρίς την στήριξή τους τίποτε δεν θα ήταν εφικτό…

Περίληψη

Ο χρόνιος πόνος αποτελεί ίσως μια από τις πιο διαδεδομένες ασθένειες, συμβάλλοντας στα ποσοστά νοσηρότητας και θνησιμότητας καθώς και στην επιβολή υψηλών κοινωνικών δαπανών. Οι μέχρι στιγμής διαθέσιμες θεραπείες για περιπτώσεις χρόνιου πόνου, αποδεικνύονται ανεπαρκείς, με τα ευρέως χρησιμοποιούμενα αναλγητικά να χαρακτηρίζονται από μειωμένη δραστικότητα και σημαντικές παρενέργειες.

Τα οποιοειδή χρησιμοποιούνται κατά κόρον σε περιπτώσεις οξέος πόνου. Δυστυχώς, η επανειλημμένη χορήγησή τους μπορεί να οδηγήσει σε σταδιακή μείωση των αναλγητικών ιδιοτήτων του φαρμάκου (αναλγητική ανοχή) καθώς και σε φαινόμενα εθισμού. Η διερεύνηση των μηχανισμών που εμπλέκονται στην ανάπτυξη αναλγητικής ανοχής και εθισμού εχεί συμβάλλει στην κατανόηση της νευροβιολογικής βάσης αυτών των φαινομένων. Παρ'όλα αυτά, οι κυτταρικοί και μοριακοί μηχανισμοί που επακολουθούν την ενεργοποίηση του υποδοχέα (μ-opioid receptor (MOR)) είναι περίπλοκοι και όχι πλήρως κατανοητοί.

Μέλη της οικογένειας των RGS πρωτεινών (ρυθμιστές της σηματοδότησης μέσω G πρωτεινών) έχει δειχθεί να σχετίζονται με την ρύθμιση των δράσεων της μορφίνης.

Μελέτες του εργαστηρίου μας συσχετίζουν την δράση των πρωτεινών RGS9-2 και RGS4 με τους μηχανισμούς ανάπτυξης αναλγησίας και εθισμού σε οποιοειδή. Στην εν λόγω μελέτη, χρησιμοποιώντας ως οργανισμό-μοντέλο τον ποντικό (M.Musculus) σε συνδυασμό με συμπεριφορικά παραδείγματα φλεγμονώδους πόνου και αξιολόγησης των δράσεων των οποιοειδών, παραθέτουμε τον ρόλο μιας ακόμη RGS πρωτεινης, της RGS20, στον φλεγμονώδη πόνο, την αναλγησία και την ανάπτυξη ανοχής σε μορφίνη. Συγκεκριμένα, η RGS20 αποτελεί αρνητικό ρυθμιστή της αναλγησίας, αλλά ταυτόχρονα και θετικό ρυθμιστή της ανάπτυξης ανοχής σε οπιοειδή μέσω της δράσης της σε συγκεκριμένη περιοχή του εγκεφάλου (PAG). Επίσης, η RGS20 φαίνεται να μην εμπλέκεται στην ανάπτυξη του εθισμού και συμβάλλει στην αποκατάσταση περιπτώσεων χρόνιου φλεγμονώδους πόνου με τρόπο εξαρτώμενο από το φύλο.

Συνοψίζοντας, η εν λόγω μελέτη παρέχει σημαντικές πληροφορίες για τον ρόλο της πρωτεινης RGS20 στους κυτταρικούς μηχανισμούς που εμπλέκονται στις δράσεις των οποιοειδών και σε περιπτώσεις χρόνιου φλεγμονώδους πόνου, συμβάλλοντας στην προσπάθεια εύρεσης νέων φαρμακευτικών στόχων για την θεραπεία του χρόνιου πόνου.

Abstract

Chronic pain is probably the most prevalent human problem, contributing to individual morbidity and mortality and imposing high societal costs. Current management of chronic, non-cancer pain is far from optimal, with existing analgesics characterized by limited efficacy and a high adverse-effect burden.

Opioid analgesics have been traditionally applied for the alleviation of severe pain conditions. Repeated opioid administration, however, may lead to a progressive decline in analgesic efficacy (tolerance) as well as the development of addiction. Investigation of the mechanisms involved in analgesic tolerance and dependence has provided a substantial insight into the neurobiology of these conditions. However, despite recent advances, the cellular and molecular alterations downstream of the receptor mediating opiate actions (the μ-opioid receptor (MOR)) are complex and still not well understood. Members of the regulators of G protein signaling (RGS) proteins have been associated with the regulation of several morphine actions, such as analgesia, reward and addiction. These proteins modulate signaling duration and desensitization of several G-protein coupled receptors (GPCRs), including the MOR.

Previous studies from our laboratory have established the involvement of RGS9-2 and RGS4 in the mechanisms of opioid-derived analgesia and addiction. Here, by using Mus musculus model organism and well-established pain/opioid related behavioral assays we report the role of another RGS protein family member, RGS20, in inflammatory pain antinociception, opioid-induced analgesia and morphine tolerance. Specifically, we show that RGS20 seems to act as a negative regulator of opioid induced analgesia, but at the same time contributes to tolerance development in a PAG-mediated manner. Regarding addiction RGS20 does not seem to affect any dependence-related behavior. Moreover, RGS20 seems to act as a positive regulator of antinociception in chronic inflammatory pain conditions in a sex-dependent way.

Together, the present data shed light on the actions of RGS20 protein providing novel information on its role in the cellular mechanisms underlying inflammatory pain conditions and morphine responses which will be important for the development of drug targets for the treatment of chronic pain.

Introduction

Chapter 1. Pain

1.1 Pain Definition & Classification

Originally pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (Merskey et al. 1994). It can be categorized based on ether its duration or its cause. Regarding duration, pain can be acute or chronic.

Acute pain is short (usually a few days) and comes as a result of an insult, trying to protect the organism from this threat and creating the memory of this dangerous stimulus for future need (Usunoff et al. 2006).

Chronic pain is generally defined as persistent pain that lasts more than the ordinary duration of time that an injury needs to heal (usually believed to be 3 months). However, due to its complexity this duration threshold in some cases is considered as 6 or even 12 months.

Based on its cause, pain can be physiological, nociceptive or neuropathic.

Physiological is the pain arising from a certain painful stimulus. Nociceptive pain is defined as pain caused by the activation of nociceptors, most of the times due to tissue damage.

Nociceptive pain can be further divided to somatic and visceral pain. Somatic pain is usually well localized but each patient experiences it and describes it in a different manner. Visceral pain, comes from the viscera via stretch receptors. It is not exactly localized, dull and cramping. Nociceptive pain can also be classified as musculoskeletal, inflammatory and mechanical or compressive pain.

Neuropathic pain is caused by abnormal function of the nervous system, due to disease, injury or any other dysfunction. The disorder can be located ether at the peripheral or at the central nervous system and thus pain can be characterized as peripheral or central neuropathic pain.

1.2 Neuroanatomy of Pain & Pain Pathways

1.2.1 Peripheral Pathways

The peripheral sensory nerves are made up of the axons of somatic and visceral sensory neurons and the connective tissue sheaths that enfold them. These axons may be myelinated or unmyelinated. Large myelinated sensory axons belong to the Aβ class and are predominantly somatic, whereas small myelinated axons belong to the Aδ group and along with the unmyelinated fibers (C fibers), they innervate both somatic and visceral tissues (Al-Chaer and Willis 2007). In general, only small myelinated and unmyelinated fibers are involved in pain processing; however, in some cases of peripheral neuropathy, large myelinated fibers have also been implicated (Kajander and Bennett 1992).

1.2.1.1 Nociceptors

Sherrington defined nociception as sensory receptors activated by stimuli that threaten to damage or actually damage a tissue (Sherrington 1906). In fact nociceptors are a subpopulation of the peripheral nervous system that is capable of transducing and encoding noxious stimuli. They have been described in most of the structures of the body that give rise to pain sensation, including skin, muscle, joints and viscera (Willis and Coggeshall 2004). Anatomically, the responsible for the body regions nociceptors are located in the dorsal root ganglia (DRG) and those responsible for the face are located in the trigeminal ganglion. All of them have two branches, a central branch targeting the spinal cord and a peripheral branch targeting each specific organ. Interestingly, due to their unique pseudo-unipolar morphology they are able to send and receive information from both directions, meaning that proteins synthesized by DRG or the trigeminal ganglion are distributed to central as well as peripheral terminals of the nociceptor.

Nociceptors are classified into three main categories (Fig. 2):

***Aβ fibres**, which are large diameter, myelinated, rapidly conducting fibres responding to light touch, projecting to lamina III, IV and V of DRG.*
***Aδ fibres**, which are medium diameter myelinated afferents and are mainly responsible for mediating the so-called "first" or fast pain (well localized), projecting to lamina I, II and V.*
***C fibres**, which are small diameter unmyelinated fibres, mainly responsible for the "second" or slow pain (poorly localized), projecting to lamina I and II.*

Human studies involving microneurography and microstimulation in peripheral nerves have demonstrated that activation of nociceptors results in pain (Ochoa and Torebjork 1989). However, the quality of pain sensation depends on the tissue innervated and the specific nociceptor activation. Not all nociceptors respond to every stimulus, some of them are sensitive to chemical, mechanical or heat stimuli (or to a combination of those). For example some nociceptors, called "silent nociceptors" are unresponsive to mechanical stimuli unless they are sensitized by tissue injury or inflammation. This specificity is mainly determined by the ion channel expression pattern of each fiber. There are specific ion channels offering sensitivity to heat (TRPV1), cold (TRPM8), acids (ASICs) and chemical irritants (TRPA1).

1.2.1.2 Peripheral Sensitization & Primary Hypersensitivity

Sensitization of nociceptors is commonly defined as an increase in the firing rate and a reduction in threshold of the nociceptor. Sensitization depends on the activation of second-messenger systems by the action of inflammatory mediators release in the damaged tissue, such as bradykinin, prostaglandins, serotonin and histamine. A hallmark of the sensitization of peripheral nociceptors is sensory hypersensitivity classified as primary hyperalgesia or allodynia (Gold and Gebhart 2010).
Hyperalgesia is defined as an increase in the painfulness of a noxious stimulus and a reduced threshold for pain. Primary hyperalgesia is felt at the site of injury and is believed to be a consequence of the sensitization of nociceptors during the process of inflammation.
Allodynia is a related phenomenon in which non-noxious mechanical stimuli produce painful responses.

1.2.2 Central Pathways

Pain is a perception subject of all the vagaries and trickery of our conscious mind. There is no simple relationship between a given noxious stimulus and the perception of pain. Psychological factors as arousal, attention and expectation can influence central nervous system (CNS) circuits involved in pain modulation. Pain transmission depends on the balance of inhibitory and excitatory influences acting on the neuronal circuits of the somatosensory system. Integration of these influences occurs at multiple levels of the CNS including spinal cord, brain stem and multiple cortical regions. Derangements of these systems are often critical in generation and maintenance of chronic pain and some of the oldest (e.g. opioids) as well as the newest analgesics access these control mechanisms.

1.2.2.1 Modulation of Pain at Spinal Cord Level

The dorsal horn (DH) of spinal cord is an important area for integration of multiple inputs, including primary sensory neurons and local interneuron networks, as well as descending control from supra-spinal centers. Repetitive stimulation of nociceptors leads to increased excitability of projection neurons within the DH, resulting in amplification in the processing of nociceptive information, a process known as "central sensitization". It has been shown that central sensitization makes an important contribution to post-injury hypersensitivity in conditions such as inflammation and nerve injury. A number of different neurotransmitters release by nociceptive afferents have been implicated in this process. Neuropeptide substance P (acting on NK-1) and glutamate (acting on NMDA) appear to be crucial. Transmission in the somatosensory system can be suppressed within the DH as a result of segmental and descending inhibitory controls. Inhibitory neurotransmitter systems within the DH include endogenous opioid peptides and cannabinoids, glycine, GABA, serotonin (5-HT) and adenosine which can act ether pre-synaptically on the primary afferent terminal, or post-synaptically on the DH neuron (Fig. 1).

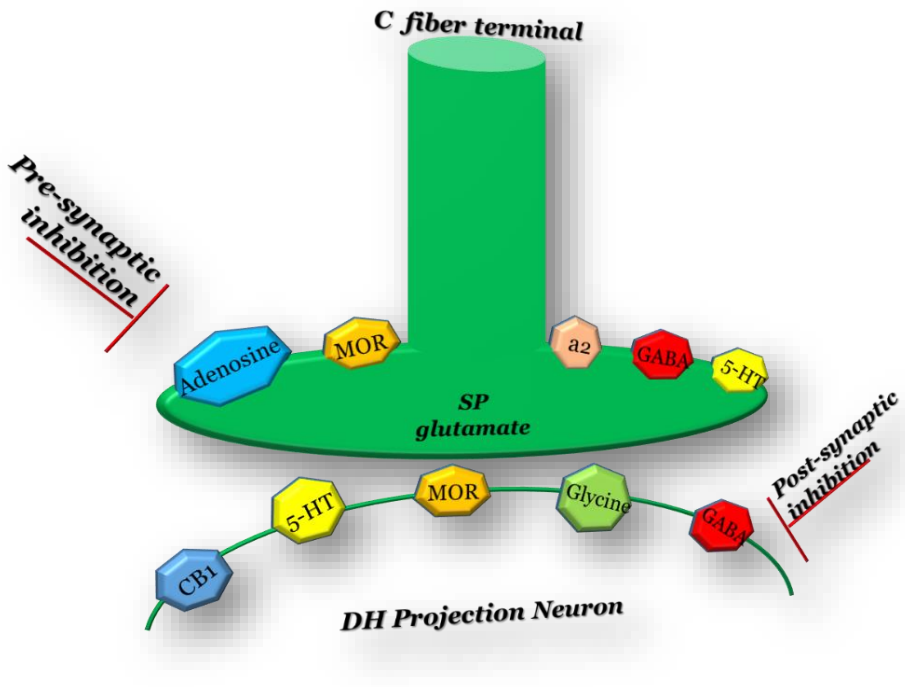


Figure 1.

Inhibition of nociceptive transmission within the DH

Opioid peptides, cannabinoids, adenosine, noradrenaline, 5-HT, glucine and GABA act via their specific receptors both pre- and post-synapricallly. This result in reduced neurotransmitter release (SP and glutamate) by C afferents and reduced post-synaptic depolarization. Therefore the DH response to a primary afferent input is reduced.

1.2.2.2 Supra-Spinal Modulation of Pain

There is a well-described descending pathway acting primarily on DH of the spinal cord, which can inhibit the central transmission of noxious information. Initial evidence for such a pain-modulating pathway was provided by stimulation produced analgesia. Electrical stimulation of periaqueductal grey (PAG) in patient with severe pain induced profound analgesia. A simplified diagram of this descending modulating network is shown in figure 2. PAG integrates information from multiple higher centers, including amygdala, hypothalamus and frontal lobe. It also receives ascending nociceptive input from the DH and it controls the processing of nociceptive information in the DH via its projections to the rostroventromedial medulla (RVM). The endogenous opioid peptides and their receptors are heavily expressed within this pathway. The actions of opioids are not restricted to the DH of the spinal cord. Opioid agonists can also stimulate PAG and RVM resulting on activation of descending pain-modulating pathways. Other neurotransmitters such as serotonin, norepinephrine and cannabinoids are also involved.

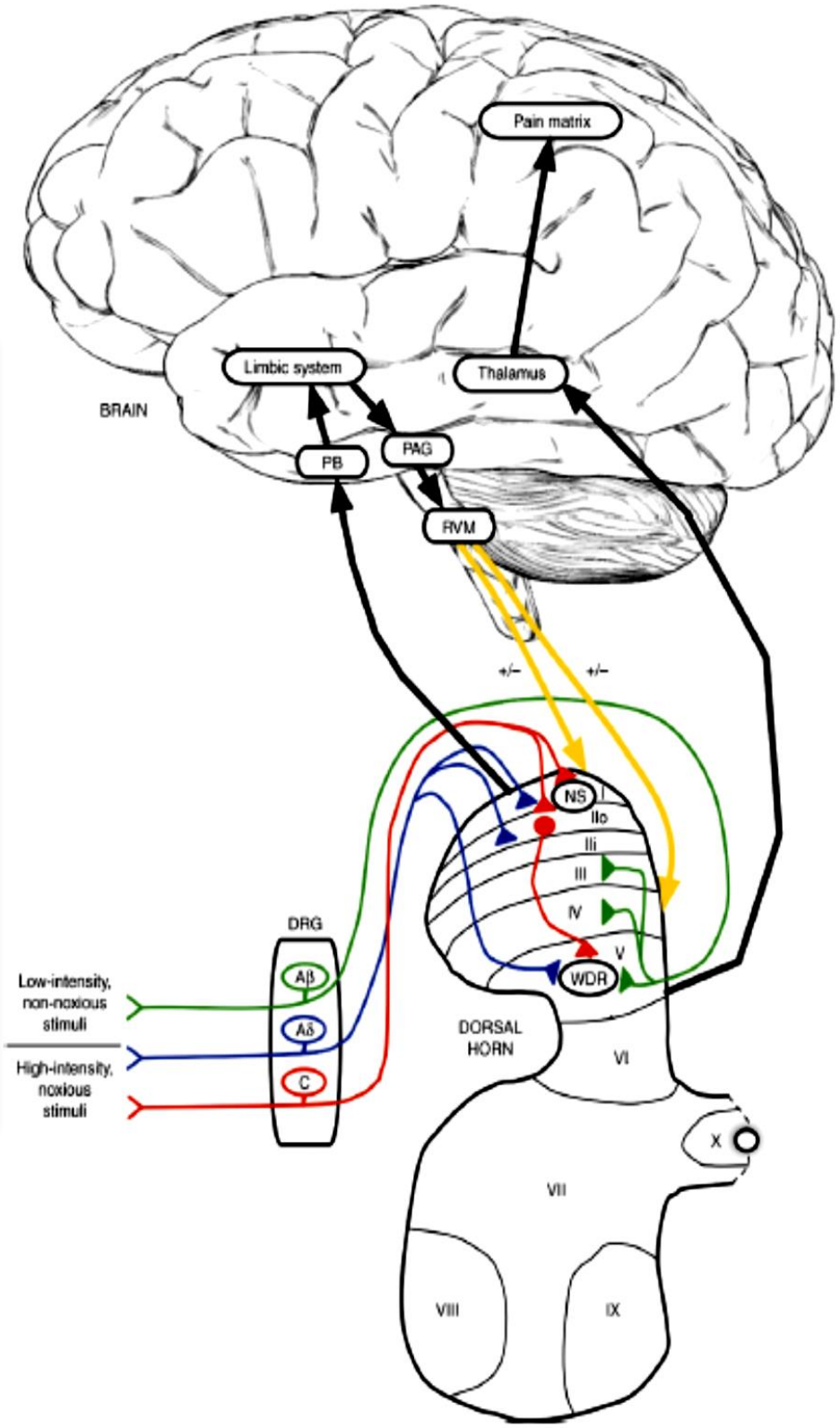


Figure 2.

Pain pathways from periphery to brain

Primary afferent fibres (Ab-, Ad-, and C-fibres) transmit impulses from the periphery, through the dorsa root ganglion (DRG) and into the dorsal horn of the spinal cord. Nociceptive specific (NS) cells are mainly found in the superficial dorsal horn (laminae I–II), whereas most wide dynamic ranges (WDRs) are located deeper (lamina V). Projection neurones from lamina I innervate areas such as the parabrachial area (PB) and periaqueductal grey (PAG) and such pathways are affected by limbic areas. From here descending pathways (yellow arrows) from brainstem nuclei such as the rostral ventromedial medulla (RVM) are activated and modulate spinal processing. Lamina V neurons mainly project to the thalamus (spinothalamic tract), and from here the various cortical regions forming the 'pain matrix' (primary and secondary somatosensory, insular, anterior cingulate, and prefrontal cortices) are activated.

D'Mello & Dickenson, (2008)

Chapter 2. Inflammatory Pain

Tissue injury, irritation or infection can induce inflammation. The classical observations of redness, heat and swelling, are invariably accompanied by pain. Each reaction contributes to the prevention of further insult and the recovery of damaged tissue. Post-operative pain exhibits the classical features of inflammatory pain. Inflammation and inflammatory pain are mediated by a plethora of diverse substances released by tissue damage itself and the subsequent cascade of inflammatory process. Some inflammatory mediators directly activate and sensitize primary afferent nerve fibers. Others stimulate the release of further mediators from immune cells, attracted by other chemicals in the inflammatory "soup", which is a term used to refer collectively to all the pro-inflammatory mediators. Immune cells are recruited to the site of injury and act as a potent source of growth factors and cytokines. These are important for the generation and maintenance of hyperalgesia. This system exhibits enormous potential for interaction and escalation between each of the contributing processes. The polymorphonuclear leukocytes (from which neutrophils seem to be most plentiful) appear to be the principle cells of immune system involved in the generation of inflammatory pain.

Different components of the inflammatory "soup" can activate or sensitize primary afferent neurons, or induce the influx of immune cells at the inflamed site that release further pro-inflammatory mediators. Sensitization of the primary afferent neurons leads to decreased threshold of pain activation and an increased response to noxious stimuli. Electrophysiologically, in the primary afferent nociceptor, this is evident not only by a lowered threshold of nociceptor activation, but also by increased spontaneous activity and an increased frequency of firing. These changes are manifest clinically as an increased response to noxious stimuli: hypersensitivity. The resultant increase in afferent input to the spinal cord leads to the development and maintenance of secondary (or central) sensitization, which may lead to chronic pain, even after inflammation has been cured.

2.1 Peripheral Mechanisms of Inflammatory Pain

2.1.1 Inflammatory Mediators Produced Locally

The list of inflammatory mediators is long and research continues to add many more. Some of the fundamental elements of inflammation and key mediators of that play a pivotal role in the generation of inflammatory pain are mentioned below.

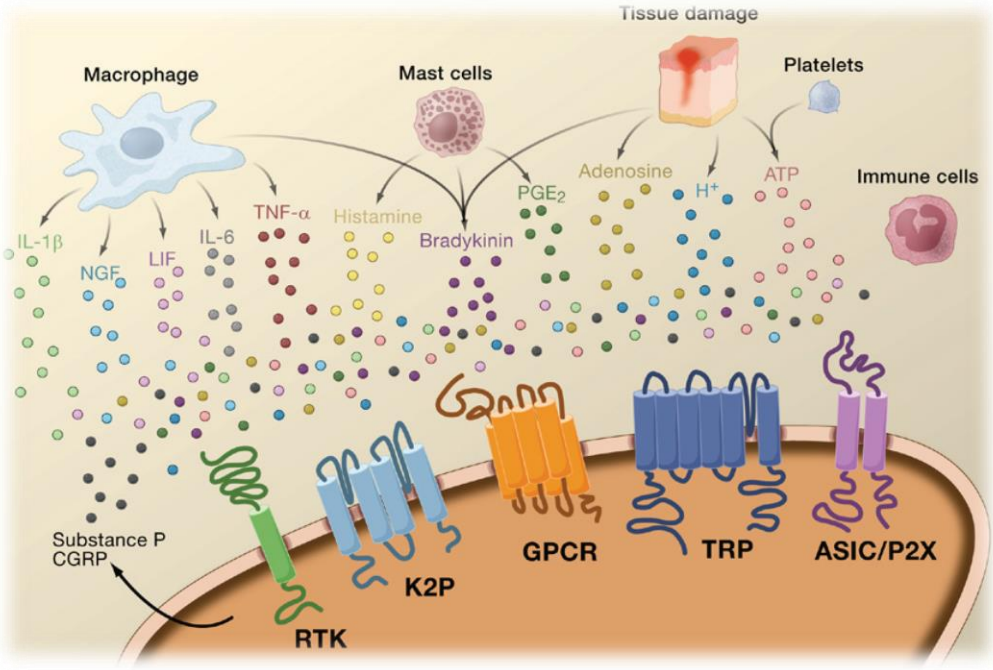
***Protons**. Tissue damage releases a number of substances directly from cells. Protons are produced in inflamed tissue and in common with serotonin (5-HT) can act directly on primary afferent neurons. This probably occurs by increasing ion permeability, a process that shares characteristics with the noxious stimulation of nociceptors by capsaicin. Exposure of C- and Aδ-fibers to pH of 6 or less can activate acid-sensing ion channels (ASICs).*

***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. As many others of the inflammatory key mediators, 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) is released locally by inflammation and like many other mediators can reproduce pain when injected locally. ATP acts upon P2X receptors contributing to hyperalgesia.*

***NGF.** NGF is released locally from a number of cells (including fibroblasts) and performs a central role in the inflammation cascade. Its increased concentration in inflamed tissue has been associated with hyperalgesia both in human inflammatory pain states and animal models. Via its receptor tyrosine kinase A (trkA), NGF can directly sensitize the NGF-dependent subset of nociceptors, in addition to potentiating the actions of other sensitizing agents (e.g. BK). NGF signaling has also been found to enhance responsiveness to heat and capsaicin by interacting with TRPV1. Moreover,*

NGF degranulates mast cells, releasing more mediators and amplifying the inflammatory signal. Some of the interactions between NGF and other pro-inflammatory systems are displayed in figure 3.



Meyer et al., (2008)

Figure 3.

Peripheral Mediators of Inflammation

Tissue damage leads to the release of inflammatory mediators by activated nociceptors or nonneural cells that reside within or infiltrate into the injured area, including mast cells, basophils, platelets, macrophages, neutrophils, endothelial cells, keratinocytes, and fibroblasts. This "inflammatory soup" of signaling molecules includes serotonin, histamine, glutamate, ATP, adenosine, substance P, calcitonin-gene related peptide (CGRP), bradykinin, eicosinoids prostaglandins, thromboxanes, leukotrienes, endocannabinoids, nerve growth factor (NGF), tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), extracellular proteases, and protons. These factors act directly on the nociceptor by binding to one or more cell surface receptors, including G protein-coupled receptors (GPCR), TRP channels, acid-sensitive ion channels (ASIC), two-pore potassium channels (K2P), and receptor tyrosine kinases (RTK),on the peripheral nociceptor terminal.

2.1.2 Inflammatory Mediators Released & Produced by Immune Cells

Products of COX and LOX metabolism

Prostaglandins (PGs) are produced by the enzymatic activity of cyclo-oxygenase (COX) and lipo-oxygenase (LOX) on arachidonic acid (AA) and perform a number of pro-inflammatory tasks. The anti-inflammatory action of corticosteroids is in part related to the prevention of AA release, by inhibition of phospholipase A2 (PLA2). During

inflammation a number of PGs are produced. Prostaglandin E2 (PGE2) is produced predominantly by the COX-2 isoform and can directly activate nociceptors via PGE2 EP receptors. PGs also enhance the effects of BK and augment neuropeptide release (including substance P). Non-steroidal anti-inflammatory drugs (NSAIDs) act by inhibiting COX and therefore reducing the production of these sensitizing PGs. Contemporary evidence suggests that PGs may be important also for the development of secondary hypersensitivity in the CNS, implicating a novel central role for the NSAIDs in the CNS. Indeed, immune-like cells in the CNS, such as microglia, appear to release similar pro-hyperalgesic to the spinal cord.

Furthermore, certain products of LOX activity (e.g. leukotriene B4 (LTB4)) sensitize nociceptors by increasing cyclic adenosine monophosphate (cAMP). Activation of adenylate cyclase by LTB4 results in the production of cAMP, which then stimulates downstream kinases, including PKA. LTB4 acts also as a chemoattractant for immune cells, recruiting them the inflammation site and stimulateing more LTB4 release and the release of other metabolites of the LOX pathways. Particular LOX metabolites produced by neutrophils have been postulated to act on TRPV1 receptors and may be responsible for the link between neutrophils and inflammatory hyperalgesia.

Cytokines

Cytokines, apart from their important role in inflammatory responses as mediators of cell-cell interactions, they also induce sensitization of the nociceptive neurons via phosphorylation of ion channels or by promoting transcriptional up-regulation of certain receptors, such as TRPV1, IL-6R, IL-1R etc. Among all cytokines, the most prominent are TNF-α, IL-6, IL-1 and IL-8. It has been shown that the use of anti-TNF-α antibodies can reduce hyperalgesia in animal models of inflammation, while mice deficient for IL-6 appear to have decreased mechanical and thermal hyperalgesia after inflammatory stimuli.

2.1.3 Neurogenic Inflammation

Part of the inflammatory process is mediated by neuropeptides released from sensory nerve endings. NGF increases neuropeptide content of sensory nerves and their local inflammation-induced release. Neurokinins (e.g. substance P and neurokinin A) and calcitonin gene-related peptide (CGRP) act via specific receptors causing vasodilation and plasma extravasation. These changes facilitate the entry of recruited immune cells to the affected area and promote the development of edema. Although these substances can directly depolarize sensory neurons, the action of neuropeptides is probably more important in the facilitation of central sensitization.

2.1.4 Endogenous Anti-Inflammatory Systems

There is a fine- regulated intrinsic inhibitory system to temper potentially damaging pro-inflammatory processes. One line of defence is afforded by release of naturally anti-inflammatory cytokines (e.g. IL-10 and IL-1ra). Endogenous opioids also act to moderate excessive inflammation. Although opioid receptors are expressed predominantly in the CNS they are also found in the periphery. Such peripheral receptors are upregulated in inflammatory states and thus increase the efficacy of endogenous agonists. Immune cells (principally neutrophils) conscripted to the inflamed tissue not only produce pro-inflammatory substances, but also release endogenous opioids in biologically significant amounts. Moreover, endogenous cannabinoids act on G-protein-coupled cannabinoid receptors (CB1, CB2) both in periphery and CNS leading to reduced hyperalgesic responses.

2.2 Central Sensitization

Central sensitization is defined as the process through which the "pain message" is led from the nociceptor terminal to the central nervous system. It is commonly known that reduced inhibition can be as effective as increased excitability. GABAergic or glycinergic interneurons are found greatly in the dorsal horn and are mostly responsible for inhibition. Studies in which GABA or glycine related inhibition was blocked have shown

that this loss of inhibition can lead to increased pain sensitivity (Malan et al. 2008). Microglia and astrocytes appear to play an important role in central sensitization.

Microglia, which is activated in cases of peripheral nerve injury releases enormous quantities of signaling molecules, including cytokines, which in turn facilitate the central sensitization process (DeLeo et al. 2007). The most prominent mechanism is that ATP binds to P2X4 receptors and this causes the release of brain derived neurotrophic factor (BDNF). Consequently, BDNF interacts with its receptor TrkB into lamina I and changes the Cl- gradient which in turn can cause GABA neurons to depolarize, thus leading to a mechanism of disinhibition (Coull et al. 2005). Recently, there are data that certain members of the Toll-like receptor (TLR) family may play a role in the activation of microglia after nerve injury. Studies in which TLR2, TLR3 or TLR4 were inhibited (genetically or pharmacologically) showed that there was reduced microglial activation as well as reduced hypersensitivity (Tanga et al. 2005, Kim et al. 2007).

As far as astrocytes is concerned, it is possible that due to their long lasting activation, their role is mostly to maintain than to induce central sensitization (Ren et al. 2008).

Persistent or repetitive activation of primary nociceptors promotes changes to the activity and function of central neurogenic pathway. Glutamate, substance P and BDNF act as co-transmitters and induce central sensitization. Primary afferent fibers release peptide transmitters, which in turn activate second messenger systems leading to an increase in calcium influx and protein phosphorylation. Thus, the responsiveness of dorsal horn cells is increased producing exaggerated responses to normal stimuli, expansion of receptive field size and reduction in the activation threshold by novel inputs (Noguchi et al. 1995, Fukuoka et al. 2001).

Apart from these, NMDA receptors seem to play a major role in central sensitization. In injury states, the neurotransmitters that are released from the activated nociceptors cause depolarization of the postsynaptic neurons, leading to the activation of NMDA receptors. This event is followed by an increase in Ca+2 influxes, which in turn enables firm connection between the nociceptors and the dorsal horn pain transmission neurons, a fact that finally, generates hyperalgesia. Consequently, metabolotropic glutamate receptors along with substance P receptors are activated, leading to further increase in cytocolic calcium levels.

2.3 Potential Drug Therapies

The treatment of inflammatory pain is a very complicated issue. At first and more importantly, the underlying cause should be faced. In terms of pharmaceutical treatment, the most commonly used agents are non-steroidal anti-inflammatory drugs (NSAIDs) and opioids (mostly morphine).

While the efficacy of opioid in the treatment of acute nociceptive and inflammatory pain is universally accepted, they are regularly associated with the stigma of abusive and addictive behaviors as long as a myriad of side effects, including sedation, nausea, itching, constipation and potentially even life-threatening respiratory depression.

On the other hand NSAIDs lack many of the adverse effects associated with opioids, but they have less analgesic potency and several important side effects of their own. Non-selective NSAIDs have the potential to increase bleeding risks by inhibiting the formation of platelet COX-1-dependent thromboxane and even COX-2 selective NSAIDs have been found to increase the risk of cardiothrombotic events.

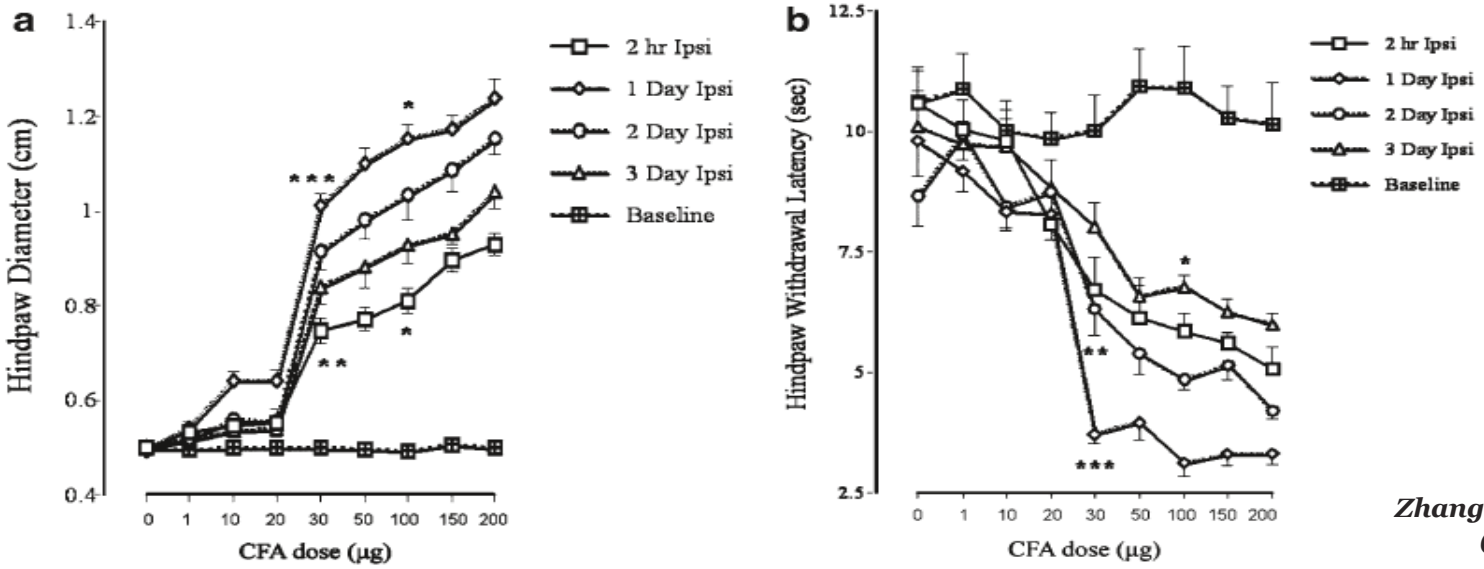
2.4 Animal Models & Experimental Approaches

Animal models of inflammatory pain have been widely used to study the mechanisms of tissue injury-induced persistent pain. A variety of inflammatory agents or irritants, including complete Freund's adjuvant (CFA), carrageenan, zymosan, mustard oil, formalin, capsaicin, bee venom, acidic saline, lipopolysaccharide, inflammatory cytokines, and sodium urate crystals, have been used to produce tissue injury and hyperalgesia in such structures as cutaneous/subcutaneous tissues, joints, and muscles. Although these models do not simulate every aspect of chronic pain, they do model key features of human inflammatory pain. Studies in animals give insight into certain aspects of human pain conditions and lead to improved pain management for patients. The most widely used ones are:

CFA model

The injection of complete Freund's adjuvant (CFA, composed of inactivated and dried Mycobacterium and adjuvant) into the footpad produces localized inflammation and

persistent pain (Millan et al. 1988, Iadarola et al. 1988). 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, an 30–200 µg 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. 4). 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 (Lao et al. 2004). The physiological and biochemical effects of CFA are limited to the affected limb and there are no signs of immune response or systemic disease. It has been shown that 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 (Iadarola et al. 1988).



Zhang et al., (1999)

Figure 4.

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 hind paw.*
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 hind paw.*

Carrageenan model

An intraplantar injection of carrageenan is also widely used to produce a model of localized inflammatory pain. When 0.5 mg of carrageenan is injected, edema develops, mainly in two phases: the first 30 min after the injection, the second beginning at the end of the first hour and lasting until the third hour after injection. The edema peaks 3–5 h after injection (Vinegar et al. 1969, Winter et al. 1962). When 6 mg of carrageenan is injected, edema peaks on day 3 and thermal hyperalgesia peaks around 4 h after injection and lasts for at least 96 h (Iadarola et al. 1988).

Formalin model

The formalin test is a popular model for studying pain mechanisms under prolonged nociception. Formalin is injected beneath the footpad and produces two phases of nocifensive behavior, characterized by licking and flinching of the paw, that are separated by a short period of quiescence (Dubuisson et al. 1978, Abbott et al. 1995). The first or acute phase occurs typically in the first 5 min; the second starts from 15 min and lasts about 40–60 min after injection. It is generally agreed that the first phase is due to the direct activation of both low-threshold mechanoreceptive and nociceptive primary afferent fibers (Puig et al. 1996). There has been disagreement about the underlying mechanisms of the second phase. Early studies suggested that the second phase resulted from an increase in the excitability of dorsal horn neurons. It has also been demonstrated that ongoing activity of primary afferent fibers is necessary for the development of the second phase (Puig et al. 1996, Pitcher et al. 2002, Taylor et al. 1995, Abbadie et al. 1997). In regard to the period of quiescence, some evidence supports the idea of an absence of activity, other evidence implicates an active inhibitory mechanism (Henry et al. 1999).

Capsaicin

Capsaicin, the pungent component of cayenne pepper that activates transient receptor potential vanilloid type 1 (TRPV1), a heat-sensitive cation channel on nociceptor terminals, has been used in humans and animals to study neurogenic inflammation and hyperalgesia. Intradermal injection of capsaicin results in flare reaction, allodynia, and hyperalgesia, the areas of which extend beyond the injection site. Intraplantar injection

of capsaicin evokes nocifensive behavior characterized by lifting and guarding of the injected paw that lasts for about 3 min. Capsaicin dose-dependently produces thermal and mechanical hyperalgesia. Thermal hyperalgesia lasts up to 45 min, whereas mechanical hyperalgesia persists up to 4 h.

Chemical	Hyperalgesia	Allodynia	Time of onset	Duration
CFA	Yes	Yes	2-6 hours	1-2 weeks
Carrageenan	Yes	Yes	1 hour	24 hours
Mustard oil	Yes	Yes	5 minutes	< 1 hour
Zymosan	Yes	Yes	30 minutes	24 hours
Formalin (phase I)	N/A*	N/A*	< 1 minute	5-10 minutes
Formalin (phase II)	N/A*	N/A*	10 minutes	1 hour
Bee venom	Yes	Yes	1 minute	96 hours
Capsaicin	Yes	Yes	1 minute	< 1 hour

Table 1.

Comparison of cutaneous/subcutaneous inflammatory pain models

** Not applicable*

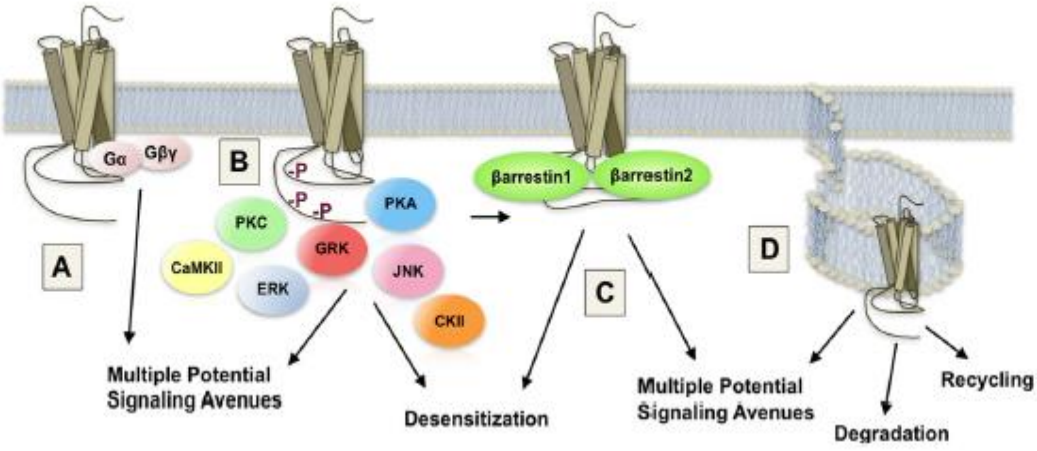
Chapter 3. Opioids and Pain Relief

Opioid analgesics are the most efficacious drugs for the treatment of moderate to severe pain and represent the largest market share of prescription pain medications (Melnikova, 2010). Although opioids are effective pain relievers, they also produce a number of adverse side effects that can limit their clinical utility, including nausea and vomiting, constipation, and respiratory suppression. Moreover, long-term exposure to opioids is also associated with the development of analgesic tolerance, physical dependence, and addiction (Cherny et al., 2001; Harris, 2008). Given their numerous effects, a major goal in opioid research is to understand the molecular and cellular mechanisms that give rise to opioid-induced physiological and behavioral responses and adaptations to develop improved analgesics that can selectively provide pain relief while reducing the onset of these unwanted side effects.

3.1 Opioid analgesic mechanisms of action

The μ opioid receptor (μOR) belongs to the superfamily of G protein-coupled receptors (GPCRs) and has been shown to be the opioid receptor subtype that primarily mediates the physiological actions of clinically used opioids (Kieffer, 1999). At the cellular level, the μOR traditionally has been described to mediate opioids drug effects by coupling to heterotrimeric G proteins (Fig. 5A), particularly pertussis toxin-sensitive $G\alpha_{i/o}$ proteins, which act to inhibit adenylyl cyclases, modulate activity of certain ion channels, and signal through several second-messenger signal transduction cascades to promote signaling. As with most GPCRs, the extent and duration of agonist-induced μOR signaling can be determined by several regulatory mechanisms including receptor desensitization, internalization, down-regulation, and resensitization. After agonist activation, μOR can be rapidly phosphorylated by G protein-coupled receptor kinases (GRKs) or other second messenger-regulated kinases, including protein kinase C (PKC) (Fig. 5B). This may facilitate β-arrestin binding and the dampening of further coupling to G proteins, despite the continued presence of agonist (Ferguson, 2001; Ahn et al., 2003) (Fig. 5C). In addition to receptor desensitization, β-arrestins can act to facilitate

receptor internalization, which can contribute to down-regulation or resensitization events (Ferguson et al., 1996) (Fig. 5D).



Raehal et al., (2011)

Figure 5.

Key points in opioid receptor signaling and regulation.

A. *heterotrimeric G proteins represent 16 individual gene products for Gα, 5 individual gene products for Gβ and 11 for Gγ proteins. Together, the diversity arising from heterotrimeric G protein subunit composition presents a gateway to potentially high diversification of agonist-directed coupling between μOR and G proteins. These interactions can determine access to secondary cascade activation.*

B. μOR can be *phosphorylated in response to agonist occupation by multiple kinases, each of which has multiple isoforms. Phosphorylation by a particular kinase may dictate secondary cascade interactions or subsequent receptor fate.*

C. *receptor interaction with scaffolding partners such as β-arrestins can be dependent or independent of receptor phosphorylation. Agonist occupancy may determine these interactions with potential binding partners. Such interactions can prevent (desensitization) or promote subsequent signaling.*

D. *μOR can be internalized in response to agonist occupancy. Endocytosis may involve clathrin- or caveolin-dependent processes and may result in the activation of subsequent signaling pathways, receptor recycling or degradation.*

The conventional understanding of receptor pharmacology has been that responses elicited by GPCR activation are determined by the "intrinsic efficacy" of the ligand acting at the receptor. In this model, a full agonist maximally activates all signal transduction pathways to which the receptor is coupled and therefore has high intrinsic efficacy. Partial agonists, on the other hand, induce submaximal activation of these

same pathways, and thus possess lower degrees of intrinsic efficacy, whereas inverse agonists reduce the basal activities of these pathways. Antagonists, which possess no intrinsic efficacy and do not shift any of the responses away from basal levels, occupy the receptor and block receptor responses induced by agonists.

Over the past several decades, numerous studies have demonstrated that not all GPCR agonists activate the same intracellular signaling pathways, even though they may be acting at the same receptor (Kenakin, 2011). It has been proposed that physical interactions between an agonist and a receptor impact upon the physical constraints of receptor conformation, which can result in a preferential or "biased" interaction with certain signaling components over others (Kenakin, 2007, 2009). Furthermore, the cellular environment, including the proteins expressed in close proximity with the receptor, can influence those interactions and thereby influence the degree of signaling induced by a particular ligand. In this way, the same ligand can induce differential signaling profiles when a receptor is expressed in different cell types (Bohn, 2009; Schmid and Bohn, 2009).

3.2 Opioid pharmacokinetics

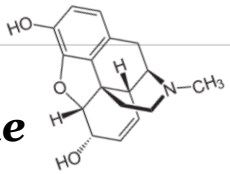
Opioids differ significantly in one measure of drug elimination: the plasma half-life value. Thus, while morphine and hydromorphone are short half-life opioids that on repeated dosing reach steady-state in 10 to 12 hours, levorphanol and methadone are long half-life opioids that on the average may require 70 to 120 hours, respectively, to achieve steady-state. During dose titration the maximal (peak) effects produced by a change dose of a short half-life opioid will appear relatively quickly, while the peak effects resulting from a change in the dose of a long halflife opioid will be achieved after a longer accumulation period. For example, a patient who reports adequate pain relief following the initial doses of methadone may experience excessive sedation if this dosage is fixed and not modified as required during the accumulation period of 5 to 10 days.

3.3 Adverse Effects, Tolerance & Addiction

There are a number of side effects associated with the use of opioid analgesics that can, depending on the circumstances, be categorized as desirable or undesirable effects. The mechanisms that underlie these various adverse effects are only partly understood and appear to depend on a number of factors including age, extent of disease and organ dysfunction, concurrent administration of certain drugs, prior opioid exposure, and the route of drug administration. The most common adverse effects are sedation, nausea and vomiting, constipation, and respiratory depression.

One of the main problems associated with opioid treatment is tolerance development. Tolerance develops when a given dose of an opioid produces a decreasing effect, or when a larger dose is required to maintain the original effect. Some degree of tolerance to analgesia appears to develop in most patients receiving long-term opioid analgesic therapy. The hallmark of the development of tolerance is the patient's complaint of a decrease in the duration of effective analgesia. For reasons not yet understood the rate of development of tolerance varies greatly among cancer patients, so that some will demonstrate tolerance within days of initiating opioid therapy whereas others will remain well controlled for many months on the same dose. With the development of tolerance, increasing the frequency and/or dose of the opioid are required to provide continued pain relief. Since the analgesic effect is a logarithmic function of the dose of opioid, a doubling of the dose may be required to restore full analgesia.

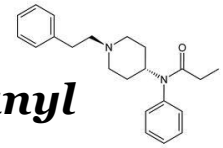
Moreover, fear of addiction is a major concern limiting the use of appropriate doses of opioids in hospitalized patients who are in pain. Addiction (also mentioned as psychological dependence) is a term used to describe a pattern of drug use characterized by a continued craving for an opioid that is manifested as compulsive drug seeking behavior leading to an overwhelming involvement with the use and procurement of the drug. Within these definitions, most but not all individuals who are addicted to opioids will have acquired some degree of physical dependence. However, the converse is not true, and an individual can be physically dependent on an opioid analgesic without being addicted to it. In fact, surveys in hospitalized medical patients and an analysis of the recent medical use and abuse of opioid analgesics suggest that medical use of opioids rarely, if ever, leads to drug abuse or iatrogenic opioid addiction.



3.4 Morphine

Morphine is a potent opiate analgesic drug that is used to relieve severe pain. In fact it is considered to be the prototypical opioid. It was first isolated in 1804 by Friedrich Sertürner, and first commercially sold by Merck in 1827. Its mechanism of action appears to mimic endorphins, endogenous opioids responsible for analgesia. It binds to and activates the μ-opioid receptors in the central nervous system leading to analgesia, sedation, euphoria, physical dependence, and respiratory depression. Morphine is a rapid-acting narcotic, it is known to bind very strongly to the μ-opioid receptors, and for this reason, it often has a higher incidence of the above mentioned side effects when compared to other opioids at equianalgesic doses.

It can be taken orally, rectally, subcutaneously, intravenously, intrathecally or epidurally. For medicinal purposes, intravenous (IV) injection is the most common method of administration. Morphine is subject to extensive first-pass metabolism (a large proportion is broken down in the liver), so if taken orally, only 40-50% of the dose reaches the central nervous system. Resultant plasma levels after subcutaneous (SC), intramuscular (IM), and IV injection are all comparable. After IM or SC injections, morphine plasma levels peak in approximately 20 minutes, and after oral administration levels peak in approximately 30 minutes. Morphine is primarily metabolized into morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) via glucuronidation by phase II metabolism enzyme UDP-glucuronosyl transferase-2B7 (UGT2B7). About 60% of morphine is converted to M3G, and 6–10% is converted to M6G, but it may also be metabolized into small amounts of normorphine, codeine, and hydromorphone. Its elimination half-life is approximately 120 minutes.



3.5 Fentanyl

Fentanyl is a strong μ-opioid receptor agonist approximately 80 to 100 times as potent as morphine. It is a highly lipophilic drug with a shorter duration of action than morphine. Fentanyl is used for the management of postoperative pain by the intravenous and epidural routes of administration, a transdermal patch device is used for chronic pain requiring opioid analgesia, and a transmucosal dosage form is used for breakthrough cancer pain.

3.6 Animal Models for Opioid Studies

Opioid antinociception, tolerance, dependence and addiction have been examined using a wide range of techniques in animal models. The main advantage of using animal models is that the nervous system is intact and opioid effects are linked to behavior. A range of different treatment schedules are commonly used to induce tolerance, dependence and addiction including repeated injections, osmotic minipumps, implantation of morphine base pellets or sustained release emulsions. In addition, the physiological relevance of the species and behavioral assessment tools are quite variable. Pain sensitivity and antinociception have been shown to vary with mouse strain (Mogil et al., 1999, Wilson et al., 2003), and morphine potency varies with the nociceptive test (Morgan et al., 2006a). Nonetheless, years of research have provided a range of methodologies to assess antinociceptive efficacy, tolerance, addiction and dependence.

3.6.1 Nociception

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 studies assessing the antinociceptive effects of opioids. These tests vary in the type of stimulus (thermal, mechanical and chemical), duration/severity of pain (acute vs. neuropathic or inflammatory) and types of response (supraspinally organized response vs. reflexive). Some of them, widely used are listed below:

Hot-Plate Test *consists of introducing the mouse/rat into an open-ended cylindrical space with a floor consisting of a metallic plate that is heated. A plate heated to a constant temperature produces two behavioral components that can be measured in terms of their reaction times: paw licking and jumping. Both are considered to be supraspinal integrated responses.*

Tail-Flick Test *involves immersing the tail in water at a predetermined temperature. Immersion of an animal's tail in hot water provokes an abrupt movement of the tail and sometimes the recoiling of the whole body. Again reaction time is measured. This reaction is considered to be a spinal reflex.*

Paw Withdrawal Test (Hargraves' Test). *Radiant heat is applied to the plantar surface of the foot and paw withdrawal latency is evaluated. One advantage of this test is that heat is applied on a freely moving animal.*

Test based on the Use of Mechanical Stimuli (Von Frey Test). *In the course of such a test, a pressure of increasing intensity is applied to a punctiform area on the hind paw. The amount of pressure needed for a pain-like reaction (paw withdrawal, flicking, licking) is measured.*

3.6.2 Tolerance

Tolerance to the antinociceptive effects of opioids in animal experiments is well documented. Tolerance has been shown with as few as a single injection (Cochin & Kornetsky, 1964, Melief et al., 2010) and in various parts of the nervous system including the periaqueductal gray (Morgan et al., 2006b), spinal cord (Stevens et al., 1988) and periphery (Aley & Levine, 1997). Tolerance appears to develop to all opioids, but the magnitude of tolerance varies depending on the route of administration and agonist efficacy. Repeated injections of morphine, fentanyl, etorphine, oxycodone and meperidine in mice produce comparable rightward shifts in the dose– response curve of the same agonist for antinociception. Continuous intrathecal infusion of morphine also produces greater tolerance than infusion of DAMGO or fentanyl (Stevens & Yaksh, 1989) indicating, as expected, that high-efficacy agonists show less tolerance than lower-efficacy agonists.

3.6.3 Dependence & Withdrawal

Although the term dependence may be used more or less interchangeably with addiction, it can be defined as the presence of withdrawal signs upon removal of the drug, as it is here. Abrupt cessation of chronic opioid use or challenge with μ-opioid receptor antagonists during continued treatment produces a highly aversive withdrawal syndrome with features that are similar in humans and a number of experimental animals. The signs of opioid withdrawal in rodents include those referred to as somatic or vegetative signs, as well as aversive signs. The distinction is somewhat artificial although some signs are clearly mediated predominantly by adaptations in

peripheral nerves while others are centrally mediated (Koob et al., 1992). In rats and mice, opioid withdrawal signs mainly include jumping, 'wet-dog' shakes, hyperreactivity, mastication, tremor, ptosis, lacrimation, diarrhoea, abrupt weight loss. These signs are readily quantified following administration of antagonists such as naloxone or after abrupt cessation of treatment with relatively short-acting opioids.

Chapter 4. Regulators of G-protein Signaling (RGS)

4.1 G-protein Signaling

G proteins comprise a diverse family of proteins implicated in several cellular functions. Their name is derived from their intrinsic GTPase activity. G proteins are expressed in many tissues and play a central role in signal transduction as well as in many cellular processes, such as membrane vesicle trafficking, cell growth, protein synthesis, neurotransmission, etc (Nestler & Duman, 2006). Mammalian G proteins are divided into heterotrimeric G proteins and small G proteins.

Heterotrimeric G proteins are involved in transmembrane signaling in the nervous system. They consist of three subunits: α, β and γ. Gα subunit has the ability to hydrolyze GTP, whereas β and γ subunits form a dimer (Gβγ) that separately activates different intracellular signaling cascades. There are over 35 heterotrimeric G protein subunits with unique distribution in the brain and peripheral tissues. The different types of heterotrimeric G proteins contain distinct α subunits (17 have been identified till now) each one with different function and specificity. As far as the Gβγ complexes are concerned, there have been identified 5 Gβ and 7 Gγ subunits. Based on their structural and functional homology, they are divided into four main categories (Neubig & Siderovski, 2002):

***Gαq**: implicated in activation of phospholipase C (PLC) and inhibition of G protein-coupled inwardly-rectifying potassium (GIRK) channels.*

***Gαi**: implicated in inhibition of adenylyl cyclase by inhibiting Gas, activation of MAPK pathway, activation of potassium (K^+) channels, inhibition of Calcium (Ca^{2+}) channels.*
***Gαs**: implicated in stimulation of adenylyl cyclase and upregulation of intracellular cAMP levels.*
***Gα12/13**: implicated in activation of guanine nucleotide exchange factor (Rho-GEF) proteins.*
In the resting state, Gα subunit is bound both to GDP and Gβγ dimer (Fig. 6a). Upon ligand's binding and G-protein coupled receptor (GPCR) activation, a conformational change occurs which triggers the exchange of GDP to GTP and the dissociation of Gα subunit from the Gβγ complex (Fig 6b). Gβ and Gγ subunits remain attached, and both the Gα and Gβγ complexes are free to interact with various downstream effector molecules within the cell. The GTP-bound Gα subunit is also capable of interacting with the receptor and reducing its affinity for ligand. Reassociation of Gα with Gβγ is triggered by the intrinsic GTPase activity of the Gα subunit.

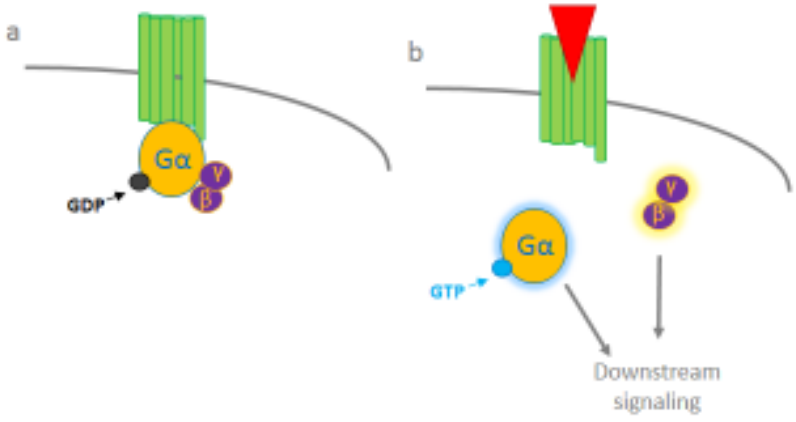


Figure 6.

Function & localization of heterotrimeric G proteins

***a.** In the resting state Gα subunit is bound both to GDP and Gβγ dimer.*

***b.** Upon ligand binding on GPCR a conformational change occurs triggering the exchange of GDP to GTP, which leads to diassociation of the heterotrimer enabling G subunits interaction with downstream effector molecules.*

Furthermore, G protein subunits, when released from their G protein receptor interactions, can directly gate (i.e. open or close) specific ion channels. For example a large amount of receptors expressed in the brain (D2-dopaminergic, α2-adrenergic, 5-HT1A-serotonergic etc.) are coupled with GIRK channels and control their activation

via Gi subunits. Responsible for this coupling seems to be the free βγ dimer. Some subtypes of Gαi have also been shown capable of opening the channel, although not to the same extent as the βγ subunits. On the other hand, these same neurotransmitter receptors are also coupled via pertussis-toxin-sensitive G proteins to voltage-gate Ca^{2+} channels, in this case leading to their inhibition. Binding of the G protein subunits to the Ca^{2+} channels reduces their probability of opening in response to membrane depolarization.

4.2 G protein-Coupled Receptors (GPCRs)

Heterotrimeric G proteins interact with GPCRs, also known as seven-transmembrane domain receptors (7TM receptors) and transduce signals to downstream signaling molecules resulting in the induction of cellular changes. There are two principal signal transduction pathways involving the G protein-coupled receptors: the cAMP signal pathway and the phosphatidylinositol signal pathway (Gilman et al., 1987). Upon ligand binding a conformational change occurs on the GPCR, which allows it to act as a guanine nucleotide exchange factor (GEF) and activate an associated G-protein by exchanging its bound GDP for a GTP. As described above, the Gα subunit-bound to GTP- can then dissociate from the β and γ subunits to further affect intracellular signaling proteins or target functional proteins directly depending on the Gα subunit type (Ritter et al., 2007).

The GPCRs are important drug targets. Many of the medications targeting neurological and neuropsychiatric disorders (including antidepressant and antiparkinsonian agents) mediate their actions via mechanisms involving modifications of GPCR function. In fact, GPCRs are involved in a variety of physiological processes such as vision (opsins), smell (olfactory receptors), immunity, behavioural and mood regulation, homeostasis modulation and many others. Opioid receptors (μ, δ, κ) are GPCRs coupled to the Gαq and Gαi/o subunits. Activation of these receptors leads to inhibition of the enzyme adenylate cyclase, increased potassium as long as reduced sodium conductance and MAP kinase activation.

4.3 RGS proteins

Regulators of G protein signaling (RGS), defined by the presence of a conserved 125 amino acid domain, comprise a diverse group of more than 40 proteins which modulate signaling amplitude and duration via receptor/G protein or receptor/effector coupling. Via their conserved domain, RGS proteins function as GTPase-activating proteins (GAPs) for the Ga subunits of the heterotrimeric G proteins, regulating thus G protein coupled receptor (GPCR) signaling (Fig. 7).

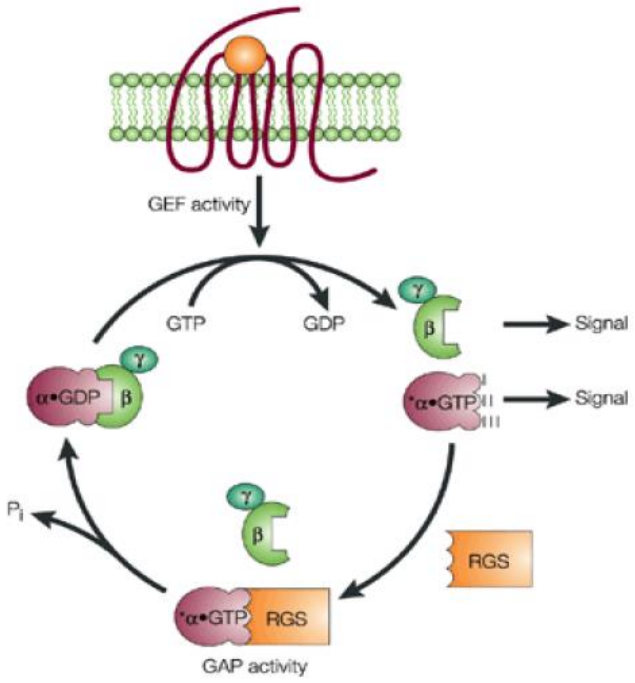


Figure 7.

RGS proteins as Ga GTPase-accelerating proteins (GAPs)

RGS proteins reduce GPCR signaling by accelerating the rate of GTP hydrolysis by the Ga subunit, which leads to Ga-Gβγ reassociation. Inhibiting the binding of the RGS-box to Ga subunit in this case would lead to a prolonged lifetime of the Ga subunit in the GTP-bound state, enhancing the receptor-stimulated response through increased levels of free Ga and Gβγ subunits.

Neubig & Siderovski (2002)

Several members of the RGS family possess unique functions, attributed to their distinct pattern of expression, regulation and structure (Abramow-Newerly et al., 2006). Most RGS are expressed in the brain, regulating essential physiological processes such as vision (Chen et al., 2000, Nishiguchi, 2004), locomotion (Rahnan et al., 2003) and working memory (Buckholtz et al., 2007), while their dysfunction is associated with several neuropathological conditions such as addiction, mood disorders and schizophrenia (Hooks et al., 2008, Terzi et al., 2009).

Some members of the RGS family have been shown to be fine modulators of opiate responses. RGS9-2 and RGS4 appear to act via distinct mechanisms, in a brain region-specific manner (Zachariou et al., 2003, 2010, Psifogeorgou et al., 2011). Particularly, RGS9-2 has been found to be a negative modulator of opiate action in vivo, as RGS9 knockout mice show enhanced behavioral responses to acute and chronic morphine, including a dramatic increase in morphine reward, exacerbated morphine physical dependence and withdrawal, increased morphine analgesia and delayed development of analgesic tolerance (Zachariou et al., 2003). On the other hand, RGS4 plays a minor role in morphine reward sensitivity in the Nucleus Accumbens (NAc), while in locus coeruleus (LC) it opposes morphine physical dependence. In contrast to what observed with RGS9-2, RGS4 does not affect morphine analgesia or tolerance (Zachariou et al., 2010). Importantly, the distribution of RGS described in rodent studies is similar to that observed in human tissue (Gold et al., 1997, Grafstein-Dunn et al., 2001, Larminie et al., 2004).

4.4 RGS20

Among RGS proteins, RGS20 is a GAP highly specific for Gαz (a Gαi subunit with very slow hydrolysis kinetics) and other Gαi subunits (Glick et al., 1998, Wang et al., 1998, 2002). Together with RGS17, RGS19 and RET-RGS1 it belongs to Rz subfamily. Flanking the RGS box domain Rz subfamily members possess an N-terminus with heavily palmitoylated cysteine string motif involved in membrane targeting (De Vries et al., 1996), and a short C-terminus of 11 or 12 aminoacid residues. RGS20 shows expression in the nervous system with a broad distribution through the brain, including areas such as caudate nucleus, temporal cortex, hippocampus, midbrain (Glick et al., 1998, Wang et al., 1998) and striatal structures (Wang et al., 2002). There is some in vitro evidence implicating RGS20 with Mu-opioid receptor desensitization (Garzón et al., 2004, Rodriguez-Munoz et al., 2007, Ajit et al., 2007), but no exact molecular pathway has been proposed.

Material & Methods

Animals

Experiments were performed on different groups of male and female adult mice. RGS20 WT and KO mice were bred in house from heterozygote RGS20 breeders. For all the assays we used 8-10 week old male or female KO mice and their WT littermates. In order to create conditional KO mice, we applied adeno-associated viruses expressing Cre recombinase (or GFP as control), in the periaqueductal gray (PAG) of floxed RGS20 mice. Stereotaxic coordinates for viral vector injections into the PAG were: anteriorposterior 6mm, lateral 8mm, and dorsoventral 28mm at an angle of 22 ^{0} from the midline (relative to Bregma). For all stereotaxic surgery procedures mice were anesthetized with avertine. Mice were genotyped with polymerase chain reaction (PCR), using DNA extracted from the tail/ear of each mouse. Mice were kept on a 12h light/dark cycle and were group-housed (4-5 per cage). Food and water were available ad libitum. Animal handling and experiments were in accordance to the guidelines of the Institutional Animal Care and Use Committee of the University of Crete.

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

Complete Freud's Adjuvant (CFA) (Sigma, Alidrich) was diluted 1:1 with saline to a final concentration of 1 mg/ml until it was emulsified. 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.

Hargreaves' test (evaluation of thermal hyperalgesia)

Mice were placed in light boxes on a glass plate (IITC Life Sciences) over a radiant heat stimulus (intensity 30%) was applied by aiming a beam of light onto the glabrous surface of the paw of the left limb though the glass plate. The light beam was turned off manually when the mouse lifted the limb, allowing the measurement of time between the start of the light beam and the paw withdrawal. A cut off of 20 sec was used in order to avoid tissue damage. The same procedure was performed also for the right paw as an internal control. There was a total of two measurements for each paw. At least five minutes were allowed between each trial, and these measurements were averaged for each limb and compared to the baseline (before injury) value.

Formalin test for inflammatory pain

30μl of 4% formaldehyde were injected to the plantar surface of the left paw of each mouse. Mice were immediately placed into light boxes and the duration of their pain-like behavior signs (licking, flicking) within an hour after the injections was evaluated.

Hot-Plate assay (evaluation of acute nociception and tolerance)

Mice were placed into a light box on a platform heated to 52.1°C with a cut-off of 40 seconds and the latency to paw lick or jump was recorded. A control response was determined for each animal before treatment. Drug was injected subcutaneously (s.c.) and analgesia was measured 30 minutes after the injection. Antinociceptive response was calculated as a percentage of Maximal Possible Effect (MPE), where MPE=100((test-control latency)/(cut-off-control)). For morphine tolerance, repeated morphine injections (15mg/kg) were given for 4 consecutive days and analgesia was measured 30 minutes after each drug dose.*

Morphine Withdrawal

Mice were injected intraperitoneally (i.p.) with escalating morphine doses (20, 40, 60 and 80 mg/kg) every 12 hours for 5 days. Three hours after the last morphine injection, naloxone (1mg/kg) was administered s.c. Withdrawal behaviors were then monitored for 30 minutes.

Drug preparation

Morphine, fentanyl and naloxone were diluted in saline (0.9% NaCl) to the desired concentration, whereas SNC80 was diluted first in HCl (1N) and then in saline. 100μl of each drug were injected ether s.c. or i.p. depending on the experimental process.

Statistical analysis

Two way ANOVA or unpaired two-tailed Student t tests were utilized to examine significant effects of treatment over genotype for all pharmacological and behavioral experiments. Significant post-hoc effects were revealed by the Bonferroni post-hoc test, and effects were considered to be significant at $p<0.05$.

Results

RGS20 is a negative regulator of morphine & fentanyl induced analgesia

Morphine induced analgesia was evaluated by Hot-plate assay on RGS20 constitutive KO mice and their WT littermates 30 minutes after s.c. injection of morphine (10mg/kg). As shown in figure 8, RGS20 KO mice of both gender show increased antinociception with the majority of KO mice tested reaching the cut-off latency.

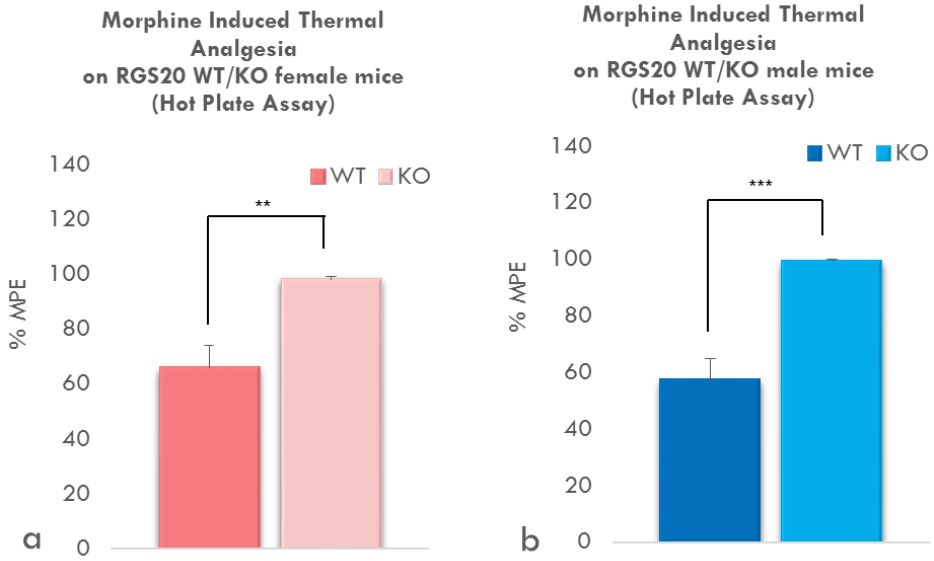


Figure 8.

RGS20 KO mice show increased antinociception upon acute morphine administration

*Both female (**a**) and male (**b**) RGS20 KO mice show higher response to morphine analgesic effects in the hot-plate assay compared to their WT controls. Mice were tested 30 min after the s.c. morphine injection.*

Dose: 10mg/kg
n=12
*t-test : **a.** p=0.0027 **b.** p=0.0002*

Interestingly this effect is presented also with a lower dose of the drug and it is consistent throughout its influence duration (Fig. 9). In fact, there is a shift in morphine dose response to the left in the case of KO mice.

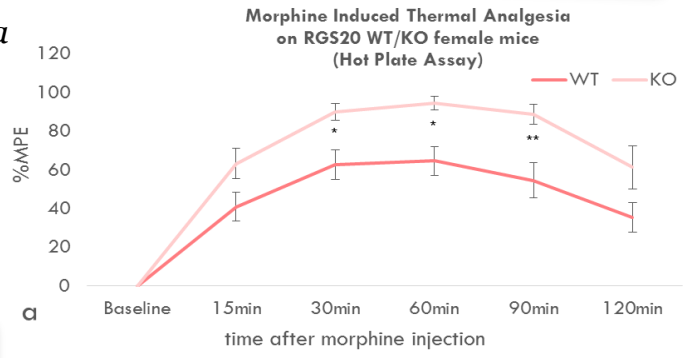
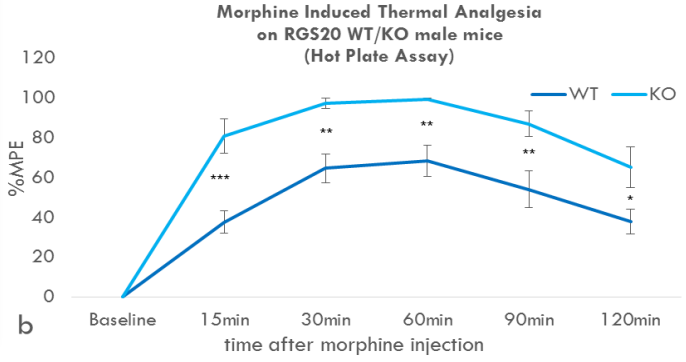


Figure 9.

RGS20 KO mice show increased antinociception upon acute morphine administration

*Both female (**a**) and male (**b**) RGS20 KO mice show higher response to morphine analgesic effects in the hot-plate assay throughout drug's influence duration compared to their WT controls. Mice were s.c. injected with morphine and tested 15, 30, 60, 90 and 120 min after that.*

Dose: 7.5mg/kg, n=12, two-way anova (bonferroni post-tests shown in graph)



Moreover, RGS20 seems to regulate in a similar way fentanyl induced analgesia. Mice lacking RGS20 expression treated even with a very low dose of fentanyl show exactly the same antinociceptive behavior on hot-plate assay with that observed after morphine administration (Fig. 10).

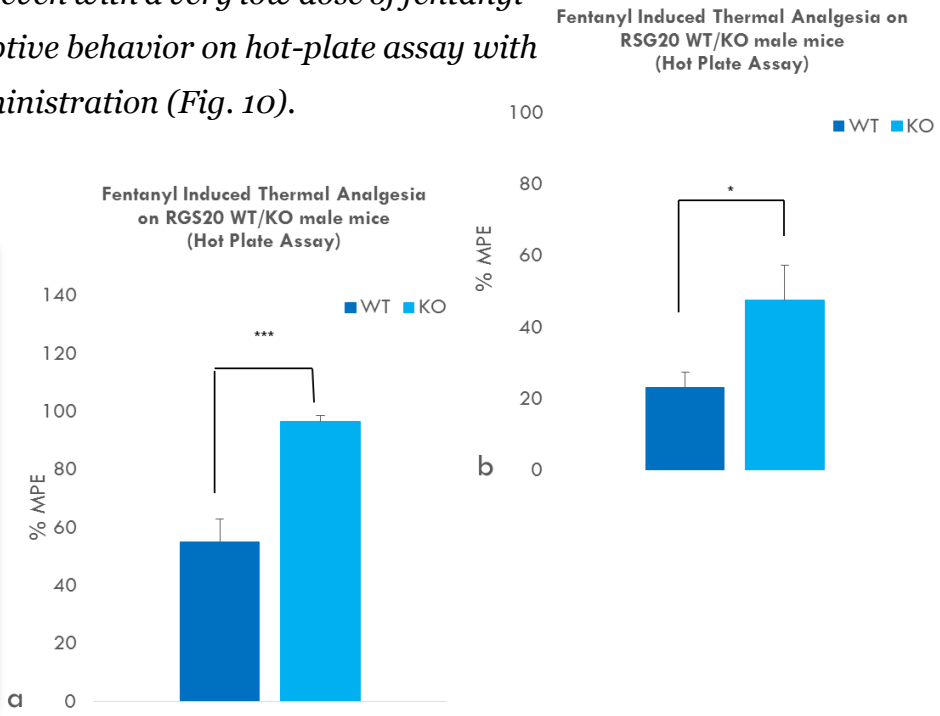


Figure 10.

RGS20 KO mice show increased antinociception upon acute fentanyl administration

*Male RGS20 KO mice show higher response to fentanyl analgesic effects in the hot-plate assay both for a low (**a**) and a very low dose (**b**) compared to their WT controls. Mice were s.c. injected with fentanyl and tested 30 min after.*

*Dose: **a.** 0.125mg/kg **b.** 0.04mg/kg*
n=10
*t-test: **a.** p=0.0002 **b.** p=0.0221*

RGS20 is a positive regulator of morphine tolerance development in a PAG specific manner

On the other hand, talking about morphine tolerance development, RGS20 seems to be a positive regulator as mice lacking RGS20 expression do not develop tolerance against the drug. RGS20 constitutive KO mice treated daily with a high morphine dose (15mg/kg) for four days continue responding to the drug exactly as they did on their first acute administration, whereas their WT littermates become unresponsive to the drug treatment (Fig. 11a,b).

In fact, this effect seems to be mediated by periaqueductal gray (PAG), since PAG-specific RGS20 KO mice show exactly the same phenotype with the constitutive KOs (Fig. 11c).

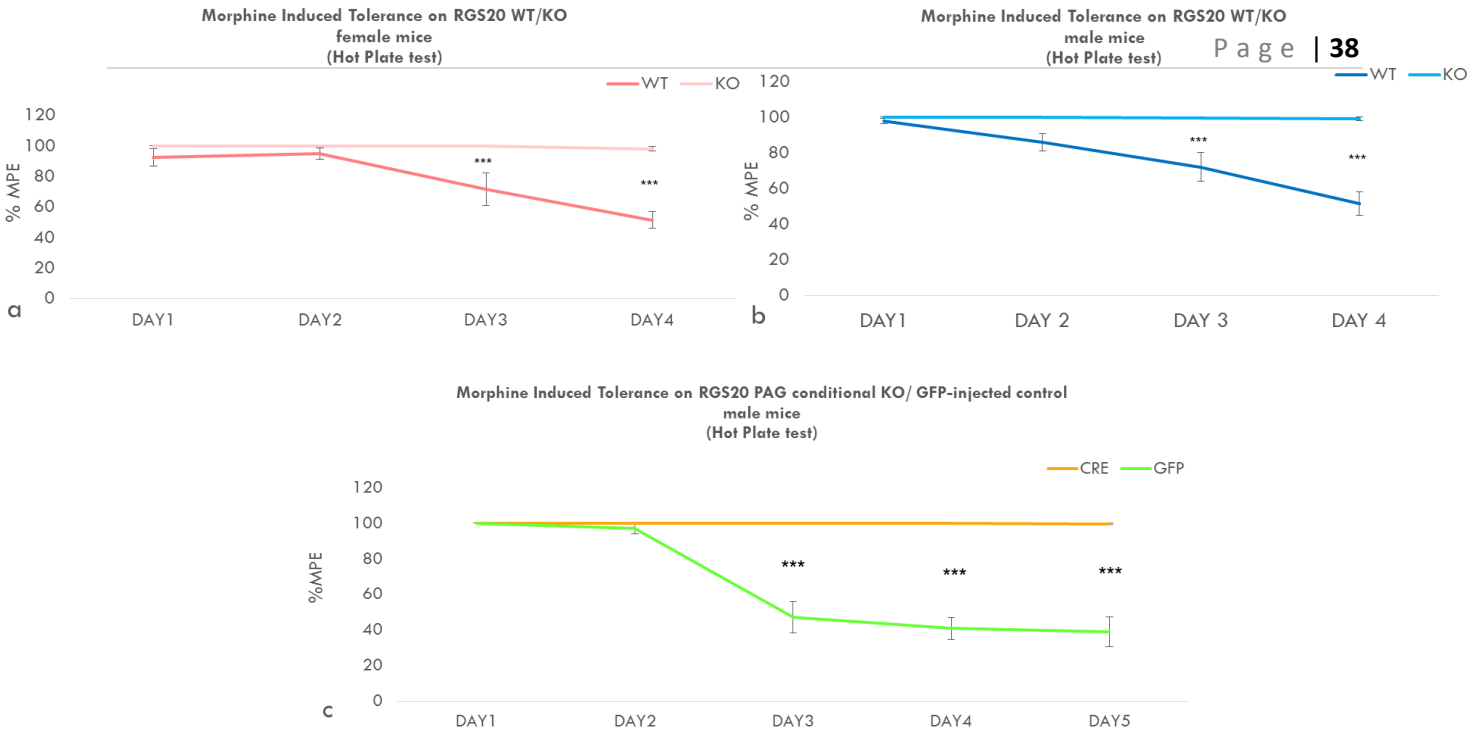


Figure 11.

RGS20 implication in morphine tolerance development

*Both female (**a**) and male (**b**) mice treated for 4 consecutive days with a high dose of morphine (15mg/kg) do not develop tolerance against the drug, whereas their WT controls become tolerant after day 3.*

***c.** PAG conditional RGS20 KO males show exactly the same phanotype compared to the GFP-injected control animals.*

*n= **a,b:** 12 **c:** 8*

two-way anova (bonferroni post-tests shown in graph)

Dependence/addiction to morphine is not affected by the absence of RGS20 protein.

RGS20 global KO mice injected for five consecutive days with escalating doses of morphine (20, 40, 60, 80 mg/kg) show no overall differences in naloxone-induced withdrawal symptoms compared to their WT controls (Fig. 12a).

Moreover, locomotor sensitization induced by daily morphine treatment for six days is identical between KO RGS20 mice and their WT littermates (Fig. 12b).

These data suggest that RSG20 protein does not affect morphine addiction development.

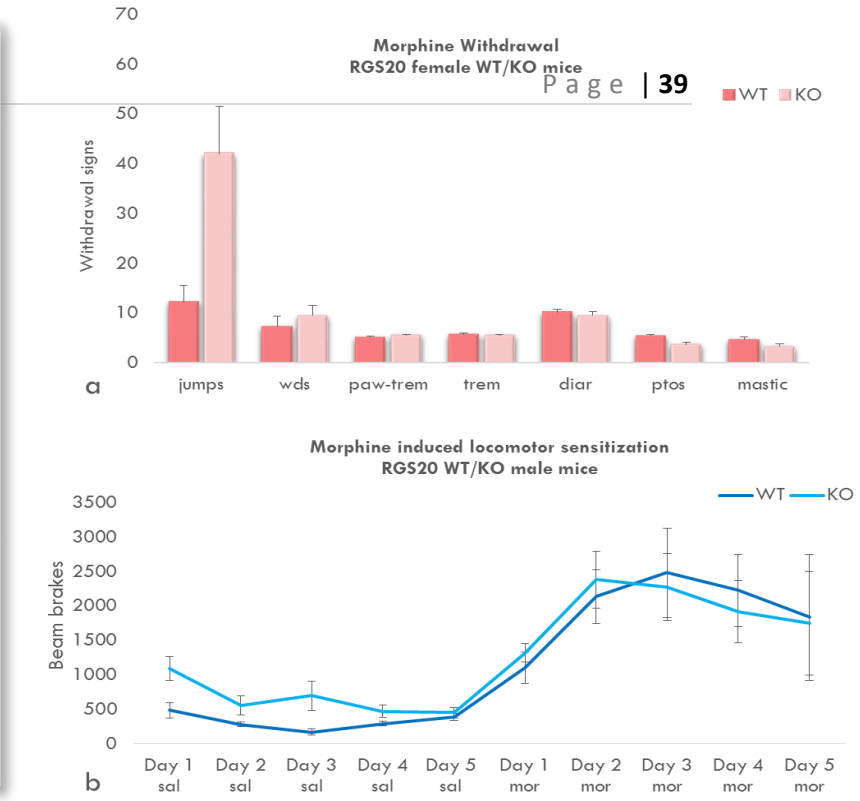
Figure 12.

Morphine addiction development on RGS20 KO mice VS WT controls

a. *Morphine withdrawal signs measured after naloxone-induced withdrawal for RGS20 KO female mice and their WT littermates. Mice were injected i.p. twice a day for 5 days with escalating morphine doses (20, 40, 60, 80 mg/kg). 3 hours after the last morphine injection, naloxone (1mg/kg) was administered s.c. and withdrawal symptoms were recorded immediately for 30min.*

b. *Morphine induced locomotor sensitization is exactly the same between RGS20 KO male mice and their WT controls. After a few days of saline injections for habituation and elimination of any baseline difference, morphine (10mg/kg) was injected daily for 5 days and mice locomotion was measured straight after each injection for 30min.*

n= ***a:*** *7* ***b:*** *10*



RGS20 regulates inflammatory derived hyperalgesia in a sex-dependent manner. Evidence for δ-opioid receptor implication.

RGS20 KO female mice show higher sensitivity during the chronic phase of inflammatory pain, both in formalin (Fig. 13a) and CFA (Fig. 13b) tests. In case of formalin, 30μl of 4% paraformaldehyde were injected to the hind paw of each mouse and licking behavior was recorded immediately after the injection for one hour. A significant increase in sensitivity was observed in the KO mice compared to their WT controls regarding the chronic phase. Similarly, RGS20 female mice injected with CFA show a delayed recovery starting at day 15 after CFA injection, whereas their WT controls start recovering from day 8.

Interestingly, this higher sensitivity was not recapitulated with RGS20 KO male mice. In this case, KO mice undergoing CFA-induced inflammatory pain seem to recover by day 8 after CFA injection exactly as their WT littermates do (Fig. 13c).

Moreover, RGS20 KO female mice undergoing CFA-induced inflammatory pain (day 3 after injection that hyperalgesia show a peak both in KO and WT mice) treated with the δ-opioid agonist SNC80 are unresponsive to the drug, suggesting an involvement of

Figure 13.

RGS20 regulates inflammatory pain in a sex-dependent manner

*RGS20 KO female mice show increased hyperalgesia during chronic inflammatory pain phase both in formalin (**a**) and CFA (**b**) tests compared to WT control animals. On the other hand, RGS20 KO male mice (**c**) show similar pain behavior to their WT littermates with full recovery after day 8.*

*n= **a:** 6 **b:** 12 **c:** 10*

two-way anova (bonferroni post-tests shown in graph)

δ-opioid receptors in RGS20 mediated regulation of inflammatory induced hyperalgesia (Fig. 14). SNC80 was injected s.c. and mice were tested for thermal hyperalgesia (Hargraves' test) 30, 60, 90 and 120 minutes after drug administration.

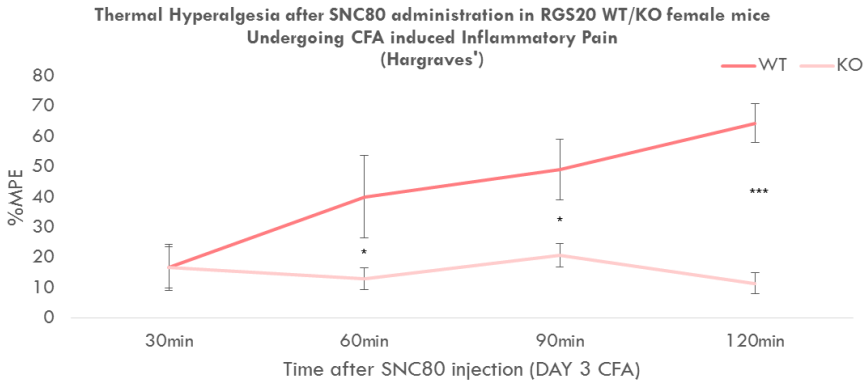


Figure 14.

RGS20 KO female mice do not respond to SNC80 treatment

RGS20 KO mice undergoing CFA induced inflammatory pain treated with δ-opioid receptor agonist, SNC80 do not show decreased levels of hyperalgesia as their WT littermates.

Drug was administered on day 3 after CFA injection when the hyperalgesic phenotype seems to have a peak both in WT and KO mice to avoid baseline differences (see fig. 13b).

SNC80 was injected s.c. and mice were tested (by Hargraves' test) 30, 60, 90 and 120 min after the injection.

Dose : 5mg/kg

n= 8

two-way anova (bonferroni post-tests shown in graph)

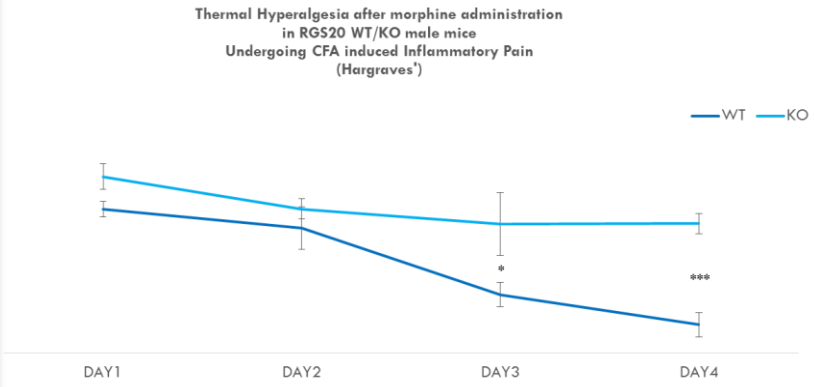
RGS20 effect on morphine tolerance development is present also under inflammatory pain condition

RGS20 KO male mice undergoing CFA-induced inflammatory pain were treated daily with a low dose of morphine (3mg/kg) and tested for thermal hyperalgesia by Hargraves' test. As shown in figure 15 RGS20 KO tolerance-resistant phenotype seem to be recapitulated also under the inflammatory pain background.

Figure 15.

Morphine tolerance development under inflammatory pain condition on RGS20 WT/KO mice

RGS20 WT and KO male mice undergoing CFA-induced inflammatory pain were injected daily with morphine and tested 1 hour after injection for thermal hyperalgesia (Hargraves' test).
Dose: 3mg/kg
n= 5
two-way anova (bonferroni post-tests shown in graph)



Discussion

Chronic pain is a major public health problem, with epidemiological studies reporting about one fifth of the general population to be affected both in USA and Europe (Breivik et al., 2006). This condition causes not only considerable personal suffering but also a huge loss of productivity resulting in enormous socioeconomic costs. In addition, pharmacological management of chronic pain has seen limited progress in the last decades with only two classical medications dominating treatment(opioids and non-steroidal anti-inflammatory drugs) and a few compounds acting on novel molecular targets having serious side effects (gabapentinoids, TprVI agonists, cannabinoids).

Agonists of the μ-opioid receptor, such as morphine, are highly effective analgesic agents for the treatment of acute pain or chronic pain associated with cancer (Stein et al., 2000, Trescot et al., 2008). However, their effectiveness for treating chronic non-cancer pain is compromised by the development of addiction and analgesic tolerance (Christie et al., 2008, Morgan & Christie, 2011).

Previous studies from our lab have enlightened the role of two regulators of G-protein signaling, RGS9-2 and RGS4 in the mouse brain, regarding the mechanisms of analgesia and addiction (Zachariou et al., 2003, 2010, Psifogeorgou et al., 2011). In this study another member of the RGS family, RGS20 seems to affect opiate analgesia and tolerance development without having any effect on addiction. These findings could be explained by the brain-region specific actions of RGS20. Certain brain areas have been associated with addiction, with most attention given to the midbrain dopamine pathway (Ventral Tegmental area dopamine neurons and their projections to the nucleus accumbens) VTA-NAc pathway (Nestler, 2005), whereas PAG-RVM descending pathway is the one highly involved in pain inhibition, potentiation of morphine-induced analgesia and tolerance development (Fitzgerald, 2005, Tortorici et al., 2001, Song et al., 2001). By using brain region-specific KO mice we revealed that RGS20 implication in morphine tolerance development is mediated by PAG, since PAG conditional KO mice show exactly the same tolerance-resistant phenotype as the global KO mice. So, it seems that RGS20 acts through PAG-RVM pathway to modulate opiate analgesia and tolerance, but it is not implicated in addiction circuits (VTA-NAc pathway).

Additionally, by applying classic inflammatory pain models, such as CFA and formalin, we noticed a sex-dependent implication of RGS20 protein in chronic inflammatory pain nociception. It is becoming increasingly clear that males and females differ in both the anatomical and physiological composition of the central and peripheral nervous system circuits that relay nociceptive information (Schenck-Gustafsson et al., 2012). In fact, other groups have also noticed gender differences in the case of inflammatory pain in rodents, which they were able to link with specific differences in PAG-RVM cell activation and signaling (Loyd et al., 2007, Loyd & Murphy 2009) and gender differences have been also noticed in pain-related PAG connectivity to other brain regions in human fMRI studies (Linnman et al., 2012). Moreover, there is ongoing evidence that some shared mechanisms between opioid tolerance development and transition to chronic pain must exist (Crain et al., 2000, Joseph et al., 2010).

All these together propose a potential PAG-mediated role of RGS20 in the regulation of nociception and morphine tolerance development, with no effect on any of the other morphine actions (reward, addiction). Moreover, RGS20 seems to be a positive regulator of inflammatory pain antinociception in a gender-dependent manner. Further work needs to be done regarding the cellular mechanisms underlying these implications that could reveal RGS20 clinical significance as a future direct target of novel non-opioid anti-inflammatory drugs or as a molecule mediating/facilitating already existing analgesic treatments.

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