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«Cellular regulation of the action of antidepressants and analgesics"

"Κυτταρική ρύθμιση της δράσης των αναλγητικών και των αντικαταθλιπτικών"

Supervisor: Venetia Zachariou Associate professor of Pharmacology

Maria Stratinaki, MD

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ΕΥΧΑΡΙΣΤΙΕΣ

Αν κάποιος μου έλεγε δύο χρόνια πριν ότι μόλις πάρω το πτυχίο μου θα περάσω δύο χρόνια κάνοντας μεταπτυχιακό στην «Κυτταρική και Γενετική Αιτιολογία, Διαγνωστική και Θεραπευτική των ασθενειών του ανθρώπου» (θέλει προσπάθεια να το πεις μόνο όχι να αποφασίσεις να το κάνεις κιόλας!) , θα δουλέψω σε εργαστήριο νευροεπιστημών και ότι θα μου άρεσε κιόλας, θα του έλεγα ότι πρέπει να επισκεφθεί έναν ψυχίατρο…

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Για πολλούς ασθενείς, ο χρόνιος πόνος (νευροπαθητικός και φλεγμονώδης) και η κατάθλιψη είναι διαφορετικές όψεις του ίδιου νομίσματος .Η πλειονότητα των ανθρώπων που υποφέρουν από χρόνιο πόνο κάποια στιγμή φαίνεται να παρουσιάζουν συμπτώματα κατάθλιψης .Θα ήταν πολύ χρήσιμο να αναπτυχθούν θεραπευτικές στρατηγικές που θα στοχεύουν τόσο τον πόνο, όσο και την κατάθλιψη. Είναι ευρέως γνωστό ότι τα αντικαταθλιπτικά μπορεί να έχουν αναλγητικές δράσεις, , αλλά ο μηχανισμός μέσω του οποίου αυτό συμβαίνει δεν είναι πλήρως κατανοητός. επιπλέον, τα αντικαταθλιπτικά δεν έχουν την ίδια αποτελεσματικότητα σε όλους τους ασθενείς και φαίνεται ότι το ίδιο ισχύει και για τις αναλγητικές τους ιδιότητες. Η μελέτη αυτή επικεντρώθηκε στο ρόλο της RGS4 (μια πρωτεΐνη 28kD που εμπλέκεται σε πολλές παθογενετικές διεργασίες ιδίως στο κεντρικό νευρικό σύστημα) στην ανάπτυξη της κατάθλιψης, του νευροπαθητικού και φλεγμονώδους άλγους καθώς και στην ανταπόκριση στα αντικαταθλιπτικά. Κατά την πρώτη σειρά πειραμάτων χρησιμοποιήθηκαν global καθώς και conditional στον επικλινή πυρήνα RGS4 KO ποντίκια προκειμένου να αξιολογηθεί η ανηδονία, ένα από τα κύρια συμπτώματα της κατάθλιψης. Τα δεδομένα μας δείχνουν ότι τα global RGS4 KO ποντίκια φαίνεται να έχουν αυξημένα επίπεδα ανηδονίας, και ότι η διαγραφή της RGS4 στον επικλινή πυρήνα κάνει τα ποντίκια πιο επιρρεπή στην κατάθλιψη . Στη συνέχεια, εξετάσαμε τις πιθανές διαφορές στην αποτελεσματικότητα της αντικαταθλιπτικής αγωγής σε μια ποικιλία από φάρμακα με γνωστή αντικαταθλιπτική δράση (δεσιπραμίνη, ριβοξετίνη, κεταμίνη. SNC80) μεταξύ RGS4 WT και KO ποντικιών. Τα ευρήματά μας υποδηλώνουν ότι η RGS4 μπορεί να λειτουργήσει ως θετικός ρυθμιστής για ορισμένα φάρμακα (δεσιπραμίνη, ριβοξετίνη) και ως αρνητικός ρυθμιστής για άλλα (κεταμίνη, SNC80). Στην τρίτη σειρά πειραμάτων, χρησιμοποιήσαμε ένα μοντέλο νευροπαθητικού πόνου για να αξιολογήσουμε τη συμμετοχή της RGS4 στην ανάπτυξη του νευροπαθητικού πόνου και την αποτελεσματικότητα των αντικαταθλιπτικών, ως αναλγητικά στη θεραπεία του νευροπαθητικού άλγους. Αν και η RGS4 δεν φαίνεται να παίζει κάποιο σημαντικό ρόλο στην εγκατάσταση του νευροπαθητικού πόνου, επηρεάζει την αποτελεσματικότητας της δεσιπραμίνης και κεταμίνης όταν χρησιμοποιούνται ως αναλγητικά, με τον ίδιο τρόπο που επηρεάζει το αντικαταθλιπτικό τους αποτέλεσμα. Για την τελευταία ομάδα των πειραμάτων, χρησιμοποιήσαμε ένα μοντέλο φλεγμονώδους πόνου, προκειμένου να εκτιμηθεί ο ρόλος της RGS4 στην ανάπτυξη της φλεγμονώδους πόνου καθώς και τα φαινόμενα άγχους που τον συνοδεύουν. Αυτό που βρήκαμε, ήταν ότι τα RGS4 KO ποντίκια είναι πιο ευαίσθητα σε φλεγμονώδη πόνο και φαίνεται να παρουσιάζουν εντονότερα συμπτώματα άγχους.

ABSTRACT

For many patients, chronic pain (both neuropathic and inflammatory pain) and depression are different aspects of the same problem. Most of the people who suffer from chronic pain at some point they appear to have depressive symptoms. Thus it would be very useful to develop therapeutic strategies that target both pain and depression. It is widely known that antidepressants can have analgesic actions as well, but it the mechanism via which it is succeeded it is not fully understand. Furthermore, antidepressants do not have the same effeciveness to all patients and the same seems to be observed for their analgesic properties. This study focused on the involvement of RGS4 (a 28kD protein involved in many pathogenetic processes especially in the central nervous system) in the development of depression, neuropathic and inflammatory pain as well as in the responsiveness to antidepressants. During the first set of experiments we used global and conditional nucleus accumbens RGS4 KO mice in order to evaluate anhedonia-one of the major symptoms of depression. Our data suggest global RGS4 KO mice seem to have increased levels of anhedonia and depression, and that deletion of RGS4 in the NAc makes mice more susceptible to depression. Accordingly, we tested the possible differences in the antidepressant efficacy of a variety of drugs with known antidepressant action (desipramine, reboxetine, ketamine, SNC80) between WT and KO mice. Our findings suggest that RGS4 can act as a positive modulator for some drugs (desipramine, reboxetine) and as a negative modulator for others (ketamine, SNC80). In the third set of experiments, we use the spared nerve injury model of neuropathic pain to evaluate the RGS4 involvement in the development of neuropathic pain and the efficacy of the antidepressants as analgesics in the treatment of neuropathic pain. Although RGS4 does not seem to play any important role in the establishment of neuropathic pain, it affects the effeciveness of desipramine and ketamine when used as analgesics, in the same manner as it affects their antidepressant properties. For the last set of experiments, we used the Complete Freud's Adjuvant model of inflammatory pain, in order to assess the RGS4 role in the development of inflammatory pain as well as the anxiety phenomena that come along. What we found out, was that RGS4 KO mice are more sensitive to inflammatory pain and appear to be more anxious as well.

INTRODUCTION

CHAPTER 1

PAIN

1.1 Pain definition and classification

The International Association for the Study of Pain (IASP) originally defined pain as *"an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage"* (1). However, the revised definition identifies pain as " a somatic perception containing: a) a bodily sensation with qualities like those reported during tissue-damaging stimulation, b) an experience threat associated with this sensation, and c) a feeling of unpleasantness or other negative emotion based on this experienced threat" (2).

Table 1. Pain in numbers
76.2 million suffer from chronic pain in the USA
63% of pain sufferers seek help from primary care
20% of outpatient visits
12% of all prescriptions
80% of chronic patients refer negative impacts on their everyday life
50 millions day/year are lost from work
$100 billion/year costs untreated or undertreated pain

Source: National Centre for Health Statistics

Pain can be categorized based on its *duration* or its *cause*. Duration based-pain can be characterized either acute or chronic. *Acute* pain is the one that protects from the variety of threats and dangers in the surrounding environment. Acute pain is short (usually a few days) and comes as a result of an insult –eg a trauma or a dysfunction of an organ-, trying to protect the organism from this threat and creating the memory of this dangerous stimulus for future need (3). *Chronic*[1] pain is defined as pain that lasts more than the ordinary duration of time that an injury needs to heal. Due to its complexity, there are several other more specific definitions, regarding chronic pain:

- ✓ The International Association for the Study of Pain (IASP) definition addresses both duration and appropriateness, defining chronic pain as "pain without apparent biologic value that has persisted beyond the normal tissue healing time (usually taken to be three months)" (4).
- ✓ The American College of Rheumatology (ACR) defines chronic pain as "widespread or regional pain for at least three months" (5). ACR criteria for chronic widespread pain include all of the following: pain present for at least three months, pain in the left and right sides of the body, pain above

[1] There is a recent debate regarding the use of the word "chronic" in terms of describing pain. There are several scientists and physicians who believe that the term "persistent" is more appropriate.

and below the waist, and the presence of axial skeletal pain (cervical spine anterior chest, thoracic spine, or low back).

- ✓ The Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) defines chronic pain as "persistent pain for six months" (6).
- ✓ The American Society of Anesthesiologists defines chronic pain as "pain of any etiology not directly related to neoplastic involvement extending in duration beyond the expected temporal boundary of tissue injury and normal healing and adversely affecting the function or well-being of the individual" (7).

Based on its cause, pain can be characterized as physiological, nociceptive or neuropathic. *Physiological* is the pain that arises from a certain painful stimulus, eg a pin prick. *Nociceptive pain* is defined as pain caused by the activation of nociceptors, most of the times due to tissue damage. It is further divided to somatic and visceral pain. Somatic pain due to tissue damage is usually well localized but each patient experiences it and describes it in a different manner (eg pain due to a broken arm). Visceral pain, comes from the viscera via stretch receptors. It is not exactly localized, dull and cramping (eg pain associated with appendicitis or cholecystitis). Nociceptive pain can also be classified as musculoskeletal, inflammatory (eg inflammatory arthropathies), and mechanical or compressive pain (eg neck pain, pain from expanding tumor masses) (8). *Neuropathic* pain is due to abnormal function of the nervous system, due to disease, injury or any other dysfunction.

1.2 Pathogenesis of pain

1.2.1 The nociceptor

Nociceptors are a subpopulation of the peripheral nervous system that is capable of transducing and encoding noxious stimuli. 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 nociceptors , have two branches , a central branch targeting the spinal cord and a peripheral branch targeting each specific organ. Nociceptors are classified into three main categories:

- ✓ *Aδ fibres* , which are medium diameter myelinated afferents-they mediate the so-called "first" or fast pain (well localized). Based on their electrophysiological properties, Aδ fibers are also subclassified as:
  a. Type I or High Threshold Mechanical nocicpetors (HTM) which respond to chemical as well as mechanical stimuli but appear to have high heat thresholds (> 50 ^{o}C)
  b. Type II , which although they respond to heat in lower temperatures , they appear to have a high threshold to mechanical stimuli
- ✓ *Aβ fibres* , which are large diameter , myelinated, rapidly conducting fibres which respond to light touch

- ✓ *C fibres*, which are small diameter unmyelinated fibres, responsible for the "second" or slow pain (poorly localized) . C fibres have some subdivisions as well :
  a. C fibres which are sensitive to both heat and mechanical stimuli (CMHs)
  b. Silent nociceptors , which are heat-responsive but develop sensitivity to mechanical stimuli only in cases of injury and are also more responsive to chemical stimuli

It should be mentioned, that not all C fibres are nociceptors. Some of them respond to cooling, and some others mediate pleasant touch.

Nociceptors can also be categorized based on the expression of specific ion channels which offer sensitivity to heat (TRPV1), cold (TRPM8) , acids (ASICs) and chemical irritants (TRPA1).

Interestingly enough, the nociceptor due to its unique pseudo-unipolar morphology is able to send and receive information to both directions , meaning that proteins synthesized by DRGs or the trigeminal ganglion are distributed to central as well as peripheral terminals of the nociceptor. Moreover, although only the peripheral terminal responds to environmental stimuli, its sensitivity is regulated by a variety of endogenous factors (pH lipids, neurotransmitters etc) , which target both the peripheral and the central terminal (10).

Anatomically, the dorsal horn of the spinal cord is organized in distinct laminae. Aδ fibers project to lamina I and V, whereas C fibers project to laminae I and II. Aβ fibers project to laminae III, IV and V. Figure 1`, shows the connection between primary afferent fibers and the spinal cord.

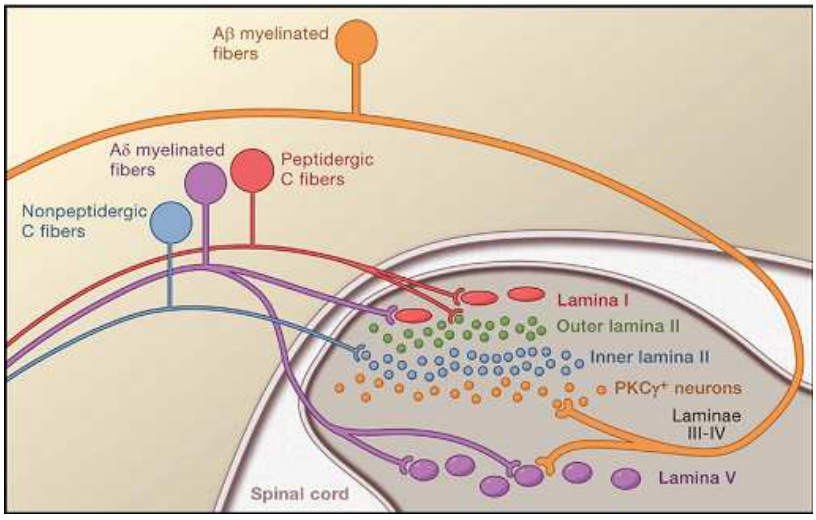


Figure 1 Connections between primary afferent fibres and the spinal cord. (Basbaum et al , 2009)

1.2.2 Ascending pain pathways

The sensation of pain starts at the periphery of the nervous system. Nociceptive information reaches the brain via multiple ascending pathways, including spinocervical, spinobullar, spinopontine, spinomesencephalic, spino-diencephalic (containing spino-thlamaic tracts) and spinotelencephalic pathways. Most nociceptive neurons project contralaterally within the spinal cord and ascend within the anterolateral quadrant, forming the spinothalamic tract which forms synapses to the thalamus. Accordingly, neurons from the thalamus project to several areas in the brain, including the primary and secondary somatosensory cortex, the cingulate cortex, the prefrontal cortex, the insular cortex, the amygdala and the cerebellum (Fig 2).

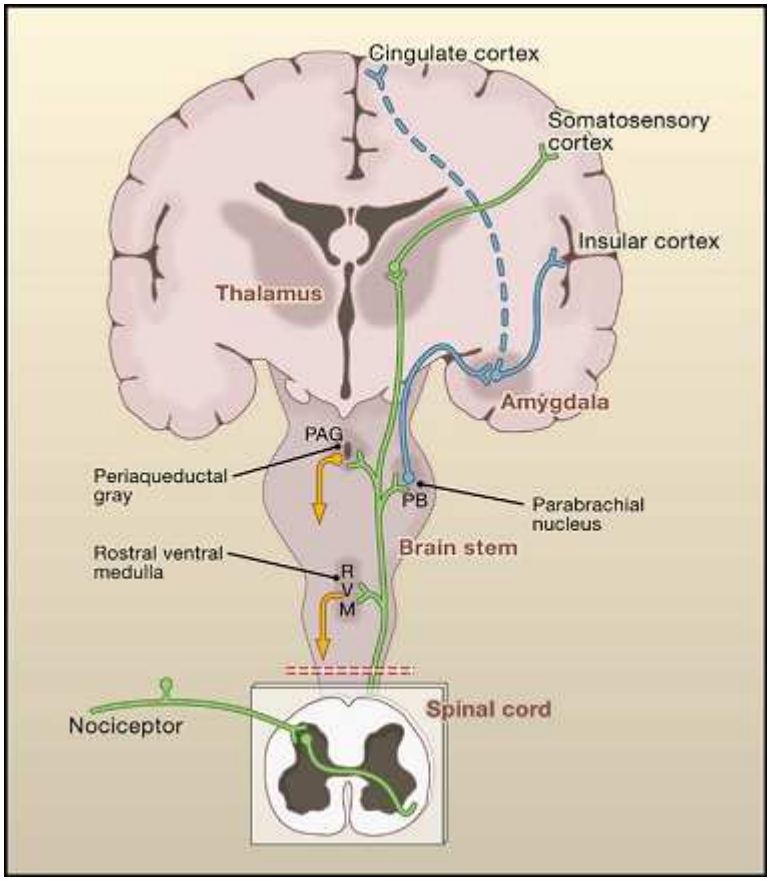


Figure 2 The pain pathway (Basbaum et al , 2009)

Regarding the physiological processes involved in pain, four are the major components transduction, transmission, modulation and perception.

- ✓ **Transduction** refers to the conversion of a noxious stimulus (thermal, mechanical, or chemical) into electrical activity in the peripheral terminals of nociceptor sensory fibers.
- ✓ **Transmission** refers to the passage of action potentials from the peripheral terminal along axons to the central terminal of nociceptors in the central

nervous system. Conduction is the synaptic transfer of input from one neuron to another.

- ✓ **Modulation** refers to the alteration (e.g., augmentation or suppression) of sensory input.
- ✓ **Perception** refers to the "decoding"/interpretation of afferent input in the brain that gives rise to the individual's specific sensory experience. (9-11).

CHAPTER 2

NEUROPATHIC PAIN

2.1 Definition and classification

According to IASP *neuropathic pain* is defined as "*pain caused by a lesion or a disease of the somatosensory nervous system*". According to the revised guidelines, "neuropathic pain is a clinical description (and not a diagnosis) which requires a demonstrable lesion or a disease that satisfies established neurological diagnostic criteria. The term *lesion* is commonly used when diagnostic investigations (e.g. imaging, neurophysiology, biopsies, lab tests) reveal an abnormality or when there is obvious trauma. The term *disease* is commonly used when the underlying cause of the lesion is known (e.g. stroke, vasculitis, diabetes mellitus, and genetic abnormality). *Somatosensory* refers to information about the body per se including visceral organs, rather than information about the external world (e.g., vision, hearing, or olfaction). The presence of symptoms or signs (e.g., touch-evoked pain) alone does not justify the use of the term *neuropathic*. (1).

As neuropathic pain is a term used to describe a wide variety of medical entities, a classification is absolutely necessary for the efficient management of the patients. In the case of neuropathic pain, two are the most commonly used classifications: the anatomy based and the disease based. Speaking anatomically, the first classification is *central* (if the neuropathy affects the CNS) or *peripheral* (if the neuropathy affects the peripheral nervous system). Peripheral neuropathies are further subdivided to *symmetrical generalized polyneuropathies* (disease affects many nerves simultaneously) and *assymetrical* neuropathies, which are further grouped to those having either *focal or multifocal* distribution.

The disease based categorization is even broader , as neuropathic pain can be a result of many pathological processes, such as mechanical default of the nerves, metabolic disorders, viral infections, neurotoxicity, inflammatory or autoimmune diseases, ischemia of the nervous system , etc .

An extra and very interesting category of neuropathic pain is the complex regional pain syndromes (CRPSs) , which are complex neuropathic disorders formerly known as reflex sympathetic dystrophies, Sudeck's atrophy or causalgia. CRPs are characterized by extreme pain, can result from trauma and usually affect the limbs. In type I, there is usually no obvious nerve lesion, whereas in type II most of the times there is lesion of a large nerve (12) . Table 3 gives a better picture of the neuropathic pain classification

Table 3. Neuropathic pain classification

Focal or multifocal lesions of the peripheral nervous system
Entrapment syndromes
Phantom limb pain, stump pain
Post-traumatic neuralgia
Postherpetic neuralgia
Diabetic mononeuropathy
Ischemic neuropathy
Polyarteritis nodosa
Generalized lesions of the peripheral nervous system (polyneuropathies)
Diabetes mellitus
Alcohol
Amyloid
Plasmocytoma
HIV neuropathy
Hypothyroidism
Hereditary sensory neuropathies
Fabry's disease
Bannwarth's syndrome (neuroborreliosis)
Vitamin B deficiency
Toxic neuropathies (arsenic, thallium, chloramphenicol, metronidazole, nitrofurantoin, isoniazid, vinca alkaloids, taxoids, gold)
Lesions of the CNS
Spinal cord injury
Brain infarction (especially the thalamus and brainstem)
Spinal infarction
Syringomyelia
Multiple sclerosis
Complex neuropathic disorders
Complex regional pain syndromes type I and II (reflex sympathetic dystrophy, causalgia)

(Baron , 2005)

2.2 Signs and symptoms of neuropathic pain

Because of its diverse etiology, neuropathic pain also manifests with a variety of symptoms. For neurologists, a key diagnostic feature for neuropathic pain is "*an area of abnormal sensation and the patient's maximum pain is coextensive with or within an area of sensory deficit*" (12). The signs of neuropathic pain can be either negative or positive. Negative signs, represent a deficit in function, are rather bothering than painful and include hypoesthesia, pallhypoaesthesia, hypoalgesia and thermohypoaesthesia. Positive signs are usually painful, -with the exception of paresthesias- and can be stimulus-dependent or stimulus-independent. The stimulus-independent signs include spontaneous shooting and electric-shock; like feelings. The stimulus-dependent signs include hypersensitivity and summation. Most of the patients experience two main types of hypersensitivity: allodynia and hyperalgesia. *Allodynia* is defined as "pain in response to a non nociceptive stimulus", whereas *hyperalgesia* is defined as "increased pain sensitivity to a nociceptive stimulus". (13).

Table 4 shows the definitions and the appropriate assessment of neuropathic pain signs and symptoms.

Table 4.Definition and assessment of signs and symptoms in neuropathic pain

Symptom/sign	Definition	Assessment	Expected pathological response
Negative signs and symptoms			
Hypoesthesia	Reduced sensation to non-painful stimuli	Touch skin with painter's brush, cotton swab or gauze	Reduced perception, numbness
Pallhypoesthesia	Reduced sensation to vibration	Apply tuning fork to bone or joint	Reduced perception threshold
Hypoalgesia	Reduced sensation to painful stimuli	Prick skin with single pin stimulus	Reduced perception, numbness
Thermohypoesthesia	Reduced sensation to cold or warm stimuli	Touch skin with objects of 10 °C (metal roller, glass of water, coolants like acetone) Touch skin with objects of 45 °C (metal roller, glass of water)	Reduced perception
Spontaneous sensations/pain			
Paraesthesia	Non-painful ongoing sensation (ant crawling)	Grade intensity (0–10) Area in cm^2	–
Paroxysmal pain	Shooting electrical attacks for seconds	Number per episode Grade intensity (0–10) Threshold for evocation	–
Superficial pain	Painful ongoing sensation, often of burning quality	Grade intensity (0–10) Area in cm^2	–
Evoked pain			
Mechanical dynamic allodynia	Normally non-painful light-pressure moving stimuli on skin evoke pain	Stroking skin with painter's brush, cotton swab or gauze	Sharp burning superficial pain in the primary affected zone, spreading into unaffected skin areas (secondary zone)
Mechanical static allodynia	Normally non-painful gentle static pressure stimuli on skin evoke pain	Manual gentle mechanical pressure to the skin	Dull pain in the area of affected (damaged or sensitized) primary afferent nerve endings (primary zone)
Mechanical punctate or pinprick hyperalgesia	Normally stinging-but-not-painful stimuli evoke pain	Manual pricking of the skin with a safety pin, sharp stick or stiff von Frey hair	Sharp superficial pain in the primary affected zone, spreading into unaffected skin areas (secondary zone)
Temporal summation	Repetitive application of identical single noxious stimuli is perceived as increasing pain sensation (wind-up-like pain)	Pricking the skin with safety pin at <3 s intervals for 30 s	Sharp superficial pain of increasing intensity
Cold allodynia	Normally non-painful cold stimuli evoke pain	Touch skin with objects of 20 °C (metal roller, glass of water, coolants like acetone) Control: touch skin with objects of skin temperature	Painful, often burning, temperature sensation in the area of affected (damaged or sensitized) primary afferent nerve endings (primary zone)
Heat allodynia	Normally non-painful heat stimuli evoke pain	Touch skin with objects of 40 °C (metal roller, glass of water) Control: touch skin with objects of skin temperature	Painful burning temperature sensation in the area of affected (damaged or sensitized) primary afferent nerve endings (primary zone)
Mechanical deep somatic allodynia	Normally non-painful pressure on deep somatic tissues evokes pain	Manual light pressure at joints or muscle	Deep pain in joints or muscles

(Baron, 2005)

2.3 Pathophysiology of the neuropathic pain

Neuropathic pain is a complex medical entity, and its pathophysiology has not been fully understood yet. However, studies in animal models have revealed many interesting aspects, concerning the peripheral and central sensitization

Peripheral sensitization

C and Aδ fibers are responsible for sensing pain; they remain silent under normal conditions and only respond to potential noxious stimuli. However, lesions in the peripheral nervous system can make these neurons abnormally sensitive and, promote robust changes at cellular as well as molecular level. First of all, following nerve injury, there is an increase in the mRNA of the voltage-gated sodium channels which are expressed in the primary afferent neurons. The accumulation of these channels to sites of ectopic stimulation may be the reason for lower threshold of the action potential (14). Nav1.8 and Nav 1.9 are greatly expressed in primary afferent neurons while Nav1.3, an embryonic channel, is upregulated after peripheral nerve lesion (15). Voltage-

gated sodium channels are accumulated not only in the area of the lesion, but also within the intact DRG. Amir et al have shown that "reciprocation between a phasically activating voltage-dependent, TTX-S sodium conductance and a passive, voltage-independent potassium leak generates characteristic membrane potential oscillations. Ectopic firing is triggered when the amplitude of oscillation sinusoids reaches the threshold" (16). Moreover, patients with erythromelalgia have a mutation in SCN9A gene which encodes Nav1.7 and this mutation can cause alterations in the firing pattern (17-19).
Peripheral nerve injury can cause a decreased expression of TRPV1 in the damaged nerve, but surprisingly, an increase in its expression in non-injured C and A fibers and also in large and medium size injured DRG cells (20, 21). The role of TRPV1 is further enhanced by the fact that TRPV1 deficient mice do not experience thermal hyperalgesia after tissue inflammation (22,23) , but it does not seem to act solely , as TRPV1 KO animals with partial sciatic nerve ligation experience almost the same levels of mechanical and thermal responses (22) .
Another member of the TRP family, TRPV4 appears to take part in taxol-induced polyneuropathy, as administration of antisense oligodeoxynucleotides against TRPV4 in the spinal cord completely ceased mechanical hyperalgesia in rats (24).
The phenomenon of cold hyperalgesia, which is found in some cases of neuropathic pain, could be explained by the upregulation of TRPM8 channel, which could lead to sensitization of cold-sensitive C fibers (25).
Emery et al have found that a special type of ion channels HCN channels and especially the subtype HCN2 is involved in the development and the maintenance of neuropathic pain (26).
A very intriguing fact is that uninjured nerve fibers seem to change their properties under the release of NGF, TNF-α and other factors (that have to do with Wallerian degeneration[2]) from the injured nerves and thus participate in pain signalling (27).

Central sensitization

As expected, peripheral nerve injury causes robust changes in the dorsal horn of the spinal cord. Abnormally sensitized C-fibers are mainly responsible for this generalized hyperexcitability. The glutamate release from these neurons along with the activation of type N voltage-gated calcium channels results to the sensitization of NMDA receptors and the increased expression of substance P (12). Once these events take place, then Aδ and Aβ fibers can be activated by innocuous tactile stimuli (27). The mechanism of disinhibition of GABA neurons together with an almost same mechanism in serotonergic and noradrenergic pathways, contribute to pain signaling and maintenance (28).
Supraspinal centers, including the mecencephalic reticular formation and more specifically the nucleus cuneiformis and the periaqueductal gray are involved

[2] Degeneration of the distal segment of a peripheral nerve fiber after it has been severed from the cell body

in neuropathic pain, as fMRI studies show that these regions are activated in individuals suffering from neuropathic pain (29).
Another possibility is that non neuronal glial cells may be activated by peripheral nerve injury and release cytokines and glutamate that further facilitate central sensitization.
Figure 3 shows schematically the pathogenetic mechanisms of neuropathic pain and table 5 shows the potential links between symptoms and mechanisms.

Table 5. Relationship between neuropathic pain mechanisms and clinical symptoms

SYMPTOM	NEURONAL PROCESSES, MECHANISMS	TARGETS	OPTIMAL COMPOUNDS	AVAILABLE COMPOUNDS
	Peripheral nociceptor hyperexcitability			
Spontaneous pain (shooting)	Ectopic impulse generation, oscillations in dorsal root ganglion	Sodium channels	Selective sodium-channel blocker	Lidocaine, carbamazepine, oxcarbazepine, lamotrigine, TCA
Spontaneous pain (ongoing)	**Peripheral nociceptor sensitization**			
	Inflammation within nerves Cytokine release	Cytokines	Cytokine antagonists Cyclooxygenase blocker	TNF-α antagonists NSAIDS?
Heat allodynia	*Reduced activation threshold to:* Heat	TRPV1 receptor	TRPV1-receptor antagonists	Capsaicin cream
Cold allodynia	Cold	TRPM8 receptor	TRPM8-receptor antagonists	Menthol?
Static mechanical allodynia	Mechanical stimuli	ASIC receptor?	ASIC-receptor antagonists	?
SMP	Noradrenaline	α receptor	α-receptor antagonists	Phentolamine, sympathetic block, TCA
	Histamine	Histamine H1 receptor	H1-receptor antagonists	TCA
	Central dorsal horn hyperexcitability			
	Central sensitization on spinal level Ongoing C-Input induces increased synaptic transmission	Presynaptic: μ-receptors Calcium channels (α_2-δ)	μ-receptor agonists Calcium-channel blocker, α2-δ ligands	Opioids Gabapentin, pregabalin
	Amplification of C-fiber input			
	Gating of Aβ-fiber input (mechanical dynamic hyperalgesia)	Postsynaptic: NMDA receptors NK1 receptors Sodium channels Intracellular cascades	NMDA-receptor antagonists NK1-receptor antagonists Selective sodium-channel blocker MAPK mediators	Ketamine, dextromethorphan? Carbamazepine?
Dynamic mechanical allodynia	Gating of Aδ-fiber input (mechanical punctate hyperalgesia)			
	Intraspinal inhibitory interneurons ↓ (functional, degeneration)			
Punctate mechanical hyperalgesia	GABA-ergic	$GABA_B$ receptors	$GABA_B$ agonists	Baclofen
	Opioidergic	μ-receptors	μ-receptor agonists	Opioids
	Changes in supraspinal descending modulation			
	Inhibitory control (noradrenaline, 5-HT) ↓	α2 receptors 5-HT receptors	α2-receptor agonists NA/5-HT-reuptake-blocker	Clonidine TCA, venlafaxine, duloxetine
	Faciliatory control ↑	?	?	?

(Baron, 2005)

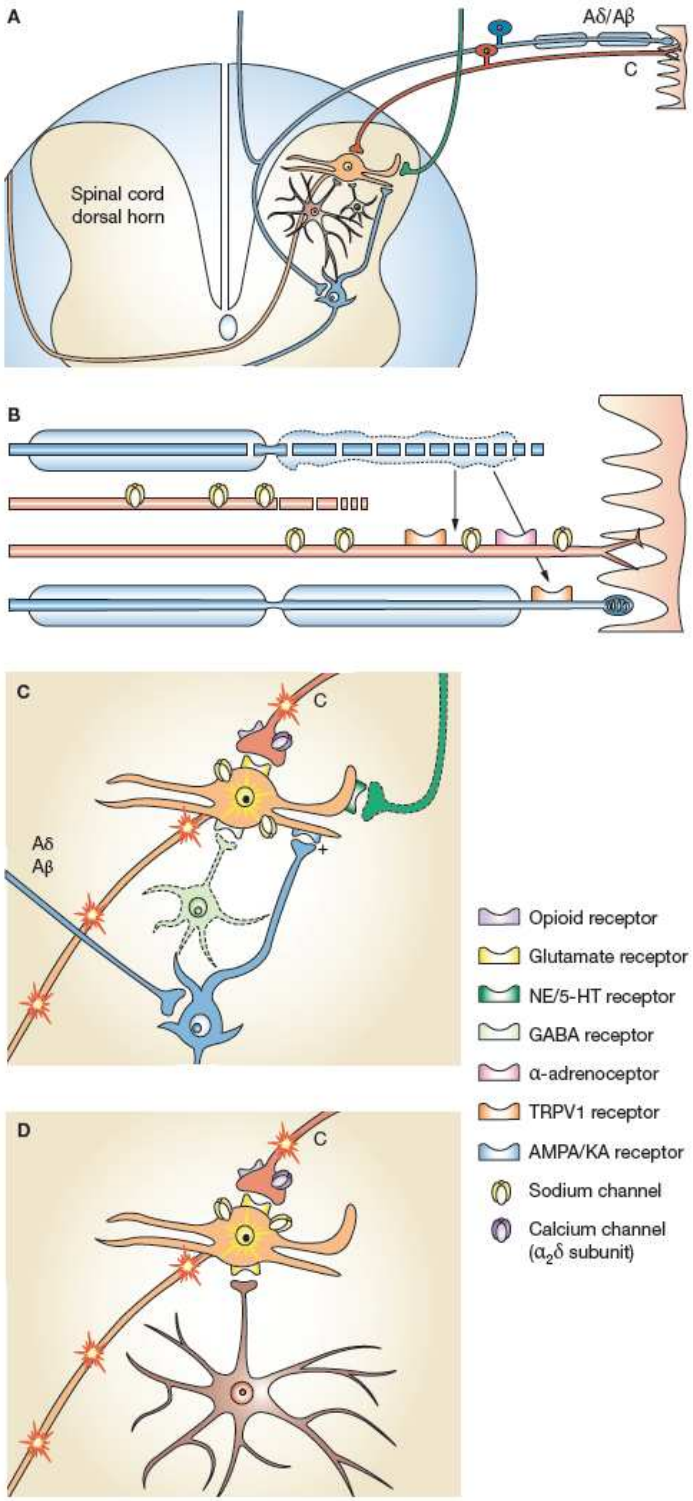


Figure 3 Mechanisms of peripheral and central sensitization in neuropathic pain. . (Baron, 2005)

2.4 Treatment of neuropathic pain

The first step to the successful management of a patient with neuropathic pain is to establish a diagnosis and identify the cause that lies beneath neuropathy. The next step is trying to face this cause-in some cases e.g. drug or toxin-related neuropathy this is enough to relief the patient. When this is not possible, pharmacological treatment comes into play. For most of the patients, first line therapy includes antidepressants (tricyclic antidepressants-TCAs or dual reuptake inhibitors of serotonin and norepinephrine) or calcium channel α2δ ligands, combined with localized therapy, in cases of localized pain.
Among TCAs, nortriptyline and desipramine are more commonly used due to the fact that they have fewer side effects, although amitriptyline appears to be equally effective. In cases of neuropathic pain, TCAs are prescribed in lower doses than in cases of depression (30).
Serotonin-norepinephrine reuptake inhibitors (SNRIs) are also considered a first line option in the treatment of neuropathic pain, with venlafaxine and duloxetine being the most effective (30).
The mechanism of action of TCAs and SNRIs will be discussed in more details later.
Regarding α2δ ligands, gabapentin and pregabalin are used in common practice. Gabapentin, originally developed for the treatment of epilepsy, is considered to be an analogue of GABA. Gabapentin mimics the structure of GABA, but it is not widely believed that it acts on the same receptors. It is yet unknown the mechanism for its analgesic actions, but there are clues that it interacts with voltage-gated Na^+ channels. Gabapentin binds to the α2δ subunit and has been found to reduce calcium currents after chronic application via an effect on trafficking of voltage-dependent calcium channels in the central nervous system (31). Gabapentin binds to the $\alpha_2\delta$ (alpha2delta) subunit of the voltage-dependent calcium channel in the central nervous system. It has also been reported that gabapentin halts the formation of new synapses (32). Pregabalin, the successor of gabapentin, decreases the release of neurotransmitters including glutamate, noradrenaline, substance P, calcitonin gene-related peptide and GABA (33). As less than 50% of the patients respond to monotherapy, it is very often to combine the above drugs with opioids. Opioids alone remain second line therapy (30).

2.5 Models of neuropathic pain

Since neuropathic pain due to an injury in the peripheral nervous system, a nerve compression or diabetes mellitus are very common in humans; most of the murine models involve peripheral injury mainly targeting the sciatic nerve.

- ✓ *Chronic constriction injury*

Three or four ligations are made around the main branch of the sciatic nerve. Some groups implant a polythelene cuff around the main branch of the sciatic nerve .

- ✓ *Partial sciatic nerve ligation*

Tight ligation of the sciatic nerve .

- ✓ *Spinal nerve ligation*

Tight ligation of L5 and L6 spinal nerves

- ✓ *Common personal nerve ligation*

- ✓ *Spared nerve injury*

The common peroneal and the sural nerve are ligated and transected, whereas the tibial nerve remains intact (34).

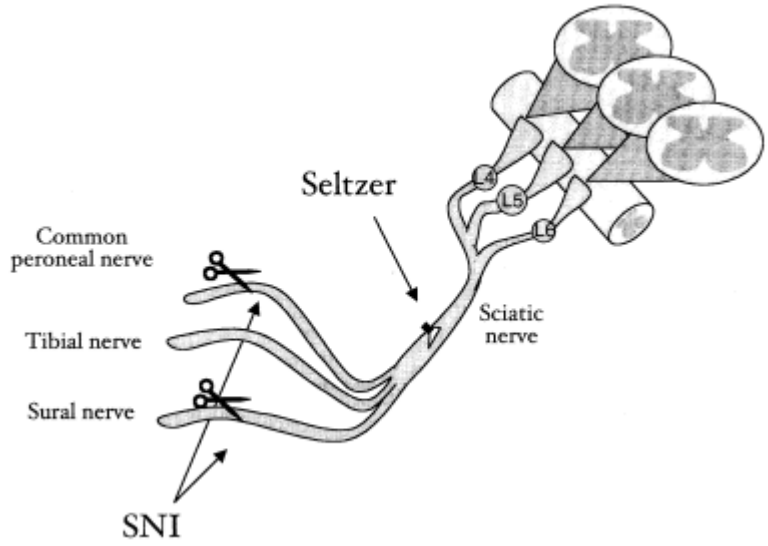


Figure 4.The spared nerve injury model (Shields et al . 2000)

CHAPTER 3

INFLAMMATORY PAIN

3.1 Inflammation

Inflammation is the body's reaction to an injury such as invasion by a microorganism or mechanical or chemical damage and is characterized by five cardinal signs: heat (calor), pain (dolor), redness (rubor), swelling (tumour) and (in extreme cases) loss of function. The response may be initiated by the release of chemicals from damaged tissue cells either as a direct response to trauma or as a result of factors released from microorganisms such as toxins. However, it is difficult to define precisely what triggers the inflammatory response since it involves a large number of different cells and mediators. All the events occurring during inflammation are geared towards increasing the local blood flow (caused by dilation of the blood vessels) and the permeability of the vasculature (blood vessels). This allows cells and serum components increased access to the area of tissue damage in order to limit the spread of infection and tissue damage and to promote healing. The inflammatory process involves the concerted action of the immune, kinin, fibrinolytic and clotting systems which interact to maintain the integrity of the vascular system and to limit the spread of infection/damage (34).
Inflammation is characterized by:
(1) Vasodilation of the local blood vessels, with consequent excess local blood flow;
(2) increased permeability of the capillaries, allowing leakage of large quantities of fluid into the interstitial spaces;
(3) often clotting of the fluid in the interstitial spaces because of excessive amounts of fibrinogen and other proteins leaking from the capillaries;
(4) migration of large numbers of granulocytes and monocytes into the tissue;
(5) swelling of the tissue cells.
Some of the many tissue products that cause these reactions are *histamine, bradykinin, serotonin, prostaglandins,* several different *reaction products of the complement system, reaction products of the blood clotting system,* and multiple substances called *lymphokines* that are released by sensitized T cells. Several of these substances strongly activate the macrophage system, and within a few hours, the macrophages begin to devour the destroyed tissues. But at times, the macrophages also further injure the still-living tissue cells (35).

3.2 Inflammatory pain

Inflammatory pain is the type of pain that is related to inflammation and accompanies most of the chronic inflammatory circumstances, such as osteoarhtirits, rheumatoid arthritis, etc. Inflammatory pain follows cell and tissue damage and the consequent release of chemical mediators, known as the *"inflammatory soup"*. This "soup", consists of a variety of molecules such as cytokines, growth factors, kinins and prostaglandins. All these molecules, apart from promoting an inflammatory response, activate the nociceptors and lead to sensitization of the somatosensory nervous system (36). The two main clinical manifestations of inflammatory pain are allodynia and hyperalgesia.
Figure 5 shows schematically the main interactions between the "inflammatory soup" and the nervous system.

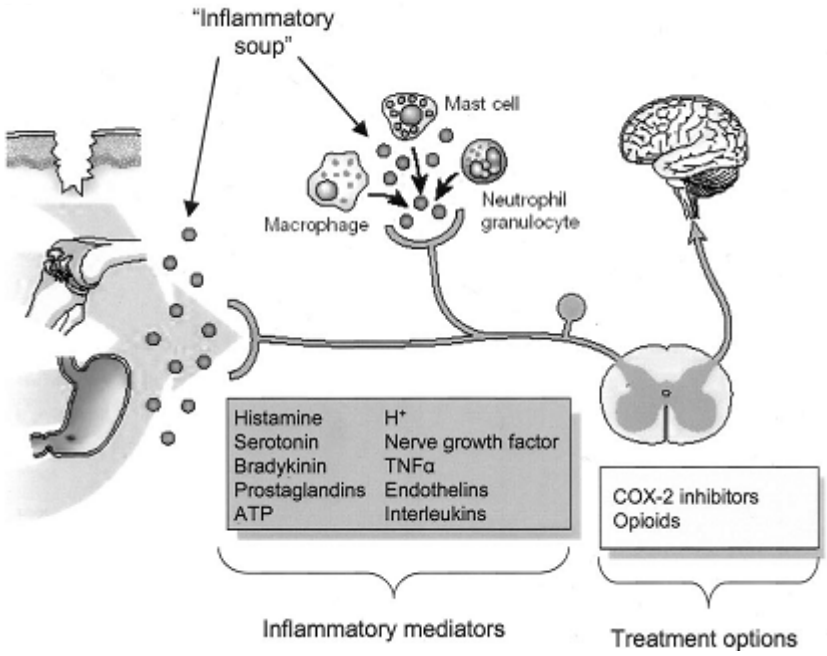


Figure 5.Interactions between inflammatory mediators and nervous system in the context of inflammatory pain.(McKey 2004)

3.3 Cellular and molecular perspectives of inflammatory pain

Peripheral sensitization

As mentioned above, tissue injury or cell damage is accompanied by the release of a variety of chemical mediators. These mediators can provoke pain in two ways: directly, via activating the nociceptors, or indirectly, by promoting the production and release of other molecules that can induce pain (37)

Two major kinins , kallidin and bradykinin are formed by high and low molecular weight kininogen precursors, following activation of tissue and plasma kallikreins by pathophysiological stimuli such as tissue trauma, inflammation, anoxia and low pH. Newly formed bradykinins act acutely on a variety of tissues, mainly through B2 receptors. Bradykinin and kallidin, along with their degradation products can provoke pain either by direct excitation and sensitization of the nociceptors, or by further stimulating the activation of the immune system (38).

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... (39). among the 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 (40) while mice deficient for IL-6 appear to have decreased mechanical and thermal hyperalgesia after inflammatory stimuli (41).

Prostaglandins, although they can activate nociceptors directly, most commonly enhance sensitization, by lowering the activation threshold of the TTX-R sodium channels, via a pathway mediated by PKA (42), as well as by sensitizing primary afferent neurons to bradykinin (43).

Nerve growth factor (NGF), is increased by many inflammatory mediators, especially TNF-α and IL-1β. In animal models of inflammation increased levels of NGF have been observed, further supporting its role in the generation of inflammatory pain. Animals that received antibodies against NGF showed reduced hyperalgesia, fact that is consistent with the involvement of NGF in inflammatory pain. (44). Neurogenic factors, such as substance P, have also been involved in inflammatory pain. Substance P is regarded to have pro-inflammatory actions. Beside this, it induces degranulation of mast cells in order to release histamine, enhances the release of cytokines from the macrophages and also has chemotactic properties for T cells, monocytes, neutrophils and eosinophils (45).

One of the major targets of the "inflammatory soup" is TRPV1, which plays central role in the production of hyperalgesia after inflammatory stimuli (22, 23). Some agents of the inflammatory soup (e.g. lipids and extracellular protons) function as direct positive allosteric modulators, whereas others (e.g. NGF, ATP, bradykinin), bind to their own receptors and modulate TRPV1 via downstream signaling pathways. In both cases, the activation threshold of the channel becomes lower and the magnitude of the responses at suprathreshold stimuli becomes higher; this is the biophysical equivalent of allodynia and hyperalgesia respectively (10, 46). However, what triggers the modulation of TRPV1 is not completely understood. It is known that many TRP channels in mammals are activated or positively modulated by PLC-mediated cleavage of plasma PIP. Consequently, these reduced membrane PIP2 levels lead to increased levels of diacylglycerol and increased cytoplasmic calcium, as well as activation of protein kinases such as PKA and PKC (47).

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 (48). Furthermore, prostaglandin PGE_2, which is released in the spinal cord after tissue injury, binds to the EP_2 receptors of the excitatory interneurons in the superficial dorsal horn leading to the activation of the cAMP-PKA pathway. This results to the phosphorylation of the GlyRα3 subunit of the glycine receptors making the neurons unable to respond to the inhibitory effects of glycine (49)

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 (50). 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 (51). 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 (52, 53). It is not known which are the factors that lead to the TLR activation, but there are several candidates, such as

mRNAs or heat shock proteins from the damaged neurons. As far as astrocytes is concerned, it is possible that due to their long lasting activation (lasts up to several weeks), their role is mostly to maintain than to induce central sensitization (54).
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 (55, 56).
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, fact that finally, generates hyperalgesia. Consequently , metabolotropic glutamate receptors along with substance P receptors are activated , fact that leads to further increase in cytocolic calcium levels (57), but due to their ubiquitous expression NMDA receptor antagonists cannot be widely used as analgesics (37 , 58).
A large number of sodium channels are expressed in the somatosensory neurons. Among them, the TTX-S Nav1.7 is highly upregulated in a variety of inflammatory pain models. Analysis of mice deficient for Nav1.7 in C fibers further support its crucial role in the genesis of mechanical and thermal hypersensitivity in cases of inflammation (59).
Furthermore, mice lacking the TTX-R Nav1.8 display attenuated mechanical and thermal hypersensitivity in inflammatory pain models (60).
Voltage-gated calcium channels seem to take part in inflammatory pain as well. Animals deficient for Cav2.2 or Cav3.2 appear to have reduced sensitization to mechanical or thermal stimuli in the context of inflammation (61).
Figure 6 summarizes the main pathways involved in inflammatory pain.

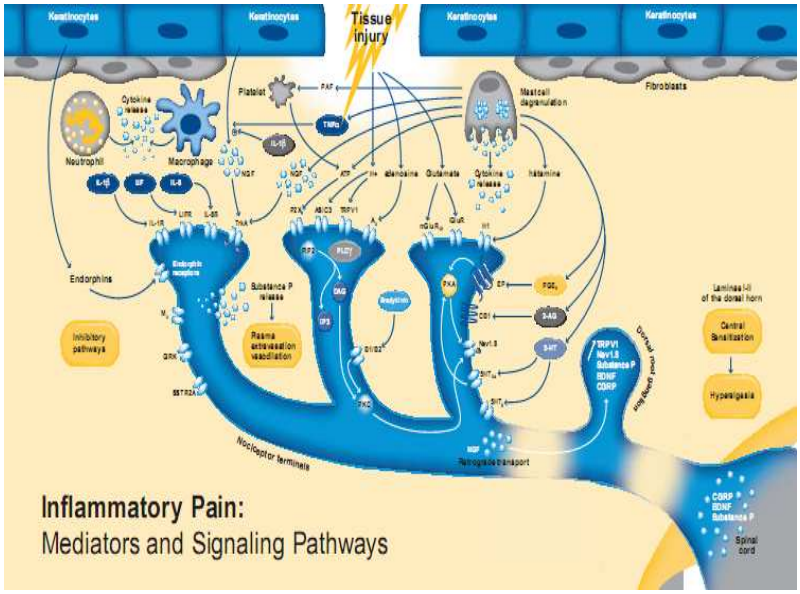


Figure 6 .Pathways in inflammatory pain

3.4 Treatment of inflammatory pain

The treatment of inflammatory 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) , which act via inhibiting cycloxygenases 1 and 2 (COX-1 and 2) and thus reducing prostaglandins and opioids (mostly morphine).
When pharmacotherapy for inflammatory pain is required, acetaminophen is typically recommended as a first-line therapy. However, acetaminophen is less effective than nonsteroidal anti-inflammatory drugs (NSAIDs) and has the potential for hepatic toxicity at doses of >4 g per day (62, 63).
An alternative first line agent is an oral NSAID. According to WHO guidelines, opioid medications should be used on a chronic basis only in patients who are assessed to be at low risk for substance abuse, and who have persistent pain despite trials of nonopioid analgesics. It should be recognized that the evidence for the effectiveness of long-term opioid therapy in terms of pain relief and improved functional outcomes is limited, and that the risk of opioid overdose increases with increasing dosing (64).

3.5 Models of inflammatory pain

- ✓ *Carrageenan*

Carrageenans are linear sulfated polysaccharides that are extracted from red seaweeds. Intraplantar injection can cause inflammatory pain of the joints and the soft tissue, as soon as 3 hours post injection.

- ✓ *Capsaicin injection*

Capsaicin is a vanilloid compound that is the hot ingredient of chili peppers. Subcutaneous injection of capsaicin can cause skin infection as soon as 60 minutes after injection.

- ✓ *Complete Freund's adjuvant*

Complete Freund's adjuvant (CFA) contains paraffin oil with mannide mono-oleate as a surfactant along with heat-killed mycobacteria (Mycobacterium tuberculosis or others). Intraplantar injections of the CFA can induce inflammatory pain 24 hours after the injection. CFA is regarded to cause inflammatory pain via the following mechanisms, as shown in figure 7:

- ✓ Enhancement of antigen uptake by the antigen presenting cells(APCs)
- ✓ Emission of danger signals resulting in Th1 skewing
- ✓ Cytokine induction (IL-2 , IFN-γ , TNF-α , IL-12 ,IL-6)
- ✓ Chemokine induction
- ✓ Granuloma formation
- ✓ Haemopoietic dysfunction (65)

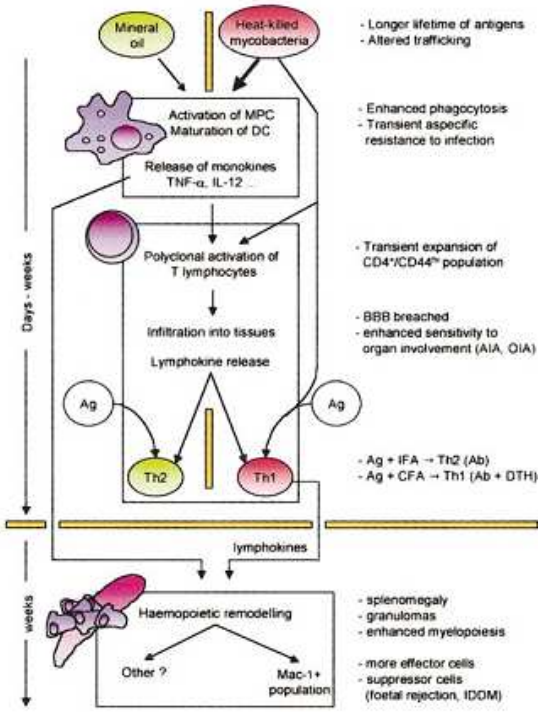


Figure 7.Synopsis of mechanisms of action of CFA (Biliau et al, 2001)

CHAPTER 4

DEPRESSION

4.1 Definition of depression

The Diagnostic and Statistical manual of Mental disorders (DSM-IV) defines *major depressive disorder(MDD)* as "a *syndrome or episode which manifests with five or more of the following symptoms, present most of the day nearly every day for a minimum of two consecutive weeks*". At least one symptom should be either depressed mood or loss of interest or pleasure:

- ✓ Depressed mood
- ✓ Loss of interest or pleasure in most or all activities
- ✓ Insomnia or hypersomnia
- ✓ Change in appetite or weight
- ✓ Psychomotor retardation or agitation
- ✓ Low energy
- ✓ Poor concentration
- ✓ Thoughts of worthlessness or guilt
- ✓ Recurrent thoughts about death or suicide

On a concept-based categorization, these symptoms could be grouped as disturbances in emotions or ideation or neurovegetative or somatic symptoms.

Several subtypes of major depression have been recognized:

- ✓ Major depression with atypical features : the combination of reverse signs with leaden paralysis or interpersonal rejection sensitivity
- ✓ Seasonal affective disorder : Recurrent MDD episodes in a seasonal pattern
- ✓ Major depression with melancholic features : Severe depression marked by profoundly depressed and non-reactive mood together with severe neurovegetative symptoms
- ✓ Major depression with psychotic features : Major depression along with psychotic symptoms

Recently, there is a debate on whether mixed anxiety and depressive disorder should be recognized as a special subtype of depression. Fava et al , found that among the cases that were studied during Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study , 53% were anxious-depressed and remission was harder to take place and took longer to be established to this specific category of patients (66,67) .

4.2 Epidemiology

Major depressive disorder is recognized as one the most common causes of morbidity in the whole world. Lifetime prevalence varies from 3% (Japan) to 17 %(USA). In most of the countries, people who would have at least one episode of MDD during their lifetime fall into the range of 8-12%.
Regarding the age of onset , studies show that depression could appear at any age , however , there are two small age peaks , one between the ages of 30 and 40 and the second between the ages of 50 and 60 (68) .

4.3 Co morbidity

Major depression can exist alone, but most of the times it co-exists with other psychiatric or medical conditions.
Psychiatric co morbidity includes:

- ✓ Anxiety disorders
- ✓ Cognitive disorders
- ✓ Eating disorders
- ✓ Somatoform disorders
- ✓ Personality disorders
- ✓ Sleep disorders
- ✓ Substance use disorders

Medical co morbidity includes:

- ✓ Diseases of the central nervous system
- ✓ Cardiovascular disorders
- ✓ Cancer
- ✓ Chronic inflammatory diseases
- ✓ Autoimmune diseases

The relationship of depression with other diseases is reciprocal: depression worsens the prognosis of other diseases and vice versa (69, 70).

4.4 Pathophysiology of depression

The monoamine hypothesis

Since the most widely used antidepressants act via increasing the levels of serotonin and serotonin in turn increases the levels of norepinephrine and dopamine, scientists were led to the hypothesis that the deficit of these neurotransmitters is responsible for the corresponding features of depression. (71) . However, there are several arguments against this monoamine hypothesis. First of all, agents that cause depletion of monoamines, although they lower the mood of depressed patients without treatment, they do not seem to affect the mood of healthy controls. Furthermore, the effectiveness of mood-stabilizing-drugs that enhance monoamine levels takes several weeks to appear despite the fact that the increase in the monoamine levels needs only hours to occur (72). Moreover, the monoamine hypothesis is not supported either by PET studies or by genetic studies involving polymorphisms in monoaminergic genes (73-75).
All these, led to the conclusion that the monoamine hypothesis is oversimplified and that although monoamines are very important for the pathogenesis and the treatment of major depression, they do not act solely.

The neural circuitry of depression-Neuroimaging studies

Regarding the brain regions which are involved in the pathogenesis of depression, many have been proposed, but their interplay it is not completely understood. The most prominent areas as proposed by postmortem and imaging studies are the raphe nuclei , the suprachiasmatic nucleus (SCN) , the ventral tegmental area (VTA) , Nucleus Accumbens (NAc) , anterior cingulated cortex (ACC) , subgenual cingulated

cortex , hippocampus , prefrontal cortex (PFC) , amygdala , thalamus and stiratum (76) .
Postmortem studies in the hippocampus and the prefrontal cortex of depressed patients have revealed significant atrophy , as documented by the decreased size of the neurons , the reduced number of the glial cells and the reductions in the density of dendrites and trophic factors as well (77,78) . The above findings are in agreement with MRI results showing volume loss of these specific brain regions in depressed patients (79, 80).
Neuroimaging studies , suggest an abnormally increased activity of the amygdala , the ventral striatum and the prefrontal cortex in depressed patients in response to negative stimuli as well as reduced activity of the same regions in response to positive stimuli , suggesting the "preference" of depressed patients to focus on the negative stimuli and ignore the positive ones (81,82) .
Other functional MRI studies indicate an increased metabolic rate in the subgenual cingulated cortex and amygdala, as well as decreased metabolic rate in the dorsal prefrontal cortex and the striatum, in patients suffering from major depressive disorder (79, 80).
Moreover, deep brain stimulation of NAc and subgenual cortex in patients with treatment-resistant depression appeared to have some antidepressant action. Furthermore, peripheral hormones such as leptin and ghrelin along with cortisol seem to play an important role in mood control, via hypothalamus (83).
Figure 8 shows a very simplified summary of the neural circuitry involved in depression.

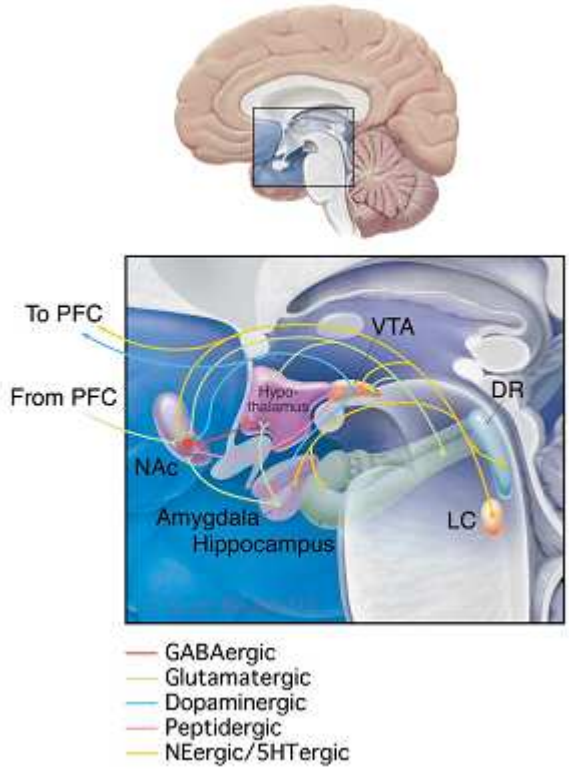


Figure 8.The neural circuitry of depression (Krishnan, 2008/Nestler, 2010)

The role of neurogenesis

During the last years, there is some evidence supporting the idea that the creation and use of new neurons in the hippocampus, could lead to antidepressant treatment. More specifically, it has been reported that known risk factors for depression such as glucocorticoids or drugs of abuse decrease the hippocampal neurogenesis, whereas factors known to confront depression, such as antidepressants, exercise and environmental enrichment seem to positively regulate this process (84).

The role of BDNF

There is a strong link between depression and BDNF(Brain Derived Neurotrophic Factor). First of all, serum levels of BDNF are decreased in patients with MDD and antidepressant therapy is able to reverse this phenomenon (85-86). Furthermore, dysfunction of BDNF signaling in hippocampus can produce depression-like phenotype and is involved in the action of antidepressants (87, 88). On the other hand, increased BDNF levels in hippocampus seem to have some antidepressant action (89). Additionally, data from postmortem brain analysis suggest decreased BDNF levels in hippocampus along with increased levels (of the same magnitude) in the Nac (90).

The hypothalamus-pituitary-adrenal axis

There are several theories regarding the implication of the hypothalamic-pituitary-adrenal (HPA) axis in the pathophysiology of major depressive disorder. At first, almost ¼ of depressed patients have increased serum levels of cortisol while cortisol levels are found to be increased in the CSF of many depressed patients as well. It has been reported that neurons which express coritocotropin-releasing hormone (CRH) are increased in patients with MDD. Animal studies have shown that CRH administration led to the development of anxiety and depression-like phenotypes. Furthermore, postmortem studies indicate decreased number of glucocoricoid receptor (GR) in depressed patients' brains (91). Figure 9 illustrates the CRH system in depression.

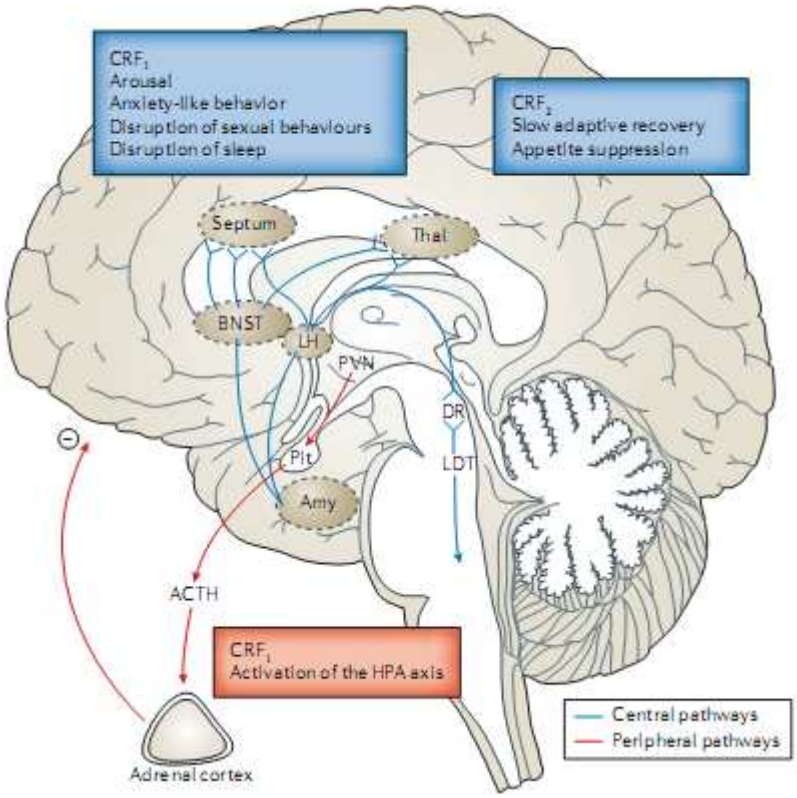


Figure 9.The CRH system in depression (Berton, Nestler, 2006)

Cytokines and depression

There are many studies regarding the role of the immune system in the generation of depression. Elevated levels of proinflammatory cytokines have been detected in the plasma and CSF of depressed patients. Acute and chronic stress can elevate IL-1β and IL-6 with consequent release of CRH and ACTH as well as decrease in GR expression (92), thus mediating hypercortisolemia and decreased sensitivity of the HPA axis, which is commonly observed in MDD.
Furthermore, patients with MDD have significantly higher levels of TNF-α, IL-1 and CRP in their blood (interestingly, CRP levels remain high even though the patients were in clinical remission) (93). Antidepressant treatment (especially TCAs and SSRIs) was able to normalize the cytokine levels (94).

Genetics and depression

Major depressive disorder is a very complicated disease and for this reason, the identification of single genes responsible for it has been proved extremely difficult. However, there are many studies with some very interesting data. Depression has been linked to polymorphisms in the glucocorticoid receptor gene NR3C1, the monoamine oxidase A gene, the gene coding for the glycogen synthase kinase-3β as well as a group-2 metabolotropic glutamate receptor gene (GRM3) (95).

Another study has shown that individuals carrying the ε2 allele of apolipoprotein E had reduced risk of MDD with respect to those carrying the ε3 allele (96).The short allele of SLC6A4 (serotonin transporter) is related to increased risk of MDD compared to the long allele and the same stands for the minor allele of MTHFR (methylene tetrahydrafolate reductase) (97) .
Additionally, several genes are related to antidepressant responsiveness. For example, the long allele of SLC6A4 is associated with better responsiveness to SSRIs, as well as several single nucleotide polymorphisms (SNPs) in serotonin type-2a receptor. Furthermore, the Met allele of the Val/Met polymorphism in BDNF is related to better responsiveness to SSRIs (98).

Glutamate

During the last years, several studies indicate a role of glutamatergic system in depression. Glutamate levels in the plasma, CSF as well as brain tissue of depressed patients have been reported to be elevated compared to healthy controls. Using magnetic resonance spectroscopy, the levels of glutamate and its metabolites were found decreased in the frontal cortex and cingulated regions of patients with MDD and increased in the occipital and parietal regions. Furthermore, agents that act via the glutamatergic system (such as NMDA receptor antagonists), appear to have strong antidepressant actions (98).

4.5 Management of depression

The three more widely used ways to manage depression are psychotherapy, psychiatric medication and eletroconvulsive therapy. In terms of psychotherapy, different forms can be useful, such as cognitive behavioural therapy, mindfulness-based cognitive therapy, interpersonal psychotherapy and psychoanalysis. Regarding medication, there is a variety of antidepressant drugs, with different mechanisms of actions:

- ✓ Selective serotonin reuptake inhibitors
- ✓ Serotonin-norepinephrine reuptake inhibitors
- ✓ Noradrenergic and specific serotonergic antidepressants
- ✓ Norepinephrine (noradrenaline) reuptake inhibitors
- ✓ Norepinephrine-dopamine reuptake inhibitors
- ✓ Selective serotonin reuptake enhancers
- ✓ Norepinephrine-dopamine disinhibitors
- ✓ Tricyclic antidepressants
- ✓ Monoamine oxidase inhibitor

Often monotherapy is not efficient and it is necessary to combine two or more antidepressant drugs. When depression is resistant to pharmacotherapy, there are other ways to be faced, such as electroconvulsive therapy, deep brain stimulation, repetitive transcranial magnetic stimulation, vagus nerve stimulation and cranial electrotherapy stimulation (34).
Table 6 shows the currently available therapeutic interventions for depression.

4.6 Animal models of depression

- ✓ *Forced swim test*

In the forced swim procedure, mice are forced to swim in un-escapable situation. The more time the animal spends immobile the more despair is regarded to be The forced swim test is very useful when validating drugs with antidepressant action. It is also useful for assessing the impact of environmental and genetic manipulations on behaviors related to depression.

- ✓ *Chronic stress*

  Animals that are put into stress have been reported to exhibit anhedonia, which is reversed under antidepressant treatment.

  -Chronic mild stress

  Mice experience a variety of stressful circumstances, such as single housing, disruption of the circadian rhythm, food or water deprivation, etc for several days

  -Social defeat

  An animal is expresses to another animal, with aggressive features. Physical contact can be allowed or not.

- ✓ *Early life stress*

  Animals can be under prenatal stress, early postnatal handling and maternal separation. It has been proposed that early life stress can lead to hyperactive HPA axis.

- ✓ *Novelty suppressed feeding test*

  Mice are food deprived for 1 or 2 days and then they are placed in the open field arena. In the centre of the arena a small amount of unfamiliar food is placed. The experimenter counts the latency to approach the centre, the latency to eat and the total time the mouse spends eating

- ✓ *Sucrose preference test*

  A test that evaluates anhedonia. Single housed mice are allowed to choose between the tab water they drink regularly and sucrose. Normal mice prefer to drink sucrose, whereas depressed mice have no difference in drinking. (99)

CHAPTER 5

ANTIDEPRESSANTS

5.1 General aspects

Antidepressants are the most commonly prescribed drugs not only by psychiatrists, but by general practicioners as well. A variety of chemical structures have been found to have antidepressant actions. Among them, monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), tetracyclic antidepressants (TeCAs), selective serotonin reuptake inhibitors (SSRIs), and serotonin-norepinephrine reuptake inhibitors (SNRIs) are the most commonly used .

Antidepressants can be administered from several months up to years and their efficacy becomes apparent 2 to 6 weeks after the initiation of treatment. Apart from depression, antidepressants are prescribed for a variety of diseases such as anxiety disorders, eating disorders, chronic pain etc. Other drugs -such as opioids and amphetamines- have been found to have antidepressant actions, but due to restrictions on their use, they are not prescribed on a regular basis.

Typical antidepressant includes several categories, such as:

- ✓ Selective serotonin reuptake inhibitors
- ✓ Serotonin-norepinephrine reuptake inhibitors
- ✓ Noradrenergic and specific serotonergic antidepressants
- ✓ Norepinephrine (noradrenaline) reuptake inhibitors
- ✓ Norepinephrine-dopamine reuptake inhibitors
- ✓ Selective serotonin reuptake enhancers
- ✓ Norepinephrine-dopamine disinhibitors
- ✓ Tricyclic antidepressants

5.2 Tricyclic antidepressants

Chemically, the *tricyclic antidepressants (TCAs)* and their demethyl derivatives are similar to phenotheiazines, with a three-ring chemical structure. Representative agents of this category are imipramine and its monodemethyl derivative desipramine, amitriptyline and its derivative nortriptyline, doxepin and protriptyline. TCAs potentiate the action of biogenic amines (serotonin and norepinephrine) presumably by blocking the inactivating reuptake of the amines after release from the presynaptic neuron. It is thought that the desmethylated derivatives (desipramine and

nortriptyline) are more selective in blocking the reuptake of norepinephrine. Due to their lipophilic nature, they become widely distributed and have relatively long half-lives. They are metabolized in the microsomal metabolizing system. T Regarding their side effects, they resemble those of phenothiazines. Anticholinergic effects can be prominent and occur both peripherally and centrally. Other common effects are sweating, dizziness and muscle tremor. However, the most serious adverse effect is that of cardiac arrhythmias, which can be fatal (99).

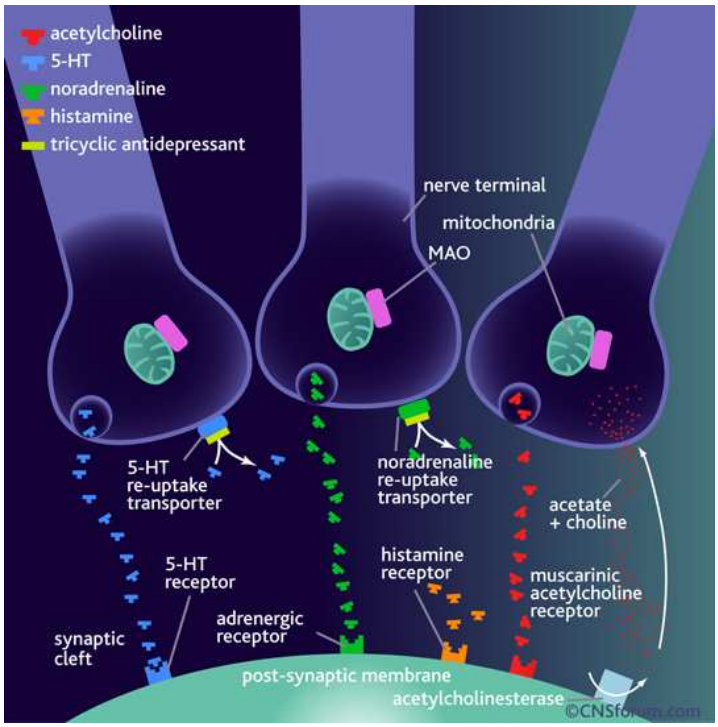


Figure 10 Mechanism of action of tricyclic antidepressants .

5.3 Norepinephrine reuptake inhibitors

Norepinephrine reuptake inhibitors (NRIs) are antidepressant drugs which act via blocking selectively the reuptake of epinephrine and norepinephrine, by inhibiting norepinephrine transporter (NET). Apart from depression, NRIs are used for a variety of other pathological conditions, such as attention deficit hyperactivity disorder, narcolepsy, fatigue and lethargy (as stimulants), obesity (as anorectics), as appetite suppressants for weight loss and as vasopressors for hypotension or orthostatic hypotension. Representative drugs of this category are tamoxetine, mazindol, reboxetin and viloxetin. Reboxetin is the first truly selective norepinephrine reuptake inhibitor. It has high affinity for the noradrenaline receptor and lower affinity for other receptors such as serotonin, dopamine, histamine, muscarinergic and α-adrenergic receptors. (100).

5.4 NMDA receptor antagonists

NMDA receptors are ionotropic glutamate receptors playing a key role in synaptic plasticity and memory. Activation of NMDA receptors results in the opening of an ion channel that is non-selective to cations with an equilibrium potential near 0 mV. A unique property of the NMDA receptor is its voltage-dependent activation, a result of ion channel blockade by extracellular Mg^{2+} ions. TAntagonists of the NMDA receptor are used as anesthetics for animals and sometimes humans, and are often used as recreational drugs due to their hallucinogenic properties, When NMDA receptor antagonists are given to rodents in large doses, they can cause a form of brain damage called Olney's Lesions(101).

Common NMDA receptor antagonists include amantadine, ketamine, phencyclidine; nitrous oxide etc In terms of depression, ketamine seems to have rapid antidepressant action. (101).

5.5 δ-receptor agonists

Delta opioid receptors are a subtype of opioid receptors whose endogenous ligand is enkephalin. When activated, they can produce analgesia, but on high doses they can lead to seizures. Delta agonists could be a novel antidepressant drug, based on data showing that they increase BDNF levels (102).

CHAPTER 6

RGS PROTEINS

6.1 G proteins

G proteins comprise a diverse family of proteins which take part in a variety of different cellular functions. G proteins owe their name to their property of binding GTP as well as GDP. G proteins are expressed in many tissues and play crucial role in signal transduction for many cellular functions, such as protein synthesis, neurotransmission, immunity, cardiovascular function etc (1). Hetrotrimer G proteins have 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. Different G proteins contain different Gα subunits, which characterize their function. More than 17 different Gα subunits have been identified, between which the most prominent are:

- Gαs-stimulation of adenylyl cyclase and increase of intracellular cAMP
- Gαi/o-inhibiotion of adenylyl cyclase by inhbiting Gas,activation of cGMP phosphodiesterase and inhibition of calcium channels
- Gαq-activation of PLC and inhibition of GIRK channels
- Gα12/13-activation of PLCε and Rho protein

Regarding the other G protein subunits, 5 Gβ and 7 Gγ subunits have been identified (103).

Under normal conditions the Gα subunit is bound to GDP. When a ligand binds to a G-protein coupled receptor, this GDP is exchanged for GTP and the Gα subunit separates from the combined Gβ and Gγ subunits. The separated Ga subunit brings about many biological effects. The Gβ and Gγ subunits do not separate from each other, and the Gβγ complex also activates a variety of effectors such as neurotransmitters-eg epinephrine,dopamine etc, peptides,hormones and a variety of others shown in table 7. The intrinsic GTPase activity of the Gα subunit then converts GTP to GDP, and this leads to reassociation of the Gα with the Gβγ subunit and termination of effector activation. Heterotrimeric G proteins relay signals from over 1000 receptors, and their effectors in the cells include ion channels and enzymes, as listed in Table 7.

Class	Ligand
Neurotransmitters	Epinephrine Norepinephrine Dopamine 5-Hydroxytryptamine Histamine Acetylcholine Adenosine Opioids
Tachykinins	Substance P Neurokinin A Neuropeptide K
Other peptides	Angiotensin II Arginine vasopressin Oxytocin VIP, GRP, TRH, PTH
Glycoprotein hormones	TSH, FSH, LH, hCG
Arachidonic acid derivatives	Thromboxane A_2
Other	Odorants Tastants Endothelins Platelet-activating factor Cannabinoids Light

Table 7Possible effectors of G proteins

6.2 G-protein coupled receptors (GPCRs)

The heterotrimeric G protein convey extracellular signals intracellularly, through activation of transmembrane proteins, the *G-protein coupled receptors (GPCRs)*, which transduce the signal to downstream signaling molecules resulting in the induction of cellular changes. (104).

There are two principal signal transduction pathways involving the G protein-coupled receptors: the cAMP signal pathway and the phosphatidylinositol signal pathway. When a ligand binds to the GPCR it causes a conformational change in the GPCR, which allows it to act as a guanine nucleotide exchange factor (GEF). The GPCR can then activate an associated G-protein by exchanging its bound GDP for a GTP. 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 ($G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q/11}$,$G_{\alpha 12/13}$) (103).

The GPCRs are important drug targets, although other compounds that bind to receptors may lead to different biological activities. 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. GPCRs also include the family of opioid receptors (mu, delta, kappa), which couple to the Gαq and Gαi/o proteins. Activation of opioid receptors leads to inhibition of the enzyme adenylate cyclase, increased potassium conductance and reduced sodium conductance as well as activation of MAP kinases (104,105)

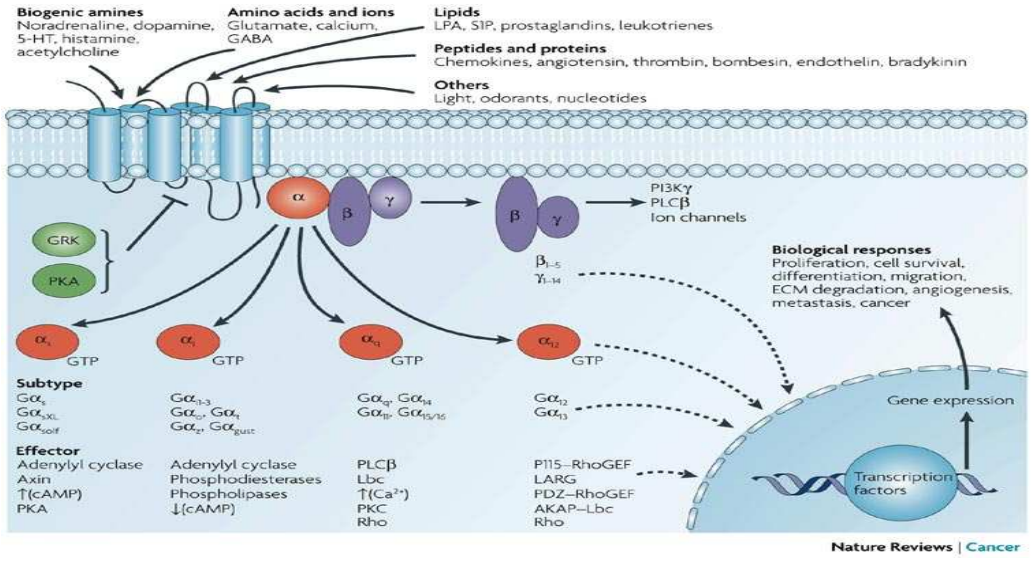


Figure 11.G-protein coupled receptors(Dorsam , 2007)

6.3 Regulators of G protein Signaling (RGS proteins)

Regulators of G protein signalling (RGS) are a structurally and therefore functionally diverse superfamily of proteins with about forty separate gene products in humans, which are divided into eight subfamilies:

- ✓ A or Rz
- ✓ B or R4
- ✓ C or R7
- ✓ D or R12
- ✓ E or RA
- ✓ F or GEF
- ✓ G, or RL (GPCR kinase)
- ✓ H or SNX (Sorting Nexin)

Additionally, D-AKAP2 and RGS22 (PRTD-NY2) are proteins that contain multiple RGS boxes but cannot be classified within the above subfamilies.

The classification is done according to homologies to the RGS domain and in many cases; RGS proteins belonging to the same subfamily also share some common territory (105). These additional areas are usually responsible for cell identification, as well as for interactions with other proteins.

The first RGS protein identified was the Sst2 in saccharomyces, and it was shown to act as a negative regulator of GPCR signalling. (106).

After analysis of the amino-terminal sequence, the presence of a common domain of about 120 amino acids was recognized to several proteins in mammals but their function was unknown. This conserved domain (RGS box) was found to be responsible for the direct binding of RGS proteins to the Gα subunit of G proteins because of its high affinity to the functional areas (107). Therefore, RGS proteins accelerate the hydrolysis of GTP not so much due to their catalytic activity, but by stabilizing the transition state leading to the hydrolysis of Gα subunit (108). Furthermore, RGS proteins do not affect the Gα subunit only through the GAP activity but also by competition for the binding site of other operators of Gα subunit (109).

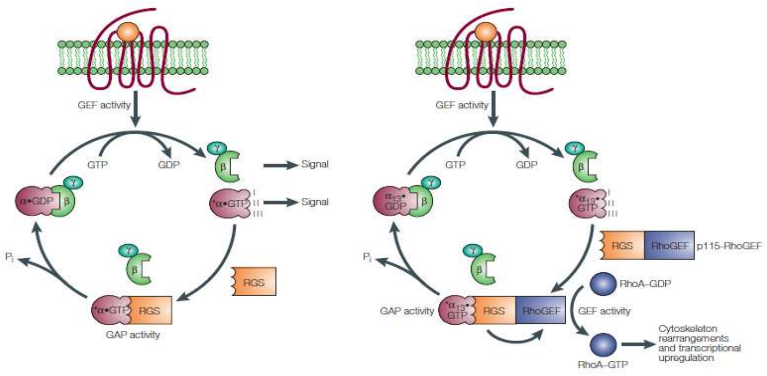


Figure 12.Regulators of G protein signalling

The RGS proteins have a much defined regulation pattern. Some of them are regulated at transcriptional level, such as RGS2 and others post-translation ally, since they are subjected to several modifications such as phosphorylation (Berman and Gilman, 1998), ubiquitination (110), and summoylation (111).

The distribution of RGS proteins in the nervous system is diverse, but usually follows the expression pattern of mRNA. Each tissue expresses a specific combination of RGS proteins and their expression levels are increased or decreased in response to extracellular signals or under certain pathological conditions

6.4 RGS 4

RGS4 protein is a member of the R4 RGS subfamily and has a size of 28 KDa. It consists of a 120 amino acids (RGS) domain that has this GAP activity and through this regulates the signaling of GPCRs, and an N-terminal cysteine-rich sequence which promotes the proteolytic degradation thus regulating the molecule's half-life (112).

5 isoforms of RGS4 have been identified in the human brain: RGS4-1, RGS4-2, RGS4-3, RGS4-4 and RGS4-5, which are expressed at high levels in the cortex. On the other hand, in the murine brain only 3 different isoforms are expressed: RGS4-1, RGS4-2 and RGS4-3 bearing the same sequence of 250 amino acid residues but differing in their 3 UTR (113).

Regarding the distribution of RGS4 in various organ systems, it has been found in high levels in the CNS and the heart. During embryonic development of the CNS, the RGS4 gene is expressed transiently in the locus coeruleus, the main noradrenergic center of the brain, the sympathetic ganglion neurons and cranial sensory and motor neurons. The dynamic expression pattern during embryonic development suggests a role in cell differentiation. Later, RGS4 is expressed in most neural layers of the cortex, and is localized in areas involved in the transmission of painful stimuli, analgesia, addiction cognitive processes, traffic, visual and responses to stress. In the amygdala, RGS4 is the most abundant RGS protein. Moreover, it is strongly expressed in the locus coeruleus, the pyramidal cells of the hippocampus, throughout the cortex and the thalamus. At spinal cord level, RGS4 is expressed at high levels in the superficial layers (I-II) of the posterior horn of the spinal cord which is particularly important for the transmission of impulses of painful and analgesic effects of opioids in the body (114).

Studies have linked low levels of RGS4 in the prefrontal cortex with schizophrenia (115) and there is evidence for its role in the action of some antipsychotic drugs. On the other hand, stress, corticosteroids and drugs seem to alter the levels of RGS4 in some brain regions, such as the superior temporal gyrus, the subventricular nucleus and pituitary gland. Recently, it has been shown that this protein acts as a negative regulator of morphine reward and promotes analgesia by opioids such as methadone and fentanyl (112). RGS4 has also been associated with Parkinson's disease, where

reduced expression levels are associated with decreased cholinergic signaling. Apart from its role in various neurological conditions, it appears to be overexpressed in cases of heart disease. Furthermore, another study (116) suggests the involvement of endogenous RGS4 in analgesic responses. The expression levels of RGS4 mRNA levels are increased in the posterior horn of the spinal cord when hyperalgesia has been fully established in models of neuropathic pain, which causes adaptive changes that lead to the development of hyperalgesia observed in chronic pain, and contribute to the reduced response to morphine, which occurs in cases of neuropathic pain (116).

AIM OF THE STUDY

The aim of this study is to investigate the role of RGS4 in chronic pain (neuropathic and inflammatory) as well as its effect in the response of antidepressants when given to treat both depression and neuropathic pain. For this reason we use RGS4 KO mice and their WT littermates in a series of behavioral assays. To explore the role of RGS4 in neuropathic and inflammatory pain at first we assessed baseline levels of RGS WT and KO mice and then we used the SNI model for neuropathic pain and the CFA model for inflammatory pain in order to evaluate any differences in mechanical allodynia or thermal hyperalgesia between genotypes. Furthermore, we used antidepressant drugs known for their analgesic properties and examined the response of WT and KO mice. Moreover, we examined the effect of RGS4 in the development of anxiety in the context of inflammatory pain. Finally, we tried a variety of antidepressant drugs in several depression tests in order to evaluate any possible differences in the antidepressant responses between WT and KO mice.

MATERIALS AND METHODS

Animals

Experiments were performed on male and female adult mice. Constitutive mutant mice were generated by breedings between heterozygous RGS4 mice (117). For all the assays we used 8-10 week old male or female KO mice and their WT littermates. Polymerase chain reaction (PCR) was used for genotyping, using DNA taken from the tail or the ear of the mice.

In order to create conditional KO mice, we applied adeno-associated viruses expressing Cre recombinase (or GFP as control), in the nucleus accumbens (NAc) of floxed RGS4 mouse as it has been described before. (118). Stereotaxic coordinates for viral vector injections into the NAc were: anteriorposterior +1.6 mm, lateral ± 1,5 mm, and dorsoventral -4.5 mm at an angle of 10^{o} from the midline (relative to Bregma). For all stereotaxic surgery procedures mice were anesthetized with avertine.(118).

Mice were kept on a 12h light/dark cycle, were group-housed (4-5 per cage) with food and water 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.

Depression tests

In order to measure depression, we used forced swim test as well as novelty suppressed feeding and sucrose preference test. For the *forced swim test (FST)* mice were transferred to the experiment room one by one, exactly before the experiment. Mice were placed in individual glass cylinders (46cm height x 18cm diameter) containing water at room temperature (25^{o}C±1) at a depth of 15cm. The assay lasted and was videotaped for 6 minutes, and then was scored manually. Two parameters were estimated, the latency to stop, referring to the first time the mouse stopped swimming for at least 5 seconds and the total immobility time, referring to the total time the mouse spent completely immobile (119). Decreased immobility time is considered to be indicative of antidepressant action.

During the *sucrose preference test*, mice were placed to cages individually, with free access to food and two 50ml falcons placed on the edges of the grade instead of their usual bottle. The first 2 days mice were drinking tap water and the two following 1% sucrose and the total volume they consumed was recorded. The side of the bottles was

changed every day in order to ensure that there was no side bias. For the next 6 days, mice had to choose either water or 1% sucrose (again the side of the bottles was changed every day). It has been referred that the normal reaction is to choose sucrose over water and depressed subjects do not seem to understand the difference (anhedonia) (120).

For the *novelty suppressed feeding test*, the following procedure was taking place: mice were deprived from food for 2 days. The third day, each mouse was transferred to the experiment room exactly before the experiment. Each mouse was placed near the wall of a square open field area (25 x 25cm) surrounded by walls to prevent mice from falling off. In the centre of this area a small amount of a special aliment (different than their usual food) was placed. Mice were videotaped for 5 minutes and two parameters were estimated: the latency to approach food-referring to the first time the animal went to the centre to the food, and the latency to eat, referring to the first time the mouse actually ate some food (121).

Anxiety tests

We used open field test as well as light-dark box test in order to estimate anxiety levels. The *open field test* was performed as follows: Mice were transported in the experiment room 30 min before testing. Each mouse was placed near the wall of an open field arena. The open field arena was an empty square arena (25 x 25cm) surrounded by walls to prevent mice from falling off. The arena was divided in three zones, the center (5 x 5cm) (lane 3/4), the periphery (10 x 10cm) (lane 2) and the borders (20 x 20cm) (lane1). The mice were videotaped for 5 minutes and were then scored manually for the following three parameters: latency to approach the centre, the time the mouse needed to enter the center for the first time, the time mice spent near the borders, both measures of animal anxiety, and the time mice spent in the periphery, indicative of less anxiety-related behavior (122).

Regarding the *light-dark box test*, a permeable black box (25 cm high x12,5cm length) was inserted into the open-field arena and covered 50% of the surface area. The experiment was performed under direct room light. Each mouse was released in the corner of the lit compartment and observed for 5 min. During this time the latency to enter, meaning the time the mouse needed to enter the black box and the time spent inside the black box were measured. Both are related to anxiety-like behaviours (123).

The spared nerve injury (SNI) model for neuropathic pain

Mice were anaesthetized with avertine and the left limb was shaved, placed in a lateral position and immobilized. Using the knee as a landmark an approximately 1 cm incision was made to the longitudinal direction (124)-. The skin was cut, the muscle layers were bluntly dissected and the sciatic nerve was exposed distal to the trifurcation. Muscles and connective tissue were cleaned and the sural, common peroneal and tibial nerves were exposed. Sural and common peroneal nerves were ligated (6.0 silk Ethicon) and transacted, whereas the tibial nerve was left intact. Skin was then closed using a 4.0 suture (34) .

The von Frey test for testing mechanical allodynia

The von Frey test was used to assess mechanical allodynia. Mice were separately placed in plastic square areas 1hr before testing, in order to habituate. Using different filaments (starting from the 0.2g filament and gradually increasing) corresponding to respective forces were applied on the central part of the left paw of the mouse. Each force was applied five times over a period of 30 seconds and the reaction of the mouse was observed. Response in three out of five stimuli was the threshold above which an animal was regarded "positive" to mechanical allodynia, As a response was regarded the sudden paw withdrawal, sudden flinching or sudden paw licking.

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 the mouse. All assays of inflammatory pain took place at least 24h after the injection.

The Hargreaves' test for measuring thermal hyperalgesia

Briefly, the mice were placed on a glass plate over a light box (IITC Life Sciences) , and a radiant heat stimulus (intensity 40%) 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 to the right paw and used as control. There was a total of three measurements for each paw. Five minutes were allowed between each trial, and these measurements were averaged for each limb and compared to the baseline (before injury) value.

Drugs

Desipramine (Sigma Aldrich) was diluted at first in sterile water to a concentration of 50mg/ml and then to its final concentration in saline. For the SNI and NSF experiments desipramine (10mg/kg) was injected intraperitoneally twice a day for 15 days. *Reboxetine* (Sigma Aldrich) was diluted to saline and doses of either 5mg/kg or 7mg/kg were given subcutaneously 30 minutes before FST. *Ketamine* was diluted to saline and a dose of 3mg/kg was given intraperitoneally 3 hours before FST. For the SNI experiment ketamine was administered intraperitoneally (doses of 3mg/kg or 5mg/kg) and von Frey test was performed 30, 60, 90, 120 min and 24h after the injection. *SNC80* (Sigma Aldrich) was at first diluted in 1N HCl to a concentration of 40mg/ml and then in saline until its final concentration of 5mg/kg and was administered subcutaneously 30 minutes before FST.

Statistical analysis

Two way ANOVA was utilized to examine significant effects of treatment over genotype for all pharmacological and behavioural 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

Depression

- ✓ *Evaluation of depression in RGS4 mice*

For the evaluation of depression we used the FST, the sucrose preference and the novelty suppressed feeding test. Previous studies in the lab had shown that naïve RGS4 KO mice have no major depressive phenotype in the FST compared to the WT mice. In the sucrose preference test, on the other hand, we observed that WT mice consumed more sucrose (as expected) while KO mice did not seem to have any significant preference (Fig. 1C). Finally, previous studies in our lab had shown that in the novelty suppressed feeding test, RGS4 KO mice showed an increased latency both to approach and to eat the unfamiliar food. In order to identify if this depressive phenotype is nucleus accumbens (NAc) mediated, we decided to test whether conditional deletion of RGS4 in the NAc could reproduce similar results. As shown in Figure 1A and B , NAc-conditional KO mice indeed appear to reach later the centre and need more time to start eating the new and unfamiliar food, just like the global KO animals. These data suggest global RGS4 KO mice seem to have increased levels of anhedonia and depression, and that deletion of RGS4 in the NAc makes mice more susceptible to depression.

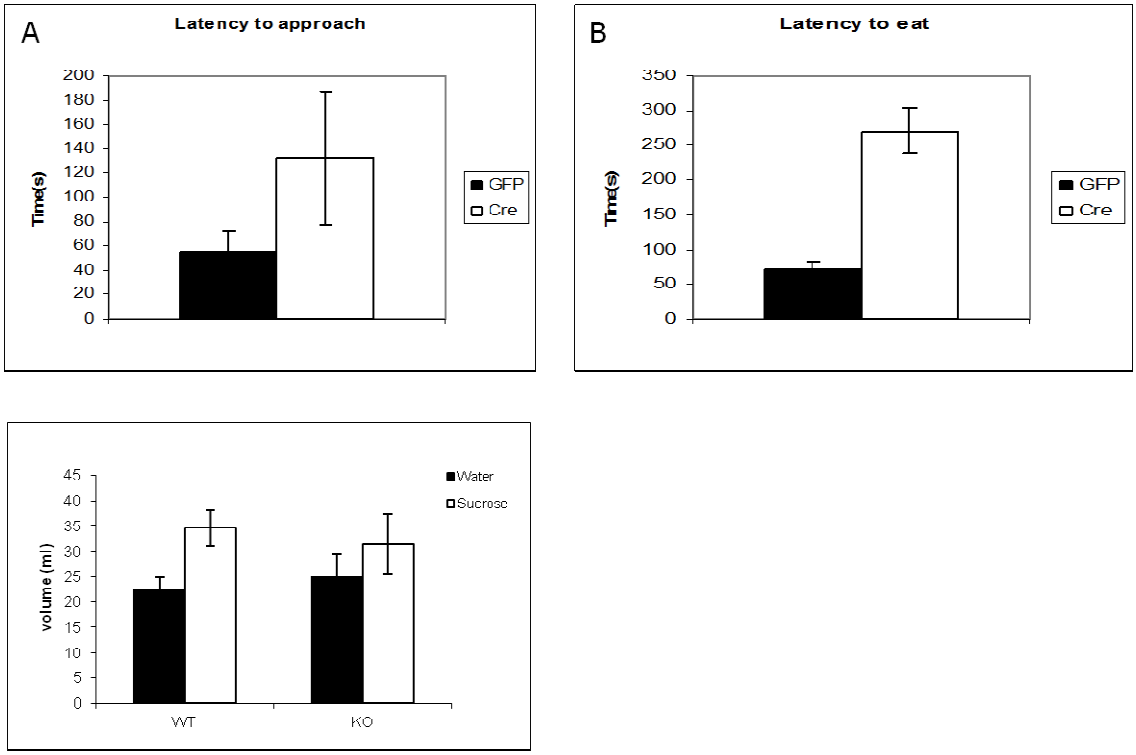


Figure 1.Estimation of depression parameters in RGS4 WT and KO mice. A,B.Conditional NAc RGS4 KO mice have increased levels of latency to approach the food for the first time(A) as well as to try the unfamiliar food for the first time (B), suggesting a more depressed phenotype.(n=6-7/group) C.RGS4 KO mice find no difference between water and sucrose, suggesting increased levels of anhedonia (n=3-4/group).Values represent mean±SE.

✓ *Estimation of the action of antidepressants in RGS4 WT and KO mice*

In order to test the potential role of RGS4 in antidepressants actions, we decided to test a variety of drugs with known antidepressant properties. We chose to test antidepressants from different categories TCAs (desipramine), NRIs (reboxetine), NMDA receptor antagonists (ketamine) and a delta opioid receptor agonist.

RGS4 and desipramine

Previous studies in our lab have shown that RGS4 acts as a positive modulator of the tricyclic antidepressant desipramine, as desipramine has a more prominent antidepressant effect in RGS4 WT mice compared to their KO littermates in the FST. To further confirm these findings, we performed novelty suppressed feeding test to RGS4 WT and KO mice that have received desipramine 10mg/kg chronically (13 days). As shown in Figure 2, the WT animals that received desipramine had smaller latency to start eating the unfamiliar food than their KO littermates suggesting increased responsiveness to the drug.

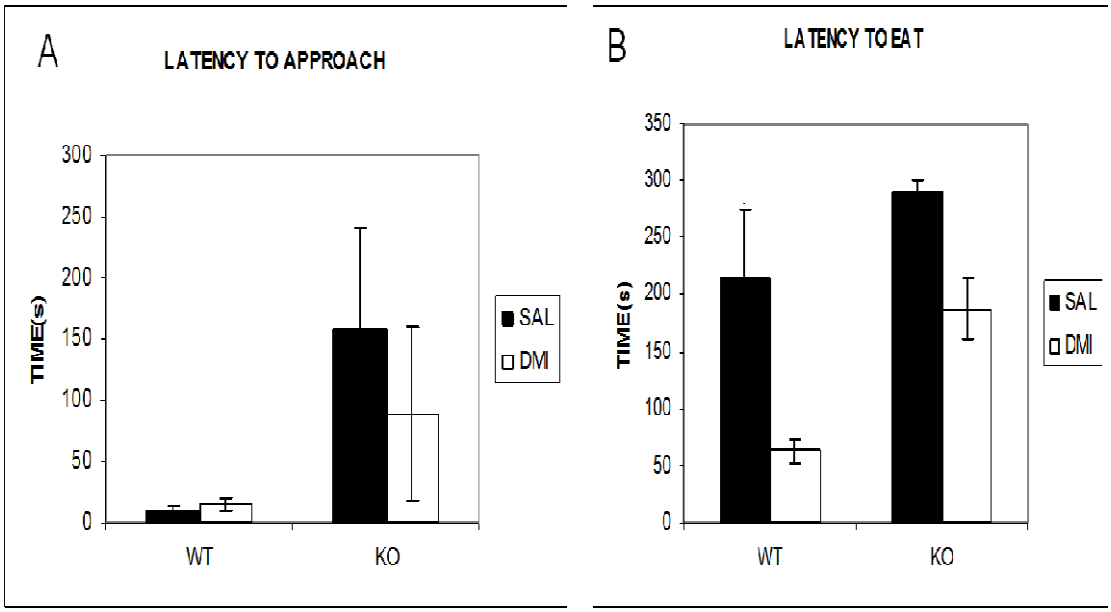


Figure 2.Novelty suppressed feeding test under chronic desipramine treatment. A. Latency to approach to the food. B. Latency to eat for the first time the unfamiliar food. WTs respond significantly better to desipramine as it is shown by the less time they need to try the unfamiliar food for the first time. (n=4/group) Values represent mean±SE.

Regulation of reboxetine responses by RGS4

In order to study the effect of RGS4 in othe mechanism of action of other drugs, we tried reboxetine, a norepinephrine reuptake inhibitor. In the low dose (5mg/kg) WT mice are more sensitive than the KO mice to reboxetine's antidepressant effect, as this was indicated by the less time they spent immobile (Fig 3A,B). As expected both WT and KO animals respond to higher dose of reboxetine (7mg/kg) (Fig 3C, D) suggesting that RGS4 is involved in reboxetine's action.

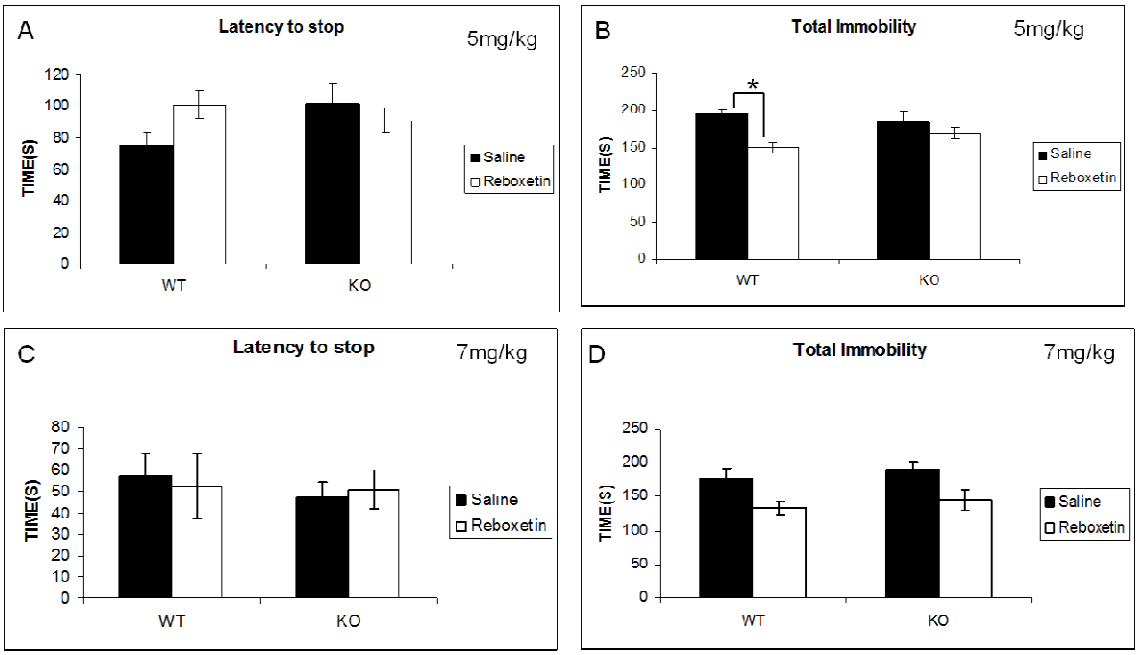


Figure 3.Evaluation of reboxetine's antidepressant actions in RGS4 WT and KO mice performing FST. A,B WT mice spent less time immobile when having received reboxetine in a dose of 5mg/kg .ANOVA $F_{(1,56)}$=12.68 ,p=0.0008 for drug , $F_{(1,56)}$=0,2714 ,p=0.0644for genotype ,$F_{(1.56)}$=3.115 , p=0.0430 for drug-genotype interaction;multiple comparisons with Bonferroni post hoc test * p<0.05 (n=15-17/group) C,D.WT and KO mice do not seem to have significant differences in their total immobility or latency to stop when received 7mg/kg reboxetine (n=5/group). Values represent mean±SE.

Regulation of ketamine responses by RGS4

Ketamine, is an NMDA receptor antagonist which has been proven to have rapid antidepressant actions (124). In this context, we decided to test whether RGS4 is involved in its mechanism of action. To do so, we administrated a single dose (3mg/kg) of ketamine 3h before FST in global and NAc conditional RGS4 KOs and their WT littermates. As shown in Figure 4A and B, the constitutively KO mice that received ketamine spent significantly less time immobile suggesting a more robust response to the drug compared to their WT littermates. The same effect but in a lesser extent was observed in the conditional NAc RGS4 KOs (Fig4 C,D), suggesting that RGS4 in the NAc could act as a negative modulator of ketamine's antidepressant action.

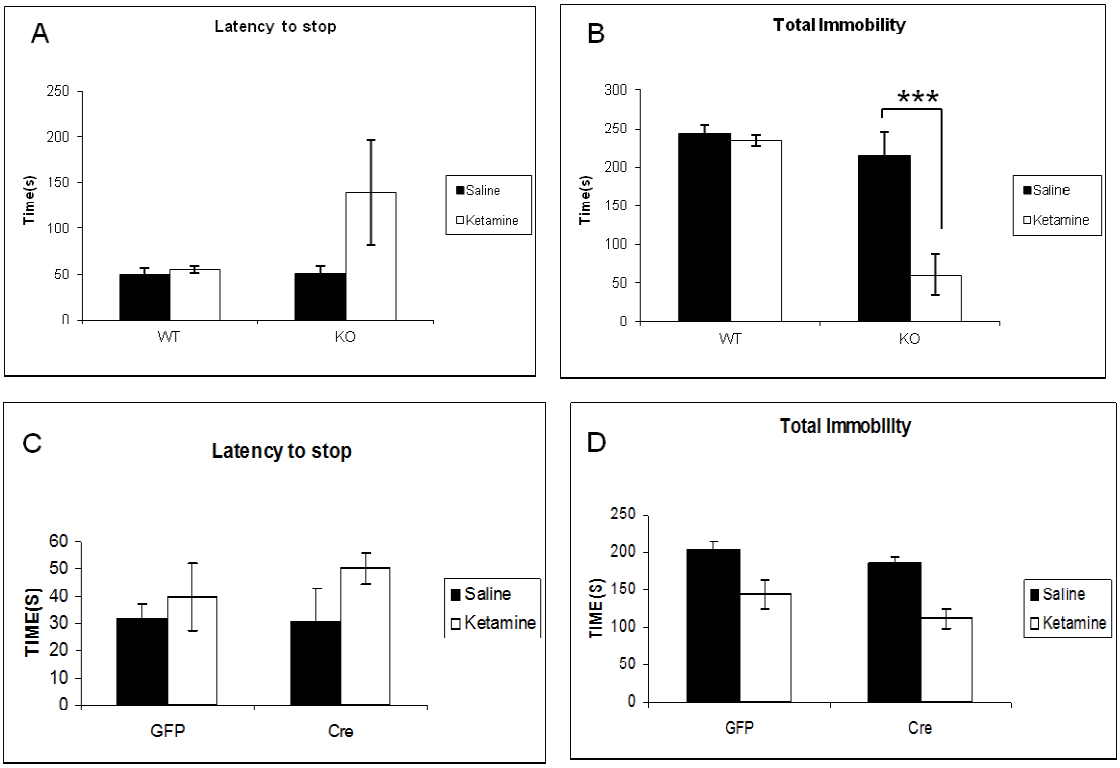


Figure 4.Forced swim test under ketamine treatment.A,B Global KOs and WTs under ketamine.A.Latency to stop.B.Total immobility time.RGS4 KOs spent signifiacantly less time immobile suggesting a strong response to ketamine. ANOVA $F_{(1,20)}$=6.783 ,p =0.0170 for the drug, $F_{(1,20)}$=23,39 , p =0.0001 for the genotype, $F_{(1,20)}$=5.051, p=0.0361 for the drug-genotype interaction;multiple comparisons with Bonferroni post hoc tests, *** p<0.001 (n=5-7/group).C,D.Conditional NAc RGS4 KOs seem to respond better to ketamine than their WT littermates (n=3-5/group). Values represent mean±SE.

RGS4 and SNC80

Consequently, we decided to test whether RGS4 could affect the antidepressant actions of SNC80, a delta opioid receptor agonist. As interpreted in Figure 5 , RGS4 KO mice that received SNC80 exhibit increased responsiveness compared to WT mice (as indicated by the smaller immobility time), suggesting that RGS4 could be a negative modulator of SNC80 antidepressant actions.

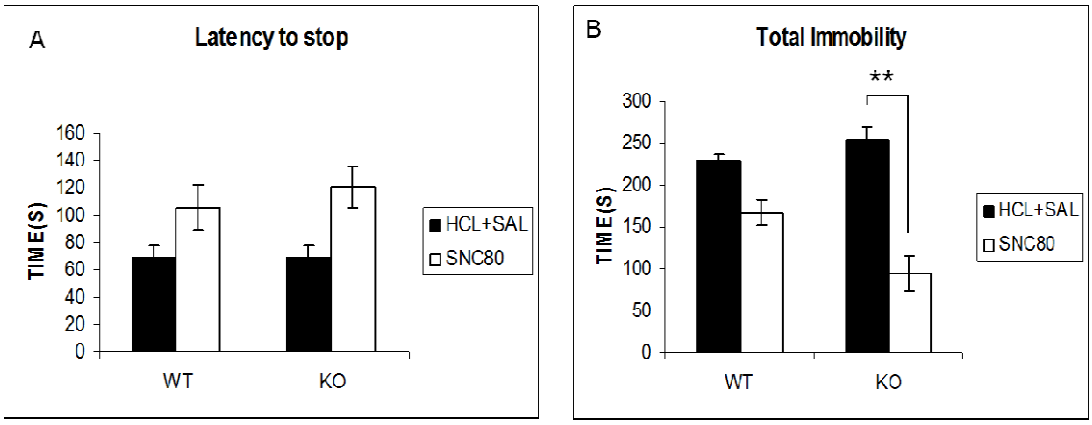


Figure 5.Forced swim test under SNC80 treatment. A. Latency to stop b. Total immobility time. RGS4 KOs that have received SNC80 spent significantly less time immobile, suggesting a better antidepressant response. ANOVA $F_{(1,23)}$=48.88,p=0.0001 for the drug , $F_{(1,23)}$=2.368 ,p=0.1375 for the genotype, $F_{(1,23)}$=9.506, p=0.0053 for the drug-genotype interaction; multiple comparisons with Bonferroni post hoc tests, ** p<0.01 (n=7-8/group). Values represent mean±SE.

Neuropathic pain

- ✓ *Evaluation of mechanical allodynia in RGS4 WT and KO mice*

We used the spared nerve injury (SNI) model of neuropathic pain and tested mechanical allodynia in order to see if there is any difference in the pain sensitization between WTs and KOs. As shown in figure 6, no significant difference could be observed.

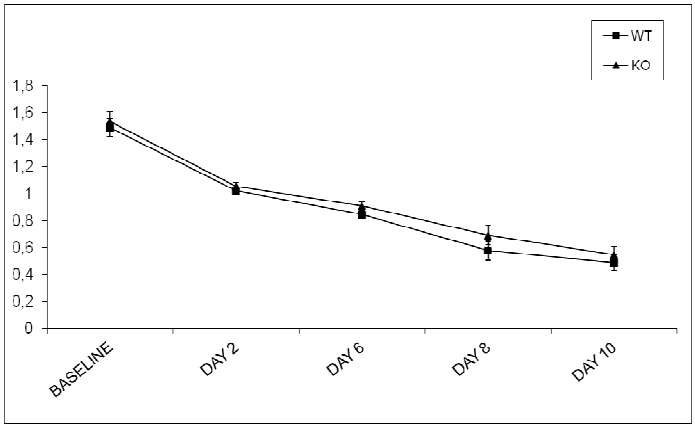


Figure 6.von Frey test evaluating mechanical allodynia in RGS4 WT and KO mice that have been through SNI. No significant difference in pain sensitization can be observed between genotypes (n=9-10/group). Values represent mean±SE.

- ✓ *Antidepressants in neuropathic pain*

Given the role of RGS4 in desipramine's and ketamine's antidepressant action, we then tested the potential role of RGS4 in the analgesic properties of these drugs in neuropathic pain conditions.

More specifically, neuropathic pain RGS4 WT and KO mice were chronically treated with desipramine (10mg/kg intraperitoneally, twice a day for 15 days) and assessed for mechanical allodynia using the von Frey test. Indeed, desipramine has an antiallodynic effect in both WT and KO animals, which is more prominent in the WT mice compared to their KO littermates (Figure 7).

Concerning ketamine, as shown in Figure 8A, low dose of ketamine (3mg/kg) relieves neuropathic pain as soon as 1 hour after the injection and lasts for 24hours, Interestingly enough, this analgesic action is more prominent in the KO mice. On the other hand administration of high dose of ketamine (5mg/kg) produces a similar analgesic effect in both WT and KO mice (Fig 8B). Furthermore, ketamine reverses thermal hyperalgesia (as this is assessed using the Haregraves' test), but no genotype difference is observed (Fig 8 C,D).

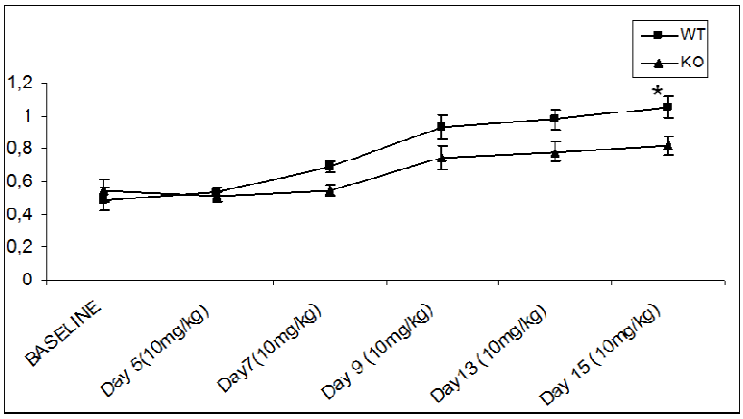
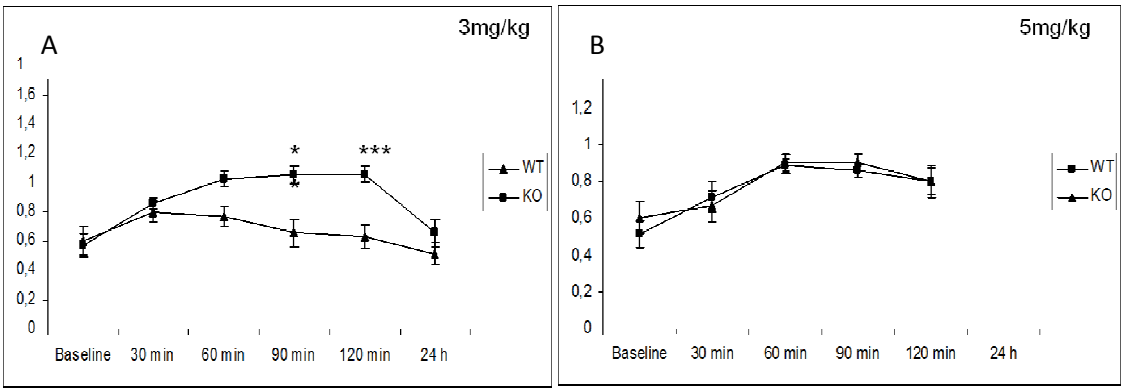


Figure 7.Mechanical allodynia under chronic desipramine treatment.RGS4 WT mice respond better to desipramine when compared to their WT littermates.ANOVA $F_{(1,90)}$=107,6 , p<0.0001 for time , $F_{(1,90)}$=3,850, p=0.0654 for genotype , $F_{(1,90)}$=5,308 , p<0.0001 for matching, $F_{(1,90)}$=2.759 , p=0.0230 for the drug-genotype interaction;multiple comparisons with Bonferrroni post hoc tests,* p<0.05). Values represent mean±SE.



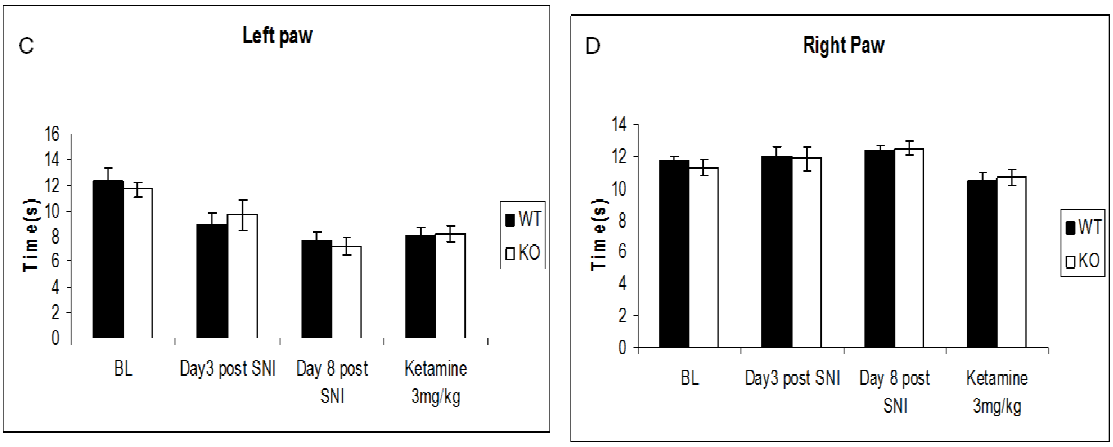


Figure 8.Mechanical allodynia and thermal hyperalgesia under ketamine treatment. A,B. RGS4 KO mice respond better to low doses (3mg/kg) of ketamine (A) than do the WTs but this difference is abolished in higher doses (5mg/kg) of the drug (B) ANOVA $F_{(1,60)}$=7.196, p<0.0001 for time, $F_{(1,60)}$=24,51,p=0.0003 for genotype, $F_{(1,60)}$=0.9564,p=0.4993 for matching, $F_{(1,60)}$=3.041,p=0.0164 for the drug-genotype interaction;multiple comparisons by Bonferroni post hoc tests ** p<0.01 , *** p<0.001. C,D Ketamine had no effect on thermal hyperalgesia .(n=7/group) . Values represent mean±SE

Inflammatory pain

- ✓ *Evaluating inflammatory pain*

The third set of experiments aimed to evaluate the differences in inflammatory pain using the CFA model and measuring thermal hyperalgesia as no differences were found when measuring mechanical allodynia Figure 9 shows that RGS4 KO mice exhibit a more severe inflammatory pain phenotype compared to their WT littermates.

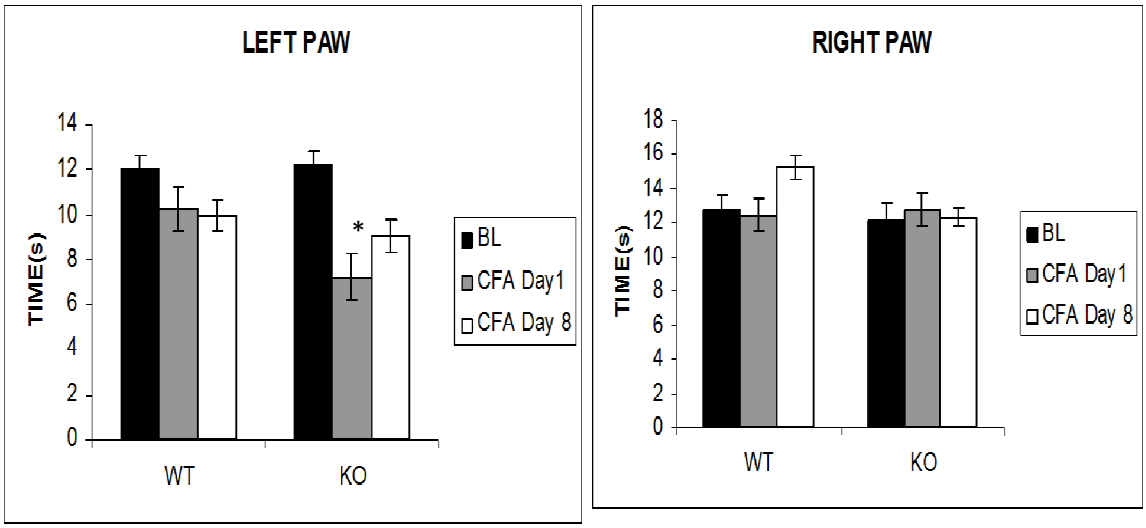


Figure 9 Evaluation of inflammatory pain.RGS4 KO mice are more sensitive to inflammatory pain as estimated by the faster paw withdrawal in the Hargreaves' test. ANOVA $F_{(1.20)}$=9,479,p=0.0013for time, $F_{(1.20)}$=6,052,p=0.0337 for genotype, $F_{(1.20)}$=1,191, p=0.3529 for matching, $F_{(1.20)}$=4.478,p=0.0247 for the drug-genotype

interaction; multiple comparisons by Bonferroni post hoc tests * p<0.05.(n=10/group). Values represent mean±SE.

- ✓ *Evaluation of levels of anxiety and depression in the context of inflammatory pain*

The literature suggests that inflammatory pain is accompanied by anxiety-like behaviors (126). Thus, we decided to test whether CFA model of inflammatory pain could induce both anxiety and depression-like behaviors, and to investigate potential role of RGS4 in these behaviors. We used the open field and the light dark box test to estimate anxiety and the forced swim test to estimate depression. During the open field test , RGS4 KO mice that suffered from inflammatory pain needed more time to approach the centre and spent more time in lane 1(boarders) than in the other zones , suggesting a more anxious phenotype (Figure 10 A-D). A similar anxious phenotype was observed during light-dark box test, during which KO mice that suffered from inflammatory pain had a higher latency to enter the dark box and spent less time in it (Figure 11). However, no significant difference between WT and KO mice suffering from inflammatory pain was observed in the forced swim test (Figure 12). Gathering together, these data suggest that RGS4 prevents the development of inflammatory pain-induced anxiety, but has no effect on pain-induced depression

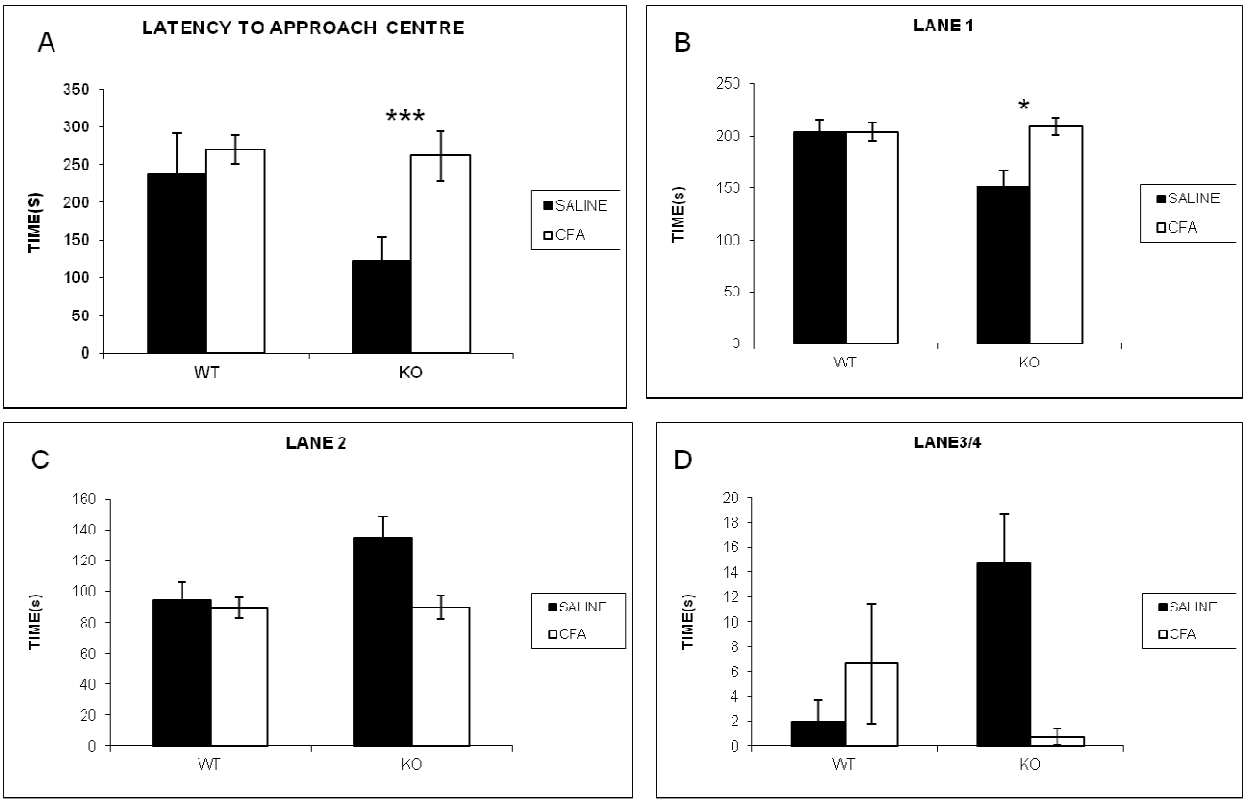


Figure 10. Open field test in mice suffering from inflammatory pain. RGS4 KO mice suffering from inflammatory pain appear to be more anxious than their WT littermates. They need more time to

approach the centre of the arena (A) and they spent more time to the boarders (B) than the peripheral zone and the centre(C,D). A : ANOVA $F_{(1,14)}$=5.541 , p=0.00337 for the drug , $F_{(1,14)}$=4,3 55 p=0.0572 for the genotype, $F_{(1,14)}$=12.68 p=0.0031 for the drug-genotype interaction;multiple comparisons by Bonferroni post hoc tests , *** p<0.001 . B : ANOVA $F_{(1,13)}$=4,862 , p=0.0461 for the drug , $F_{(1,13)}$=4,355, p=0.0572 for the genotype, $F_{(1,13)}$=6,119, p=0.0279 for the drug-genotype interaction;multiple comparisons by Bonferroni post hoc tests , * p<0.05.(n=4-5/group). Values represent mean±SE.

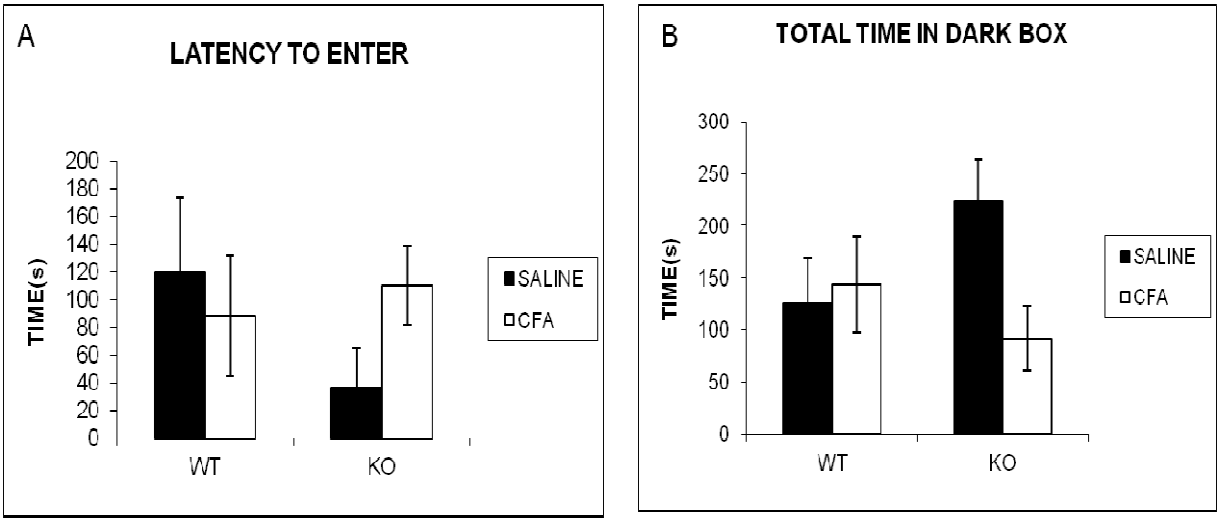


Figure 11.Light-dark box test in mice suffering from inflammatory pain.RGS4 KO mice need more time to enter the dark box (A) and spend less time in it (B) , suggesting a more anxious phenotype (n=4-5/group). Values represent mean±SE.

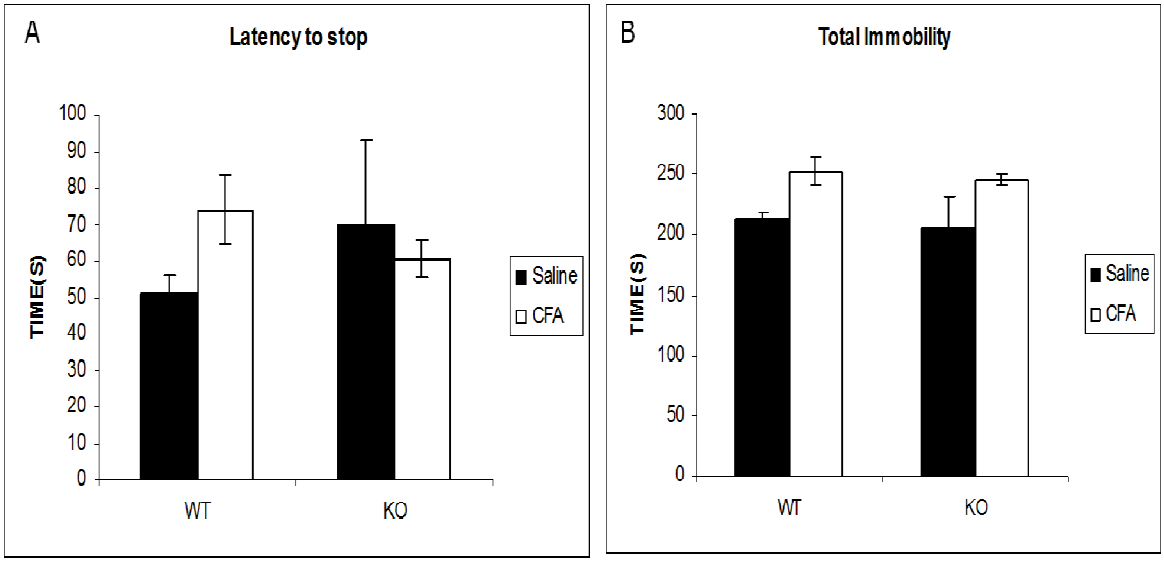


Figure 12.Forced swim test in mice suffering from inflammatory pain. No significant differences were observed between KO and WT mice neither in their latency to stop (A) nr to their total immobility time (B) (n=4-5/group). Values represent mean±SE.

DISCUSSION

One of the major problems that physicians who treat depression deal with, is that among patients, there is a large number that does not respond to certain drug categories or to specific drugs from one category (but interestingly enough respond to other substances from the same category). Furthermore, antidepressants have not the same efficacy to all the patients. Thus, it is very important to understand the mechanisms via which the antidepressants act and to identify specific molecules that are involved.

Previous studies in our lab suggested the important role of RGS4 in antidepressants' actions. More specifically, using the FST it had been shown that RGS4 is a positive regulator of tricyclic antidepressants (TCAs-desipramine) and selective serotonin re-uptake inhibitors (SSRIs-fluoxetine), something that in the case of desipramine was confirmed in the novelty suppressed feeding test.

Similarly, RGS4 seems to be a positive regulator in one more category, the norepinephrine re-uptake inhibitors. Reboxetine, one of the most widely used drugs of the category had a better efficacy in the RGS4 WT mice than in their KO littermates when used in low doses. Recently, several studies have shown that ketamine, a non-competitive NMDA receptor antagonist, mostly known for its use as an anesthetic agent, has a very strong and rapid antidepressant action (127). Furthermore, there are clinical studies supporting the efficacy of ketamine as a rapid and effective drug (128). In this context we decided to see whether ketamine's antidepressant efficacy could be affected by RGS4. Unlike TCAs, SSRIs and NRIs, we observed that mice lacking RGS4 responded significantly better after acute administration of a low, subanaesthetic dose of ketamine Another drug category that has been referred to exert antidepressant properties is delta opioid receptor agonists. When we administered such an agonist, SNC80, to RGS4 WT and KO mice we observed that RGS4 negatively modulated its antidepressant properties, as this was indicated by the less time the KO mice spent immobile during the forced swim assay.

Taken together, these data suggest that RGS4 seems to be important for the response to a broad spectrum of antidepressants by either positively, or negatively regulating these responses. These finding could be very useful in the clinical practice, when trying to identify a suitable antidepressant for a patient. Another perspective could be the identification of specific polymorphisms in the RGS4 gene that could affect its activity and thus the responsiveness to antidepressant treatment. Moreover, strategies

that improve RGS4 activity could be valuable in improving the antidepressant efficacy of certain drugs that act via mechanisms including RGS4.

Chronic pain is one of the most complex issues in medicine as it affects large numbers of people and as there are not specific treatments, it costs a lot both to the patients in terms of suffering and to the hospital in terms of money. A very complicated issue for a physician to come across is that of the treatment of neuropathic pain. Many approaches have been mentioned to be effective but there are not clear guidelines on how to treat patients who suffer from neuropathic pain. Since chronic pain very often comes together with depression, and antidepressants have been found to act as analgesics, it would be good to have a drug able to face both pain and depression. Antidepressants (mostly TCAs) are widely used in the treatment of neuropathic pain (30). Having noticed the difference that RGS4 produces in the antidepressant potential of desipramine and ketamine (two drugs that have been mentioned to have analgesic properties), we wanted to examine if it affects the analgesic properties of these drugs in any way. Using the SNI model for neuropathic pain, we observed that as in the case of depression, desipramine when administered chronically was more potent to relieve mechanical allodynia in the RGS4 WT mice than in their KO littermates. In the case of ketamine, again the analgesic properties were affected the same way as its antidepressant ones, meaning that this time the KOs responded better than the WTs. There was however a difference between the action of these drugs: ketamine acted acutely and its effect lasted for a very short period of time, as 24h after the injection mice had returned to their baseline levels. Notably, ketamine had no effect on thermal hyperalgesia.

Apart from neuropathic pain, chronic pain also refers to pain caused by inflammation either local or systematic. Previous studies in our lab have shown that RGS4 is implicated in the process of the development of pain, as RGS4 KO mice exhibit a more severe pain phenotype in the formalin test (a model of inflammatory pain). Using the CFA model of inflammatory pain (a model that causes local inflammatory response in the site of the injection), we wanted to examine whether RGS4 could affect the development of inflammatory pain in this model too. When estimating thermal hyperalgesia, we found that RGS4 KO mice developed more intense hyperalgesia and in a shorter period of time than the WT animals. This implies that RGS4 could be involved in the establishment of inflammatory pain.

It is widely known that chronic pain triggers anxiety and depression phenomena and for this reason we decided to test whether RGS4 could be a part of it. As it was expected, the RGS4 KO mice, suffering more, were more anxious than their WT littermates as estimated by the open field and the light dark box tests. However, RGS4 WT and KO mice had no significant differences in the forced swim test, suggesting that RGS4 probably does not impair the development of depression in the context of inflammatory pain.

Summing up, RGS4 is a molecule that takes part in a variety of processes. It is very interesting that affects the antidepressant potency of a broad spectrum of drugs from different categories, both as a positive modulator (as in case of desipramine (TCA) and fluoxetine (SSRI)) and as a negative modulator (As with ketamine (NMDA receptor antagonist and SNC80 (delta-opioid agonist)). Certainly, more studies are needed in order to fully understand the underlying mechanisms but strategies which affect RGS4 could be a target for the improvement of antidepressant therapies or for the screening of patients in order to find the most suitable antidepressant. Furthermore, RGS4 appears to affect the analgesic properties of antidepressants in the same way as their antidepressant actions. Finally, RGS4 is involved in the development of inflammatory pain and the intensity of the anxiety phenotype that comes along.

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