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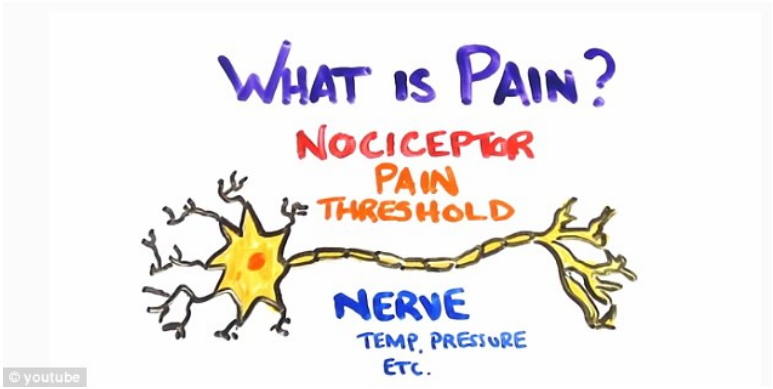
Graduate Program in Neuroscience

Effect and mechanism of action of neurosteroids on inflammation-induced analgesia



Poulaki Smaragda

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Supervisor

Dr. Maria Venihaki

Assistant Professor of Clinical Chemistry

Ευχαριστίες

Αρχικά θα ήθελα να ευχαριστήσω θερμά την επιβλέπουσα καθηγήτρια κ. Μαρία Βενυχάκη, για την απόλυτη εμπιστοσύνη που μου έδειξε και για την κατανόηση που επιδείκνυε σε τυχόν προβλήματα που αντιμετώπισα κατά την παραμονή μου στο εργαστήριο, και για την ενθάρρυνση που μου παρείχε τις στιγμές που πραγματικά το χρειαζόμουν. Οι συμβουλές και οι γνώσεις που μου προσέφερε κατά τη διάρκεια της συνεργασίας μας, θα με συνοδεύουν και θα με βοηθήσουν στη μετέπειτα σταδιοδρομία μου. Ένα πολύ μεγάλο ευχαριστώ θα ήθελα και οφείλω να απευθύνω στα μέλη του εργαστηρίου Καραγιάννη Ευθυμία, Ρασούλη Όλγα που είναι υπεύθυνες για μεγάλο μέρος της τεχνικής και θεωρητικής μου κατάρτισης, αλλά και στις Παυλίδου Άττικα και Δασκαλάκη Ακριβή για την άψογη συνεργασία και το ευχάριστο κλίμα που δημιούργησαν σε όλη τη διάρκεια της παραμονής μου στο εργαστήριο. Θα ήθελα επίσης να ευχαριστήσω τον κ. Αχιλλέα Γραβάνη, τον κ. Ιωάννη Χαραλαμπόπουλο και τα μέλη του εργαστηρίου τους που μας προσέφεραν το μόριο με το οποίο δούλεψα καθώς και γνώσεις για το σωστό χειρισμό του. Ακόμη οφείλω ένα μεγάλο "ευχαριστώ" στα μέλη του εργαστηρίου του κ Χρήστου Τσατσάνη και στη Ρένα Δερμιτζάκη για την συνεργασία και την βοήθεια στην επίλυση τυχόν τεχνικών προβλημάτων κατά την εκπόνηση της παρούσας μελέτης. Από τον κατάλογο των ευχαριστιών δε θα μπορούσα να παραλείψω τους γονείς μου για το σεβασμό τους στις μέχρι τώρα επιλογές μου, για την πολύτιμη ηθική και υλική υποστήριξη τους καθ' όλη τη διάρκεια των σπουδών μου και για την πίστη που έδειξαν σε μένα, η οποία και με βοήθησε να συνεχίσω.

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Summary

Summary

Pain is an unpleasant perception often caused by intense or damaging stimuli. Many types of pain have been described according to its different components, such as duration, location, and type of system that influences. In this study, we focused on inflammatory pain that occurs after tissue injury or infection, and is interwoven with immune system response. This situation causes discomfortness, stress and fear to the individual, but pain is also protective by preventing the individual to further use the injured member.

Neurosteroids, like DHEA and Allopregnanolone have been demonstrated to exert positive effects in pain and hyperalgesia, as has been shown in pain models like caraggeenan-induced inflammation. A new synthetic analogue of DHEA has been recently developed, which conserves the immunomodulatory effects of DHEA, but lacks the endocrine properties of it (BNN27). Thus, the aim of our study was to examine the effect of BNN27 on inflammatory pain using the mouse model of inflammation and hyperalgesia induced by Complete's Freund's Adjuvant (CFA) in hind paw.

Specifically, we focused on the contribution of BNN27 in basal and inflammatory pain, and edema and the mechanism BNN27 likely mediates its effects, through. Our results demonstrated that BNN27 has positive effect on basal and inflammatory pain, as it increased pain thresholds in treated mice, but it did not influence edema. BNN27 increased protein levels of TNF-a, IL-6, and NGF at 6 hours following the induction of inflammation, while it decreased TNF-aat 24 hours following inflammation. IL-6 and NGF were unchanged at 24 hours, while IL-10 was elevated in BNN27-treated mice at all time points examined. In DRGs, NGF was decreased at 6 and 24 hours after CFA injection. The analgesic affect of BNN27 seems to be mediated by μ-opioid receptor (MOR), as its mRNA expression levels were elevated in inflamed tissue and DRGs. M-opioid receptor ligand, β-endorphin and its precursor POMC were also elevated at 6 hours, while the mRNA of PENK, the precursor of enkephalins, was elevated at 24 hours.

Our data indicate a potential role of BNN27 on inflammatory pain. Most studies with this compound so far were focused on its beneficial effect in neuroprotection. Here we show its positive contribution in another system, that of

pain and inflammation. Further studies are in progress to clarify its role and the mechanism in these conditions.

Περίληψη

Ο πόνος είναι μια δυσάρεστη αίσθηση οποία συχνά προκαλείται από έντονα ή επιβλαβή ερεθίσματα. Διάφοροι τύποι πόνου έχουν περιγραφεί σύμφωνα με τις διάφορες συνιστώσες του, όπως η διάρκεια, η τοποθεσία, και τον τύπο του συστήματος που επηρεάζει. Η μελέτη αυτήεπικεντρώθηκε στον φλεγμονώδη πόνο που εμφανίζεται μετά από τραυματισμό ιστού ή μόλυνση, και είναι συνυφασμένος με την αντίδραση του ανοσοποιητικού συστήματος για την επιδιόρθωση του ιστού. Ο πόνος προκαλεί δυσφορία, άγχος και φόβοστο άτομο, αλλά έχει και προστατευτικό ρόλο, εμποδίζοντάς το να χρησιμοποιήσει περαιτέρω το τραυματισμένο μέλος.

Ο κύριος στόχος αυτής της μελέτης ήταν να προσδιοριστεί ο ρόλος των νευροστεροειδών σε καταστάσεις φλεγμονώδους πόνου. Τα νευροστεροειδή όπως η DHEA και η αλλοπρεγνανολόνη έχει δειχθεί ότι συμβάλλουν θετικά στην αντιμετώπισητου πόνου και της υπεραλγησίας, όπως έχει παρατηρηθεί χρησιμοποιώντας μοντέλαεπαγωγής πόνου και φλεγμονής όπως το ¨Carraggenan-induced pain model¨. Το νέο συνθετικό ανάλογο της DHEA που στερείται των ενδοκρινικών ιδιοτήτων της, BNN27, μελετήθηκε σε ένα μοντέλο φλεγμονής και υπεραλγησίας σε ποντίκια όπου προκαλείται από το ¨Complete Freund'sAdjuvant (CFA)¨. Επικεντρωθήκαμεστο ρόλο του BNN27 στα βασικά επίπεδα πόνου και στον φλεγμονώδη πόνο και οίδημα χρησιμοποιώντας τη συσκευή του Hargreaves και το πληθυσμομέτρο, αντίστοιχα. Επιπλέον, μετρήσαμε τα επίπεδα κορτικοστερόνης ορού, και εξέτασαμετο φλεγμαίνοντα ιστό για διείσδυση ανοσοκυττάρων. Διερευνήσαμε επίσης την απελευθέρωση κυτοκινών όπως TNF-α, IL-6, IL-1β, IL-10, σε διαφορετικά χρονικά διαστήματα. Στη συνέχεια αξιολογήθηκε ο ρόλο του NGF ο οποιος εμπλέκεται στον πόνο και τη φλεγμονήστον τοπικό φλεγμαίνοντα ιστό και στα DRGs, και η συμμετοχή του οπιοειδούς συστήματος στην αναλγησία.

Τα αποτελέσματα έδειξαν ότι το BNN27 έχει θετική επίδραση στην βασικό και το φλεγμονώδη πόνο, καθώς αυξάνει τον ουδό του πόνου στα ποντικια που τους χορήγηθηκε, αλλά δεν επηρεάζει οίδημα. Εξέταση των φλεγμαίνοντωνπελμάτων έδειξε ότι το BNN27 αυξάνει τα επίπεδα της πρωτεΐνης του TNF-α, IL-6, και του NGF σε 6 ώρες μετά την επαγωγή της φλεγμονής, ενώ μειώνει TNF-α 24 ώρες μετά από φλεγμονή. Η IL-6 και ο NGF δεν επηρεάστηκαν στις 24 ώρες, ενώ η IL-10 ήταν αυξημένη σε όλα τα χρονικά σημεία που εξετάστηκαν στα ζώα που χορήγηθηκε το νευροστεροειδές. Τα επίπεδα της IL-1β παρέμειναν αμετάβλητα. Στα DRGs ο NGF

μειώθηκε 6 και 24 ώρες μετά την επαγωγή της φλεγμονής στα ζώα που έλαβαν την ουσία. Η αναλγητική επίδραση του BNN27 φαίνεται να διαμεσολαβείται από μ-υποδοχέα οπιοειδών (MOR), καθώς τα επίπεδα του mRNA του εμφανίστηκαν αυξημένα στον φλεγμαίνοντα ιστό και στα DRGs. Τα επίπεδα της β-ενδορφίνης και του προδρόμού της μορίου POMC ήταν επίσης αυξημένα στις 6 ώρες, ενώ το mRNA του προδρόμου της εγκεφαλίνηςPenk, ήταν αυξημένο στις 24 ώρες στα ζώα που έλαβαν BNN27.

Τα δεδομένα αυτά υποδεικνύουν έναν ευεργετικό ρόλο του BNN27 σε ένα διαφορετικό σύστημα, καθώς οι περισσότερες μελέτες με την ένωση αυτή έχουν επικεντρωθεί στη νευροπροστατευτική του δράση. Εδώ δείξαμε τη θετική συμβολή τουστην ανοσολογική απάντηση μετά από φλεγμονή και πόνο. Περαιτέρω μελέτες βρίσκονται σε εξέλιξη για την αποσαφήνιση του ρόλου του στην απόκριση του ανοσοποιητικού συστήματος μετά από φλεγμονώδεις ή αυτοάνοσες καταστάσεις.

Introduction

1.1 History and classification of pain

According to the International Association for the Study of Pain (IASP) founded in 1973, "pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Backonja, 2003). Despite the fact that pain is an unpleasant feeling, it is essential for the individual in order to avoid a harmful stimulus or to protect a damaged part of the body. Pain can be determined by four components: nociception, perception, suffering, and pain behavior (Loeser*et al.,* 1999). Nociception is the detection of tissue damage by transducers attached to A delta and C fibers. Perception of pain is triggered by noxious stimuli such as injury, disease or lesions to the central or peripheral nervous system. Suffering is a negative response of pain that coexists with fear and stress, leading to pain behaviors, things a person does or does not do that can be ascribed to the presence of tissue damage (Loeser*et al.,* 1999).

Over the centuries many theories have been developed to determine pain. In an ancient Chinese book 3000 years ago, pain was described as an imbalance between "yin" and "yang". When "yin" dominates results in "han" (cold) and damage to the "xing" is caused, leading to edema that is known as tissue injury. Domination of "yang" results in "re" (heat) and damage to the "qi' is caused leading to pain (Chen, 2011). Aristotle student of Plato, referred to pain as an emotion experience and not a sensory one, that comes when evil spirits enter injured parts of the body. He considered pain as a punishment from "Gods" or a test of faith.

During Renaissance in 1644, René Descartes supported that the body is a machine, and pain comes out from disturbance in machine's function, that reaches the brain through nerves. This was the first time that pain is considered as a physical mechanical sensation, instead of spiritual experience. The specificity theory developed in 1811 by Charles Bell, proposed that specialized sense organs (nociceptors) increase their activity when a strong noxious stimulus reaches their threshold. These organs are connected to particular spinal and brainstem projection neurons.The intensive theory developed in 18^{th} and 19^{th} centuries, argues that pain is an emotional state resulting from stronger than normal stimuli, such as intense light, temperature or pressure. In 1955 peripheral pattern theory proposed that pain is produced by stimulations of skin fiber endings, while gate control theory in 1965, proposed that information from an injured member are carried in the dorsal horn of

spinal cord, by thin and large diameter nerve fibers responsible for pain and touch/pressure/vibration respectively. The more large fiber activity relative to thin fiber activity at the inhibitory cell, the less pain is felt. All these theories have been replaced by the discovery of neurons and their role in mechanisms of pain (Chen, 2011).

Classification of pain is complicated and depends on the location, duration, frequency, intensity and cause. Depending on the site of pain, there are two systems of classification. The anatomic pain classification system focusing on sites of pain such as head, while the body system pain classification method focuses on body systems such as musculoskeletal (Cole, 2002). Another important parameter of pain is duration. When pain lasts less than 30 days it's characterized as acute pain, in contrast to chronic pain that lasts over 6 months and can be divided to non-cancer related pain and cancer related pain. Subacute pain involves the interval between the first months of pain until the seventh month (Cole, 2002). Acute pain has a protective role for life, as it warns for pathology or limits the use of damaged members. On the other hand chronic pain has no protective role, but lowers and complicates patient's life.

According to Woolf, pain can be divided into nociceptive, neuropathic and inflammatory pain as seen in Figure 1a. **Nociceptive** pain is a high-threshold pain, which activates nociceptors that transmit pain signals from damaged tissue to the brain through peripheral nerves and the spinal cord (Woolf, 2010). It may appear as spontaneous pain, or as hyperalgesia and/or allodynia. It is produced by intensive thermal, mechanical or chemical stimuli that cause non-neuronal tissue damage (Klasser*et al.,* 2012), and its role is to protect the individual by forcing it to avoid contact with noxious stimuli, which when removed and the damaged tissue heals, pain is also removed. Visceral pain is the subtype of nociceptive pain that involves the internal organs, and usually it's episodic and poorly localized.

Neuropathic pain is a consequence of abnormal function of the nervous system, and it's defined as "pain caused by a lesion or disease of the somatosensory nervous system" according to IASP (Klasser*et al.,* 2012). This kind of pain can occur not only from lesions in the nervous system, but also from non-neuronal and non-inflammatory damage too, a situation called dysfunctional pain (Costigan*et al.,* 2009; Woolf, 2010). Neuropathic pain is characterized by pain and other sensory symptoms that persist beyond the healing period, the presence of other neurological signs including motor, manifesting as negative and positive motor phenomena or autonomic

signs (Backonja, 2003).

Inflammatory pain is a situation evoked from tissue injury or infection followed by activation of the immune system. This process promotes the healing procedure of the damaged tissue by causing pain hypersensitivity, so the individual is discouraged to further use the injured part of the body (Woolf, 2010).

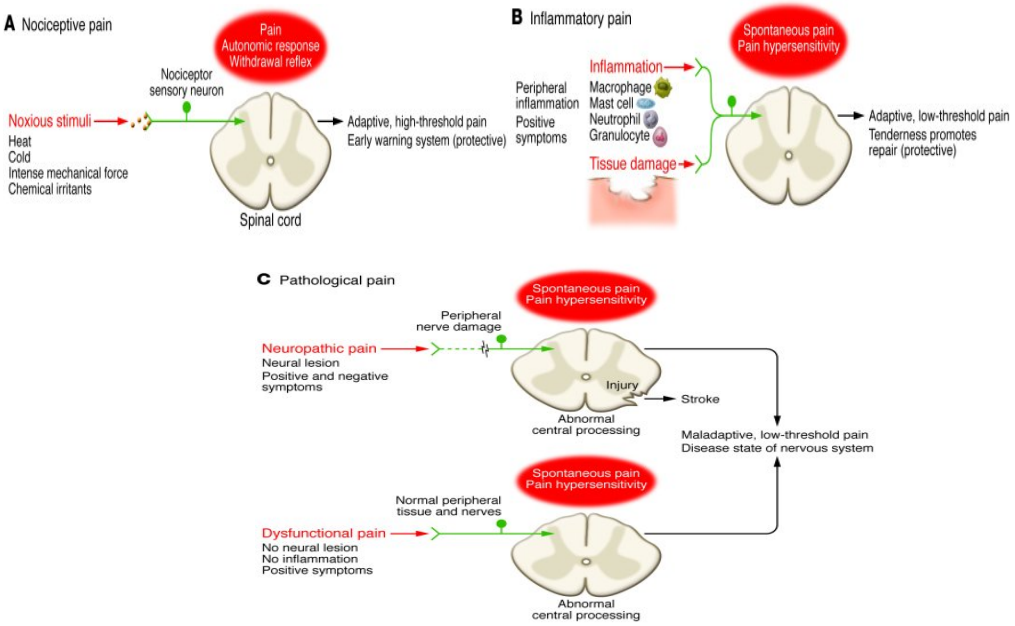


Figure 1a. Classification of pain (Woolf, 2010)

1.2Mechanisms of Inflammatory pain

Within the inflamed tissue noxious stimuli are transmitted as action potentials that activate nociceptors in the terminals of primary afferent sensory neurons. The cell bodies of primary sensory neurons are located in the trigeminal and dorsal root ganglia (DRGs), and their axons are either Aδ or C fibers (Stein et *al.,* 2009). The role of primary afferent sensory neurons is at first to recognize a damaging stimulus, a process called transduction. Then, the action potential is conducted from the periphery to the spinal cord, and finally the signal is transmitted to neurons of the dorsal horn and DRG (Kidd *et al.,* 2001). From the dorsal horn, sensory information is transmitted to the thalamus and the brainstem, where the sense of pain is being perceived (Figure 1b).

DRGs are pseudo unipolar, having an axon with two branches that acts as a single axon. A branch extends toward the periphery, and the other reaches the gray matter of the spinal cord. DRG neurons are divided into three categories. Large neurons with myelinated axons that express neurofilament are present. It is also found a population that contains cell surface glycoconjugates with terminal D-galactose, their axons are not myelinated and are possible to innervate predominantly nociceptors. The last group includes neurons that constitutively synthesize neuropeptides (Kandel, 2006).

Transduction

At the site of inflammation many inflammatory mediators such as cytokines, chemokines, growth factors, ions, bradykinin, histamine, ATP, nitric oxide are released (Kidd *et al.,* 2001). The source of these mediators it's not entirely clear, even though evidence show that immune cells play important role. Cells located at the site of inflammation including mast cells, macrophages dendritic cells, and cells from other organs of the immune system such as T cells, neutrophils, circulating macrophages are thought to produce many proinflammatory mediators like TNF-a, IL-1β, NGF (Denk *et al.,* 2012). Release of these peripheral pain mediators results in activation of nociceptors located in the terminals of peripheral primary afferent neurons. Nociceptors can also release neuropeptides such as substance P from their sensory endings. In this way they can lead to vasodilatation, plasma extravasation,

attraction of macrophages or degranulation of mast cells. This kind of inflammation is called neurogenic inflammation (Schaible *et al.,* 2005).

Activation of nociceptors by these mediators produces inputs transduced by two kinds of fibers with their cell bodies located in DRG and trigeminal ganglion. Large diameter myelinated fibers respond to thermal or mechanical stimuli such as cold and pressure. Their conduction velocity ranges from 2 to 30 m/s and activate low-threshold thermal and mechanical nociceptors, Aδ fibers. C unmyelinated fibers carry sensory information with conduction velocity no more than 2 m/s, and activate high-threshold polymodal nociceptors that respond to a variety of chemical, mechanical and thermal stimuli (Woolf *et al.,* 1999; Kidd *et al.,* 2001). Their cell bodies and axons have small diameter, and they can be classified in two categories: those that contain proinflammatory peptides such as substance P, calcitonin gene-related peptide, and are regulated by nerve growth factor, and those that can be identified histological because of the presence of enzymes (Caterina *et al.,* 2001).

Conduction

The action potential from peripheral nerve terminals is conducted to the spinal cord by voltage-gated sodium channels, and potassium channels. Voltage-gated sodium channels in neurons, have two subtypes: those that are sensitive to nanomolar concentrations of tetradotoxin (TTXs), and those that are resistant to all except micromolar concentrations of tetradotoxin (TTXr) (Woolf *et al.,* 1999). Within the DRG, neurons express several types of these sodium currents. A rapidly inactivating, fast TTXs current and a slowly activating and inactivating TTXr current are expressed by neurons with small diameter and high-threshold nociceptors. TTXs current is identified only in large diameter neurons, which express tyrosine kinase receptors for brain-derived neurotrophin factor (BDNF) and neurotrophin-3 (NT3), TrKB and TrKC respectively (Kidd *et al.,* 2001). This kind of neurons is divided in two different populations, those that produce neuropeptides, and those that bind the lectin IB4 and express the receptor for NGF, TrKA, in development, but not in postnatal stages.

Transmission

From nociceptors the action potential is travelling to the gray matter of dorsal horn, and second-order neurons are activated to pass the input to the brainstem and thalamo-cortical system, so the sense of pain is perceived. Other neurons of the spinal

cord are responsible for the activation of motor system and the generation of autonomic reflexes to avoid the noxious stimulus. Primary afferent neurons encode for the sensation, location and intensity of the stimuli. The main neurotransmitter of this process is glutamate via AMPA and NMDA receptors, when the noxious stimuli is not tissue damaging and when the stimuli is tissue damaging, respectively. Activation of these receptors by glutamate increases the excitability of dorsal horn neurons. Depending on the intensity of the stimuli, neuropeptides are released from vesicles to generate a greater postsynaptic response. Substance P that acts via neurokinin-1 receptor (NK1), located on dorsal horn, is such an important neuropeptide that is released from central C fiber terminals. Substance P enhances the activity of NMDA receptors through protein kinase C (Kidd *et al.,* 2001).

Peripheral sensitization

During inflammation, inflammatory mediators trigger nociceptors to reach their excitation threshold. These mediators can act even directly by activating peripheral nociceptors, even indirectly by activating the release of more algogenic agents via inflammatory cells. Activation of nociceptors results in a situation where excitation threshold reaches a state where even normally non-harmful stimuli can activate them and evoke pain. Noxious stimuli produce stronger responses in this sensitized state. Nociceptors located in skin surface are stimulated by thermal or cold stimuli, while nociceptors in deeper layers of the skin are stimulated by mechanical stimuli (Costigan*et al.,* 2000; Kidd *et al.,* 2001).

Central sensitization

When the presence of the noxious stimulus is persistent and the activation of nociceptors continuous, there is also activation of central neurogenic pathways. In addition, it is possible that C fibers will fire spontaneously, producing an unstable state in the dorsal horn, were many neurotransmitters, substance P, glutamate the main communicator between the spinal cord at the periphery, and neurotrophic factors like BDNF, are released and activate AMPA, NMDA and tyrosine kinase receptors. This cascade of events leads to activation of second messenger systems and increased calcium influx and protein phosphorylation. Because of these events, prolonged inflammation produces transcriptional changes, exaggerating responses to normal stimuli, reduction in the threshold for activation by novel inputs (Kidd *et al.,* 2001). Changes are observed in blood-brain-barrier (BBB) too, during central and peripheral

inflammatory pain. In 2000 Huber and his colleges have shown that peripheral inflammation leads to increased BBB permeability, as e result of changes in tight junctional protein expression (Huber *et al.,* 2000)

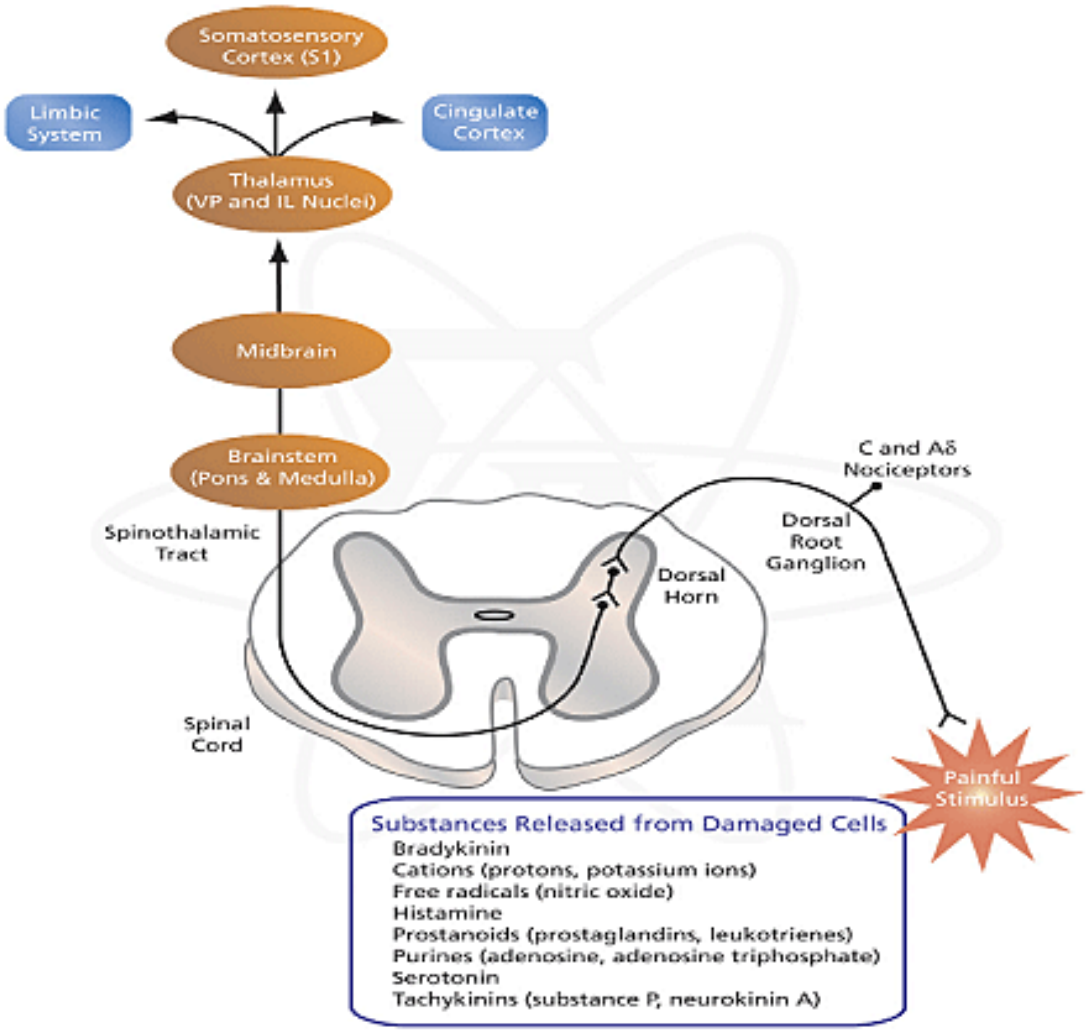
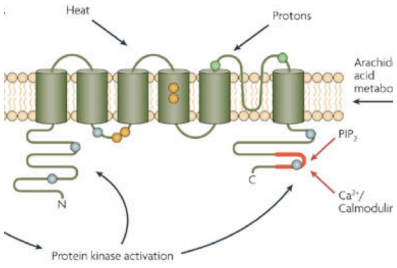


Figure 1b. Mechanism of inflammatory pain generation. Peripheral inflammation and sensitization of nociceptors by inflammatory mediators leads to amplification of pain signals in DRG and spinal cord neurons, to activate different brain regions responsible for pain perception.
(http://www.cgl.ucsf.edu/Outreach/bmi219/slides/images/ascending_pain_pathway.Par.0001.File.png)

1.3Important receptors in pain perception

Vanilloid receptor



(Szallasi *et al.,* 2007)

Sensory neurons in dorsal root ganglia express predominantly the vanilloid-receptor 1 (VR1), a ligand-gated, non-selective cation channel. VR1 consists of four subunits that form homo-tetrameric or hetero-tetrameric structures, arranged to create an ion pore. Each subunit contains a six-trans-membrane domain, which forms a pore between the fifth and sixth segments. Its family includes the vanilloid receptor-like protein 1 (VRL1) and the stretch-inactivating channel (SIC). VRL1 is not sensitive to capsaicin, but responds to thermal stimuli (approximately 52 ^{o}C). Protons activate VR1, so it is possible that its activation is enhanced inside the acidic environment of inflamed tissues (Kidd *et al.,* 2001). VR1 are activated by noxious stimuli, especially thermal (approximately 43 ^{o}C), and are also sensitive to capsaicin, component of chilly peppers (Davis *et al.,* 2000).

The majority of capsaicin sensitive sensory neurons are C fibers, but Aδ fibers exist too. VR1 has been observed in skin, cornea, mouth, muscles and joints. Such fibers innervate also parts of the cardiovascular and respiratory systems, and are responsible for the perception of visceral discomfortness (Caterina *et al.,* 2001). Many electrophysiological studies have been done to elucidate the function of VR1. These studies revealed that VR1 depolarizes sensory neurons by promoting the influx of sodium and calcium ions, with a preference for divalent cations. The response to pain is achieved by peripheral activation of VR1 that results in release of glutamate, substance P and other neuropeptides to the dorsal horn and spinal cold (Caterina*et al.,* 2001).

Tyrosine kinase receptor

Tyrosine kinase receptors are receptors for neurotrophins, a family of growth factors that activate pathways for survival, development and differentiation of neurons. In this family, belong the nerve growth factor (NGF), the brain-derived

neurothophic factor (BDNF), the neurotrophin-3 (NT-3), and neurotrophin 4/5 (NT4/5) (Barbacid, 1994).

Neurotrophins bind to the family of tyrosine kinase receptors TrK, and p75 ^{NTR}. Tyrosine kinase receptors bind to tropomyosin and have three types, TrkA that binds NGF, TrkB that binds BDNF and TrkC for the other neurotrophins. They consist of an extracellular domain that binds the ligand, an intracellular domain with kinase activity, an intracellular regulatory region and a transmembrane domain. These enzymes, autophosphorylate their tyrosine residues and activate pathways of phosphorylation and activation of other kinases like MAPK, ERK, Act (Figure 1c) (Segal, 2003). These pathways result in activation of different transcription factors and subsequent activation of a variety of target-genes that provide survival via regulation of anti-apoptic Bcl-2 proteins (Charalampopoulos *et al.,* 2004). Trk receptors are spread through the nervous system and in peripheral tissues, and play role in pain mechanisms.

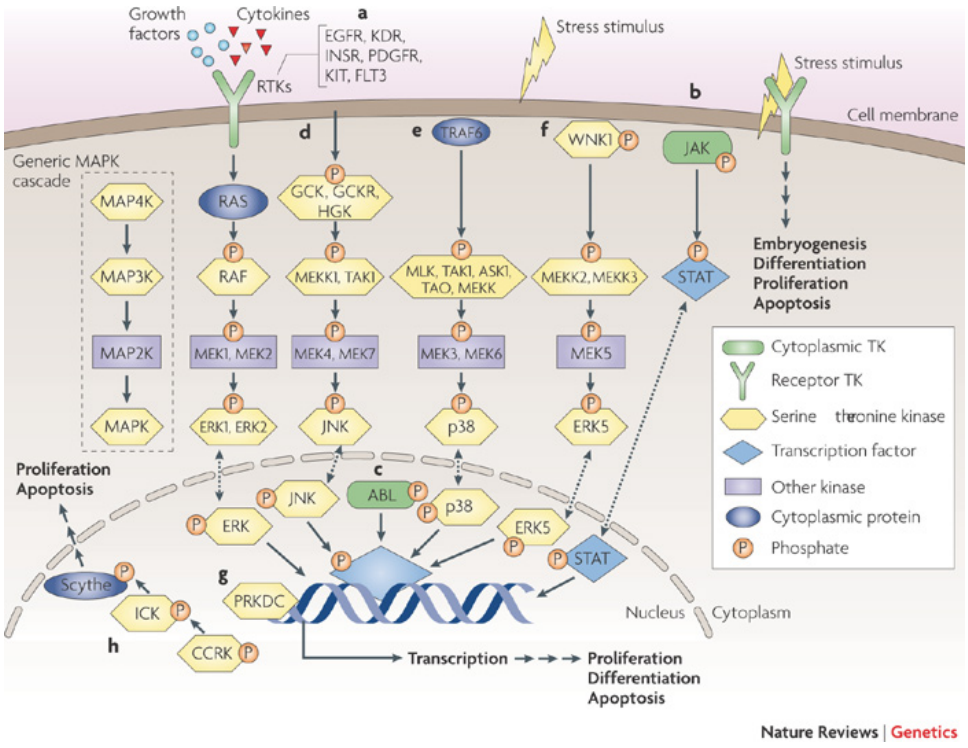


Figure 1c. Pathways through tyrosine kinase receptors (Lahiry *et al.,* 2010)

Glutamate receptors

Glutamate is the most important neurotransmitter in the central nervous system of vertebrates. Glutamate acts by binding to two types of receptors, the metabotropic and iontotropic receptors (Figure 1d).

Iontotropic glutamate receptors (iGlu) are ion channels that mediate fast synaptic transmission. They are classified in two groups, N-methyl-Daspartate (NMDA) and a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), according to the agonists that activate and the antagonists that inhibit. NMDA receptors are activated by the analog of amino acid NMDA, and inhibited by APV. They are also permeable to Na^+, K^+, Ca^+. AMPA receptors are activated by AMPA, kainic acid and quisquilic acid, and inhibited by CNQX. They are permeable to Na^+, K^+, but not to Ca^+. Iontotropic receptors consist of four subunits, M1, M2, M3, and M4. M1, M3 and M4 form three transmembrane domains, while a loop is generated by M2 subunit. The pore is formed by M2 subunit. The amino-terminal residues are located in the extracellular domain, whereas the carboxy-terminal residues in the intracellular domain. It is shown that iGlu receptors are expressed by primary sensory neurons, and they are depolarized by kainate, quisqualate, AMPA and NMDA. In DRG neurons there are two types of NMDA receptors, one that is present only in C fibers, and another located in C and A fibers too. Glutamate receptors can be transported to the periphery, contributing to inflammatory pain responses (Carlton, 2005).

Metabotropic glutamate (mGlu) receptors belong to the family of G-protein-coupled receptors. Eight mGlu receptors have been identified yet, and they can be divided to three groups. The first group contains mGlul and mGlu5, which are linked to phosphoinositide hydrolysis and intracellular calcium mobilization via phospholipase C. To the second group belong mGlu2 and mGlu3, and the third group contains mGlu4 and mGlu6-8, which are negatively coupled to adenylyl cyclase. Seven transmembrane a-helix domains characterize their general structure, and their amino-terminal and carboxyterminal residues are located in the extracellular and intracellular environment, respectively. Group 1 and 2 receptors are found in C fibers, and small neurons of DRG, but no evidence for group 3 in the periphery has been observed yet (Carlton, 2005).

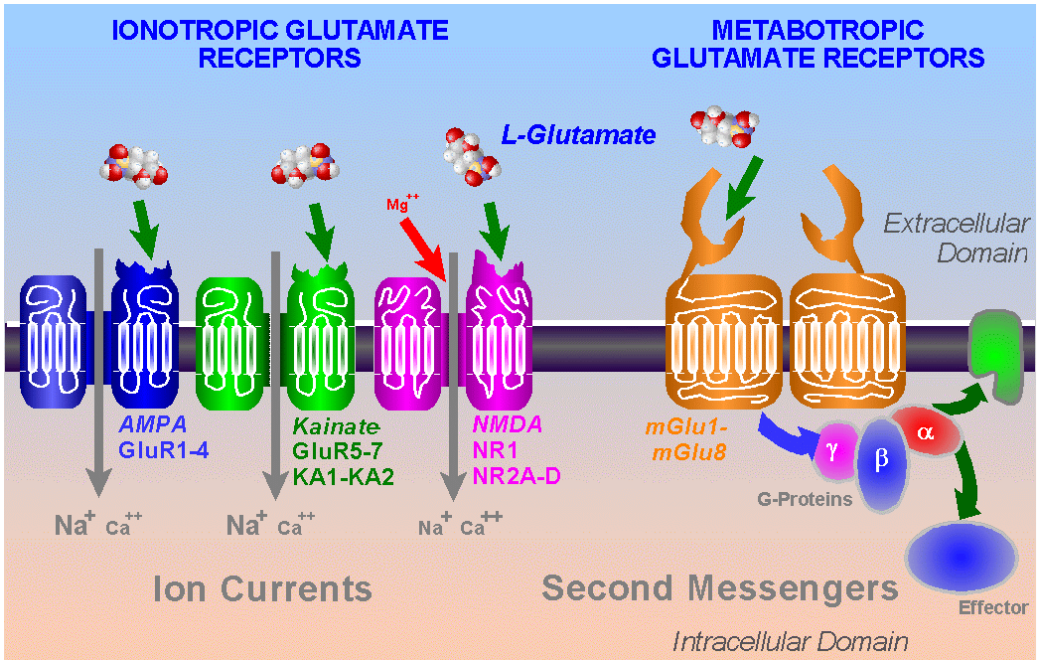


Figure 1d. Structure of iontortopic and metabotropic glutamate receptors (http://www.ucl.ac.uk/~smgxt01/frameh.htm?page=glutamat.htm)

Calcium and sodium channels

Calcium channels are believed to be involved in mechanisms of pain, but little data supports this hypothesis. It is found that the anticonvulsant gabapeptin, effective in patients with diabetic and post-herpetic neuralgia, has affinity for the a2δ subunit of voltage-gated calcium channels. It's not effective in inflammatory pain, though (Kidd *et al.,* 2001). Blockade of calcium channels has analgesic effects, but they are not used as therapeutic agents.

It has been shown that sodium channels play role in pain pathways. They can be classified in two categories based on their source. Large diameter neurons express only channels that are sensitive to the puffer fish toxin tetradotoxin (TTX-S), while small diameter neurons express sodium channels that are not only sensitive to tetradotoxin, but also channels resistant to tetradotoxin (TTX-R). Within the DRG during inflammatory pain, it has been observed increased amount of SNS/PN3 channel, a subcategory of TTX-R sodium channels. Drugs that blockade sodium channels have been developed, but their application is limited because of their side effects in the nervous system (Kidd *et al.,* 2001).

1.4 Immune system and pain

During tissue injury and inflammation, immune cells are activated to initiate immune response, to protect and repair the tissue. Another aim of immune cells is also to sensitize peripheral nociceptors. Immune cells synthesize and release cytokines and other inflammatory mediators that interact with many molecules and affect neurotransmitters and their receptors in afferent neurons. This process modulates the excitability of nociceptors, and starts the pathway of pain perception. Not only pro-inflammatory mediators are released from immune cells. Synthesis and release of anti-inflammatory and analgesic molecules also occurs (Ren*et al.,* 2010).

Activation of immune system is accomplished via Toll-like receptors (TLRs) located in immune cells (macrophages, dendritic cells, monocytes), which recognize and bind pathogens and molecules that release damaged cells. When a ligand binds to TLR, activation of NF-kB signaling occurs and cytokines, chemokines, bradykinin, vasodilators are released. T-lymphocytes, neutrophils and other cells, migrate at the site of inflammation, and contribute to peripheral sensitization of nociceptors by releasing a variety of factors.

The role of immune cells in inflammatory pain can be summarized in Figure 1e. **Mast cells** are granulated immune cells that contain histamine. They contribute to allergic reaction, and immunity as well. In the presence of an antigen, mast cells degranulate and release their content, thus participating in inflammatory events. Degranulation of mast cells occurs through interaction with peripheral nerve terminals. The cell-adhesion molecule N-cadherin that is found in mast cells and primary sensory neurons mediates this interaction and results in increased thermal hyperalgesia (Ren*et al.,* 2010). Monocytes in the presence of inflammation form **macrophages**. They migrate and proliferate at the site of injury to phagocytose. Macrophages are implicated in nerve injury, where they are responsible for allodynia, and pain induced from non-noxious stimuli. Polymorphonuclear **neutrophils** granulocytes migrate at the site of inflammation and associate with inflammatory pain. They produce and release a wide range of mediators to activate immune response (R Amann - Encyclopedia of Pain, 2007 – Springer). Infiltration of **lymphocytes** during inflammation is also observed. Lymphocytes contribute to sensitization of peripheral nociceptors by releasing a number of pro-inflammatory and anti-inflammatory cytokines.

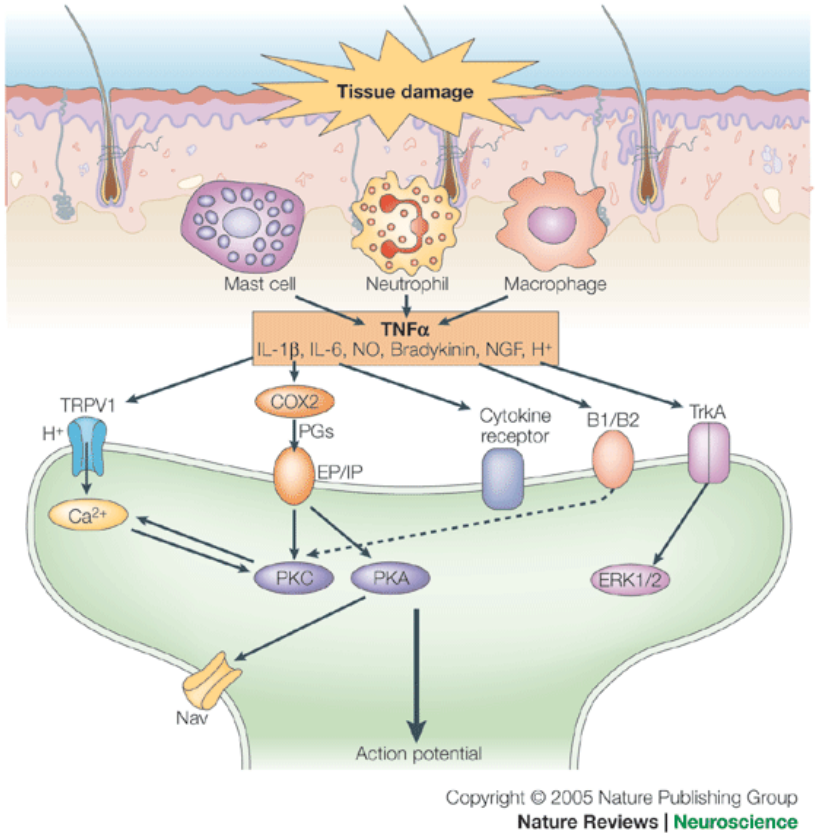


Figure 1e. Pathways activated by immune cells during inflammation (Marchand *et al.,* 2005)

I.5 Inflammatory mediators

During pain generated by tissue damage or infection, there is a state of inflammation in which a variety of chemical mediators are released and alter the functions of peripheral afferent fibers. Inflammatory mediators can also alter vascular permeability and blood flow, and activate the immune cells to migrate at the site of inflammation and produce other chemical mediators. This cascade of events can lead to overt activation of sensory neurons, or sensitization to non-noxious stimuli, even changes in their phenotype and structure. Some mediators alter membrane permeability and cell excitability by acting directly to membrane's ion channel proteins, but the majority acts through G-protein coupled receptors and activate second messenger pathways. Here are the most widespread mediators of inflammation:

Reactive oxygen species

Reactive oxygen species (ROS) are produced by the electron transfer reaction within the cell, and include hydrogen peroxide, superoxide, and hydroxyl species. During ischemia caused by tissue injury, concentration of ROS is decreased leading to suppression of enzymes with anti-oxidative properties such as superoxide dismutase and catalase. The consequence of this is oxidative stress, where oxygen and nitrogen species are overproduced and activate Nf-kB, AP-1 and other pathways. This results in activation of tissue-repair enzymes, production of cytokines, growth factors, and adhesion molecules (Dray, 1995).

There is not many information about direct activation of nociceptors by ROS during inflammation, but it has been observed that ROS enhance activation of other inflammatory molecules such as bradykinin, prostaglandin E_2 (PGE_2) and nitric oxide (NO). NO is synthetized from L-arginine by the enzyme nitric oxide synthases (NOS). It is responsible for a delayed burning pain during an injection, and activates cerebral sensory fibers resulting in release of vasodilator calcitonin gene-related peptide (CGRP). During inflammatory pain or nerve injury a calcium-independent form of NOS, is produced and enhances further NO synthesis. NO also changes the responsiveness of sensory neurons to bradykinin, but its role must be further studied (Dray, 1995).

Bradykinin

Kinins and especially bradykinin are produced from blood and other tissues. They are present on tissue injury and contribute to inflammatory pain situations. They promote the release of cytokines, prostanoids, and free radicals from many cell types, and stimulate postganglionic sympathetic neurons to affect blood vessel caliber. Bradykinin causes degranulation of mast cells and histamine release, and extravasation by contraction of vascular endothelial cells (Dray, 2005; Kidd *et al.,* 2001). This molecule contributes to inflammatory pain, as its administration to humans causes pain, inflammation and hyperalgesia.

Bradykinin, callidin and their degradation products, affect primary afferent neurons, direct or indirect. Antagonists for B2 receptor, which has affinity for bradykinin and callidin, block inflammatory hyperalgesia in animal models of inflammatory pain (Dray *et al.,* 1993). B2 receptor in produced from neurons, and other cells. The degradation product of bradykinin, des-Arg^{9}bradykin binds to B1 receptors. Agonists for B1 receptors, induce pain only in the presence of inflammation, so it's possible that sensitization or enhanced expression of B1 receptor is required. B2 receptors are GPCRs that act through activation of protein kinase C (PKC). They are present on sensory neurons, and the pathway of PKC contributes to excitation of afferent fibers.

B1 receptors are not expressed in abundant in healthy tissue, but in prolonged inflammation or infection their number is rapidly increased. B1 antagonists produce also analgesia. Contribution of B1 receptors in pain is thought to be through other molecules that are released from leucocytes and macrophages, as there is no evidence of direct activation of sensory neurons by them (Dray, 1995).

Prostaglandins

Prostaglandins belong to prostanoids, agents that play role in inflammation, pain, and fever. They come from arachidonic acid by the enzymes cyclo-oxygenase 1 (COX-1), and cyclo-oxygenase (COX-2) that is expressed in peripheral tissues by inflammatory mediators. Prostaglandins usually directly affect nociceptors by reducing their activation threshold, but they can act as sensitizing agents too, by reducing the activation threshold for TTX-R sodium channels, through PKA pathway. Prostaglandin-2 (PGE_2) evokes the release of substance P from neurons in culture, maybe due to an increase in membrane conductance to Na^+ (Kidd *et al.,* 2001). EP

receptor for PGE_2, and IP for prostacyclin (PGI_2), are the most important for their effect on sensory neurons. Subtypes of EP receptors have been fount in small sensory neurons.

Adenosine triphosphate (ATP)

Adenosine triphosphate when administered intradermal induces sharp pain, via activation of sensory neurons and causes an increase in their permeability to cations. Adenosine triphosphate binds to A_2 receptors, GPCRs that are connected with adenylatecyclase, so their activation increases cAMP, and leads to events that cause overt activation of afferent fibers. Activation of A_1 receptors, results in reduced afferent excitability because of the reduced conductance to Ca^+ and increased permeability to K^+.

Histamine and serotonin

Inflammatory mediators such as substance P, NGF, trigger degranulation of mast cells, resulting in histamine and serotonin release. Histamine binds to its receptors H1 and induces increased membrane permeability to calcium in sensory neurons. This process it is possible to evoke the release of neuropeptides and other inflammatory mediators responsible for pain and hyperalgesia when the concentration of histamine is high, and itching when the concentration is low (Dray *et al.,* 1995). Serotonin binds to 5-HT_3 receptor, and causes an increase in sodium permeability, resulting in excitation of sensory neurons.

Cytokines

Many cytokines are released from immune cells at the site of inflammation, enhancing mechanisms of initiation and maintenance. Their action seems to be mostly indirectly by increasing the expression of NGF and neurokinin receptors, triggering the release of prostanoids. There is a hypothesis that suggests their direct binding to nociceptors of sensory neurons, but there no many data to support this. In acute inflammation, their role correlates to phosphorylation and sensitization of ion channels, while in chronic pain cytokines contribute to transcriptional up-regulation of receptors and second messenger pathways. Administration of pro-inflammatory cytokines like TNF-a, IL-6, causes hyperalgesia, which is diminished by antibodies against them (Dray, 1995; Kidd *et al.,* 2001).

Neuropeptides

The neuropeptides substance P and CGRP can be released from the periphery, and the terminals of small sensory neurons too. In the periphery they contribute to inflammation and hyperalgesia, while in the center they are responsible for pain signal transmission. CGRP causes vasodilatation, and together with substance P, plasma extravasation.

Substance P was discovered from Euler and Caddum in 1993 and is a member of tachykinin neuropeptides, derived from preprotachykinin-A gene. It exerts its effect by binding to three types of neurokinin receptors (NK-R), NK-1R, NK-2R, and NK-3R. Neurokinin receptors are GPCRs that belong to the family of guanine nucleotide binding-coupled receptors. Their structure consists of seven trans-membrane domains that form a-helix, an extracellular amino-terminal domain, and an intracellular carboxyl-terminal domain. In low ligand concentration, substance P has high affinity for NK-R1, while in high concentration tachykinins including substance P, bind to all neurokinin receptors (O' Connor *et al.,* 2004). Synthesis of substance P takes place in DRG neurons. It is released from primary afferent neurons to transmit pain signals that produced by the activation of peripheral nociceptors from noxious stimuli (Tang *et al.,* 2007).

The involvement of substance P in inflammation becomes clear from the extremely high concentration of the peptide and its receptors at the site of inflammation. This peptide induces proliferation of immune cells, vasodilatation and migration of leucocytes and macrophages to the inflamed tissue. Additionally, it causes chemo-attraction and degranulation of neutrophils, and triggers the release of cytokines, serotonin and histamine from mast cells, thus it enhances inflammation. Substance P implicates also to proliferation of endothelial cells, fibroblasts, smooth muscle cells, and plays a role in angiogenesis, thus suggesting its role to tissue repair too (O' Connor *et al.,* 2004).

Growth factors

Growth factors like NGF, contribute to the inflammatory process. The mRNA and protein of NGF and its receptor TrkA is found in many cell types, and many inflammatory mediators increase its concentration. The role of NGF in pain is obvious, thus topically administration of NGF produces hyperalgesia, and this effect seems to be via direct sensitization of nociceptors, as many of them express TrkA, but

also through activation of other molecules too. Mast cells and sympathetic ganglionic neurons express TrkA, and as a result NGF may causes degranulation and release of histamine and other molecules, enhancing inflammation and pain indirectly. In addition, NGF knockout mice appear to have no small caliber sensory neurons, and hypoalgesic behavior, whereas animals with over-expression of NGF exert hyperalgesic behavior. The same behavior exhibits mice lacking NGF receptor gene, TrkA (Macmahon, 1996).

NGF released from keratinocytes, increases within few minutes at the site of inflammation and in later stages its levels are found elevated in sensory neurons and DRG cells. At early stages of inflammation, tyrosine phosphorylation of many targets occurs, because of the activation of TrkA by NGF. Later in sensory neurons it has been observed that NGF causes up-regulation of substance P and CGRP via TrkA, and affects expression of many genes in these neurons (Macmahon, 1996). This indicates that NGF is implicated in central sensitization mechanism. Activation of TrkA also is associated to the ras/MEK/ERK pathway, and some studies have shown that ERK inhibitors block the actions of NGF on TRPV1. Exogenous administration of NGF causes an increase in the levels of BDNF that is synthetized by small neurons in DRG (Kidd *et al.,* 2001).

1.6 Inflammatory pain models

To study mechanisms involved in acute and chronic inflammatory pain many experimental pain models have been developed. Their aim is to understand pain at molecular, cellular, electrophysiological and anatomical level, and find out more effective therapeutic approaches. Two of the most famous inflammatory pain models are Carrageenan-induced inflammation and CFA inflammation.

Carrageenan-induced inflammation

Carrageenan belongs to polysaccharides, which are obtained by extraction from certain species of red seaweeds of the class Rhodophyceae. This agent has diverse biological activities including immunomodulatory, anticoagulant, antithrombotic, antiviral and antitumor effects. The antivirus properties of carrageenan include inhibition of the synthesis of viral proteins inside the cells (Necas&Bartosikova, 2013).

Carrageenan-induced paw edema and hyperalgesia is a widely used model to study pain and inflammation, without any injury or damage to the inflamed paw. An intraplantar injection of 1–3% carrageenan in saline in doses of 50–150 μl is commonly used. High dose is immunosuppressive. It produces thermal and mechanical hyperalgesia/allodynia for hours to days post injection. Many inflammatory mediators such as histamine, NO, serotonine and bradykinin are detected in the early phase of carrageenan-induced inflammation. Prostaglandins are detected in the late phase of inflammation, and cause an increase in vascular permeability. Pro-inflammatory cytokines such as TNF-a, IL-1, and IL-6 are also released (Necas&Bartosikova, 2013).

Complete Freund's Adjuvant (CFA)

Complete Freund's adjuvant (CFA) injection is an experimental animal model used to study acute and chronic pain, and also to test the effect of analgesic and anti-inflammatory drugs. CFA is composed of inactivated and dried mycobacteria, usually *Mycobacteria Tuberculosis*. The optimal dose for rat is 100-150 μl, whereas for mice an intraplantar injection of 20-25 μl is used.

Within 2-3 hours after CFA injection, edema and swelling of the injected paw is developed. Redness, allodynia, thermal and mechanical hyperalgesia are also

present, and can last more than seven from the injection. CFA elicits time-dependent spontaneous activity of primary afferent fibers that may underlie injury-induced pain (Okun*et al.,* 2011), and stimulates cell-mediated immunity and robust infiltration of immune cells in injected tissue. It elicits the release of pro-inflammatory mediators such as TNF-a, IL-1β, and NGF in the hind paw (Woolf *et al.,* 1997).

1.7 Inhibition of peripheral sensitization

Inhibition of peripheral sensitization is achieved by different compound categories. Some compounds act to reduce Cox enzymes, responsible for the formation of prostaglandins. Cox blockers exert important analgesic effect, however their clinical application is limited because of their gastrointestinal side effects. Other drugs belong to the category of serotonin-uptake blockers, or inhibitors of pro-inflammatory cytokines, like anti-TNF-a drugs. Cannabinoids are another approach for pain relief. Their receptors CB1 and CB2 are spread through the immune system, while CB2 is expressed from neurons too. Agonists for cannabinoid receptors and systemically administration of their endogenous ligand anandamide, provide analgesic affects (Kidd *et al.,* 2010). The most important category of analgesic molecules includes opiates. Immune cells and neurons produce opioid peptides, and their receptors have been found in peripheral sensory neurons. Their role is being obvious, as agonists for opioid receptors, show anti-nociceptive activity during inflammation.

Opioid receptors

Four main types of opioid receptors have been identified in cellular, molecular and pharmacological level yet: **mu- (MOR)**, **delta- (DOR)**, and **kappa- (KOR)** and **opioid receptor like-1 (ORL1)** (Al Hasani*et al.,* 2013). Additional receptors have been defined, such as epsilon, orphanin, sigma, but they are not included to classical opioid receptors. Opioid receptors belong to the superfamily of G-protein coupled receptors that activate inhibitory G proteins. Their structure consists of a seven transmembrane domain, an intracellular carboxy-terminus domain, and an extracellular amino-terminus domain (Figure 1f). Their genes have 50-70% homology especially in their transmembrane domains and intracellular loops (Jordan *et al.,* 1998), and they have been found in central and peripheral neurons, in immune neuroendocrine and ectodermal cells (Stein *et al.,* 2009). They are found on C and A fibers, on DRG neurons expressing transient receptor potential vanilloid subtype-1 (TRPV-1) and G-protein-coupled inwardly rectifying K^+ (GIRK) channels, and on fibers expressing isolectin B4, substance P, and/or calcitonin-gene-related peptide (Stein, 2013). They can form homodimers and heterodimers with opioid receptors and others like cannabinoid receptors too. Studies have shown that opioid receptors are implicated to analgesia, reward, depression, anxiety, and addiction (Table 1). Their

best-studied role is in pain control, where they inhibit the release of neurotransmitters from dorsal root ganglia in the dorsal horn of spinal cord.

Table 1. Opioid receptors and their actions.

Receptor	Location	Function
delta (δ)	Brain	Analgesia
		Antidepressant effects
		Physical dependence
kappa (κ)	Brain	Spinal analgesia
	Spinal cord	Sedation
		Inhibition of antidiuretic hormone release
mu (μ)	Brain	Supraspinal analgesia
	Spinal cord	Respiratory depression
		Euphoria
		Reduced gastrointestinal motility
		Physical dependence

Table1. Actions of opioid receptors

(http://www.remedicajournals.com/Companion-Animals/Volume-1-Issue-1/Recognition-and-Management-of-Pain-in-Cats-View-PaperOfTheMonth.aspx)

Opioid receptor signaling

When an endogenous or exogenous ligand binds to opioid receptors, the complex GαGβγ subunits that is linked to the receptor, dissociates as a result of GDP replacement by GTP in Gα subunit, and second messenger pathways are activated (Al Hasani *et al.,* 2013; Stein *et al.,* 2009; Jordan *et al.,* 1998). GTP modulates agonist binding to opioid receptor. It was determined that in the presence of GTP the association and dissociation rates of agonist are increased, especially the dissociation rate (Jordan *et al.,* 1998). Agonists and endogenous ligands, stimulate GTPase activity. In addition, it has been observed that stimulation by agonists like morphine, inhibit the production of cyclic adenosine monophosphate (cAMP) (Al Hasani *et al.,* 2013; Stein *et al.,* 2009; Jordan *et al.,* 1998). GTPase activity is obvious in hippocampus and cortex, whereas in frontal cortex inhibition of cAMP occurs. In striatum both events have been observed.

Negatively modulation of calcium, and positively modulation of potassium

channels by opioid receptors is widely accepted too. Activation of the receptor is followed by dissociation of Gα subunit from the complex, which then interacts with G protein gated inward rectifying potassium channel $K_{ri}3$. Deactivation of the channel takes place as GTP is hydrolyzed to GDP. In this way hyperpolarization of the cell occurs and tonic neural activity is inhibited. Activation of opioid receptors causes inhibition of calcium conductance, as a result of Gβγ subunits binding to the channel and reduces the activation of the pore opening. Previous studies have reported also reduction in Ca^{+2} content in synaptic vesicles and synaptosomes, in the presence of opioid receptor agonists. Inhibition of adenylyl cyclase activity results in reduced cAMP-dependent Ca^{+2} influx (Al Hasani*et al.,* 2013). Release of neurotransmitters is also achieved by opioid receptors. For instance substance P release from central and peripheral sensory neurons is inhibited by activation of mu and delta receptor by ligands in analgesic doses, as observed from neurokinin-1 receptor internalization (Kondo *et al.,* 2005).

Internalization of opioid receptors is exerted by arrestins, proteins that bind phosphorylated GPCRs leading them to desensitization. Enzymes such as phosphokinase C, GRK2 and 3, and GPCR kinases increase affinity for arrestins, by phosphorylating opioid receptors. This process desensitizes the receptor by preventing G-protein coupling, and internalization via clathrin-dependent pathways is promoted. The C-terminal of opioid receptors is important for arrestin binding. Resensitization of receptor occurs again by recycling to the membrane. Down-regulation occurs through targeting to the lysosomes (Stein *et al.,* 2009).

Activation of opioid receptors activates MAPK signaling as seen in Figure 1f, which is implicated in cell proliferation, apoptosis, differentiation, transcription factor regulation, and channel phosphorylation. Gene products of MAPK pathway are ERK1/2, JNK, and p38 kinases. ERK1/2 phosphorylation by MOR requires phosphokinase C activation and lasts 5-10 minutes. It also requires ERK1/2 signaling, arrestin and GRK phosphorylation. Activation by DOR requires integrin signal transduction through transactivation by epidermal growth factor. KOR phosphorylates ERK1/2 in two phases, 15 minutes and 2 hours after the exposure to agonist. Activation of the pathway includes an arrestin-dependent late phase, and an arrestin-independent early phase. Gβγ subunit is important in the early phase, and arrestin 3 is required for the late phase. Activation of ERK1/2 BY KOR is mediated by PI3-kinase, PKC, and intracellular calcium (Al Hasani*et al.,* 2013). The JNK and p38

pathways are activated by environmental factors like stress, inflammation, and cytokine production. PI3 is necessary for MOR to activate JNK pathway, but not for KOR, while DOR has been proposed to be PI3-dependent and independent too. MOR and KOR activation, affects p38 pathway through GRK phosphorylation and arrestins (Al Hasani*et al.,* 2013).

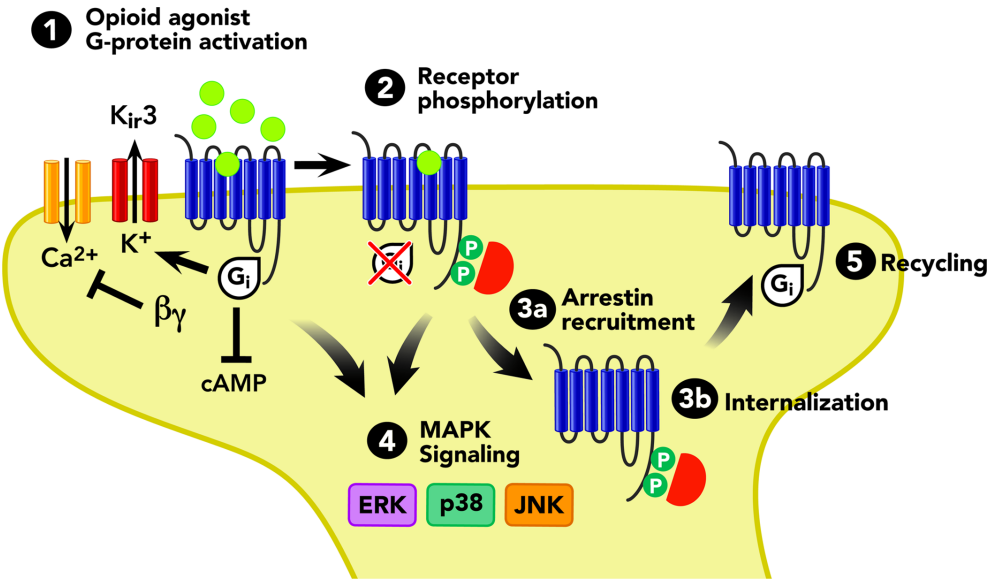


Figure 1f. Summary of pathways activated by opioid receptors (http://www.bruchaslab.org/research/)

Opioid receptors in pain

Opioid receptors are found in small, medium, and large diameter neurons in DRG, together with the neuropeptides substance P and CGRP. During peripheral inflammation and pain, it has been detected up-regulation of the mRNA and the protein of opioid receptors in DRG, and then they are transported to the peripheral nerve terminals. This up-regulation is likely to be mediated by transcription factors, induced by cytokines, which bind to the promoters of the receptor. The axonal transport of opioid receptors is mediated by cytokines and growth factors. They are coupled to inhibitory G proteins, and modulate ion channels activation. Especially, inhibition of calcium channels appears to be of great importance for the inhibition of sensory neurons firing. Opioid receptors also affect tetradotoxin Na^+ currents, and TRPV1, via inhibitory G proteins and the cAMP pathway (Stein *et al.,* 2009). All these information, make it clear that activation of opioid receptors by endogenous or

exogenous ligands, can decrease the excitation of sensory neurons and the release of inflammatory neuropeptides, leading to analgesia.

Opioid peptides

Opioids are used as therapeutics for centuries, for many pain types from mild acute nociceptive pain to chronic advanced or end-state pain. The endogenous ligands for opioid receptors are opioid peptides, enkephalins, dynorphin and β-endorphin. They are derived from three precursor molecules, pro-opiomelanocortin (POMC) for β-endorphin, pro-dynorphin (PDYN) and pro-enkephalin (PENK), for dynorphin and enkephalin, respectively (Mcnally*et al.,* 2002; Stein *et al.,* 2009) (Figure 1g).

The precursor molecules are cleaved by peptidases into smaller molecules, and then a series of post-translational modifications take place to form the final structure and biological activity of the peptides (Froehlich, 1997). Opioid peptides have conserved N-terminal sequence of Tyr-Gly-Gly-Phe- (Met or Leu), which is followed by a variety of C-terminal extensions that produce peptides ranging from 5 to 31 residues in length. Opioid peptides exhibit different affinities for opioid receptors; β-endorphin exhibits high affinity for MOR and DOR and low for KOR, enkephalins are characterized by high affinity for DOR and very low for KOR, while dynorphins bind strongly to KOR, and low to the other two (Stein *et al.,* 2009; Lesniak*et al.,* 2001). Two additional ligands for MOR have been isolated from bovine brain, endomorphin-1 and endomorphin-2, but their precursor has been not extensively studied yet. Other peptides that show affinity for opioid receptors are amphibian dermorphin and deltorphin, milk digestions like casomorphins, gluten digestions such as gluteomorphins.

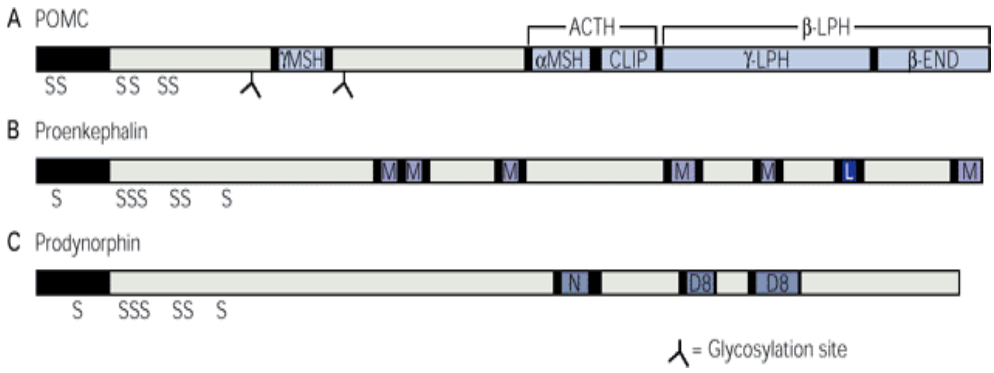


Figure 1g. The three precursors of opioid peptides

(http://www.ib.cnea.gov.ar/~redneu/2013/BOOKS/Principles%20of%20Neural%20Science%20%20Kandel/gateway.ut.ovid.com/gw2/ovidweb.cgisidnjhkoalgmeho00dbookimagebookdb_7c_2fc~30.htm)

Opioid peptides are distributed in different regions of the brain, so they are implicated in many different physiological functions. Enkephalins are key elements in pain perception by acting in the spinal cord and in the periaqueductal gray (PAG) region of the brain. They affect emotional responses by acting in limbic areas, like the amygdala, while in cardiovascular or respiratory system they trigger responses by acting in the hypothalamus and the brain stem. Neurons that contain enkephalins have short axons, so enkephalins are possible to act near their site of production. Neurons that express β-endorphin are located in the hypothalamus and the brain stem. The dynorphin-containing neurons are found in the hypothalamus. These neurons have long axons that extend to distant brain regions as well as to the pituitary gland, brain stem, and spinal cord. This shows that they can act far from their site of production (Froehlich, 1997). There is evidence that sensory neurons contain opioid peptides, as previous studies support that in DRGs have been identified dynorphin and enkephalins. It is suggested that they can be transported to central and peripheral nerve terminals, and modulate neuron's function (Stein *et al.,* 2009).

All types of opioid peptides are found not only in the brain, but also in non-neuronal tissues, where they activate opioid receptors, avoiding the central side effects (Stein, 2013). During inflammation it is known that opioid receptors on sensory nerve terminals are up regulated. This supports the hypothesis that the endogenous ligands, opioid peptides, are present in inflamed tissue. Indeed, many studies describe several transcripts of POMC and PENK mRNA in leucocytes of vertebrates and invertebrates, as well as the prohormone convertases PC1/3 and PC2, necessary for their posttranslational processing. In addition, in lymphocytes obtained from rats with inflamed paw, POMC has been detected too (Stein *et al.,* 2009). It is also important that the endorphin knockout mice are unable to exhibit analgesia during a short stressful swim challenge, indicating that endorphins are crucial for endogenous anti-nociception.

The migration of opioid containing cells at the site of inflammation is exerted by the endothelial cell wall and L-selectins, P-selectins, and E-selectins. Endothelial and inflammatory cells contain and release chemokines that activate leukocytes. As a

result, integrins are increased and mediate the adhesion of leukocytes to endothelial cells via molecules such as the intercellular adhesion molecule-1 (ICAM-1). The secretion of opioid peptides from leukocytes is triggered by corticotropin releasing hormone (CRH) and interleukin-1β, in a process that is calcium-dependant (Stein *et al.,* 2009). Release of opioid peptides from inflammatory cells is triggered by many endogenous and exogenous factors; viruses, endotoxins, cytokines, corticotropin releasing hormone (CRH) and adrenergic agonists, but the most important of them is stress. Stress has been indicated to inhibit pain, as a study conducted by Macelska in 2003, showed that in rats with unilateral hind paw inflammation stress, induced by cold water swim, potent antinociception in inflamed but not in the contralateral non inflamed paws is elicited. The contribution of the opioid system at early stages of inflammation is mostly central, while prolonged inflammation activates peripheral mechanisms of opioid anti-nociception. The peptide that is involved in greater extend to peripheral anti-nociception is β-endorphin, but Met-enkephalin, dynorphin and endomorphins also contribute (Stein *et al.,* 1990).

1.8 Neurosteroids

Hans Selye in 1941 was the first to study neurosteroids and he found that progesterone, dehydroepiandrosterone (DHEA) and many metabolites exhibit hypnotic and anesthetic action. In 1987 Baulieu & Robel in experiments with rats where they removed adrenal glands, the main tissue of production and secretion of steroid hormones, observed that the concentration of these steroids was higher in the brain than in the blood of rats (Corpëchot *et al.,* 1981, 1983). This led to the conclusion that these steroids are synthesized in the brain. The term neurosteroids refers to those steroids, synthesized in the nervous system by neurons and glial cells, either de novo from cholesterol or metabolic precursor molecules that reach the brain with blood circulation. These steroids accumulate in the nervous system at concentrations which are at least partially independent of the rate of excretion of steroids from glands primarily responsible for steroidogenesis. Another term used to describe neurosteroids is the neuroactive steroid. This refers to all natural or synthetic steroids that can alter the excitability of neurons by binding to membrane receptors. This term is used to indicate particular steroids with biological activity in the nervous system (Paul & Purdy, 1992).

Biosynthesis

Neurosteroids are produced in the central and peripheral nervous system from specific types of cells such as oligodendrocytes, the Schwann cells, astrocytes and neurons. They derive from cholesterol (Figure 1h) which is synthesized in adrenal glands and is capable to cross the blood-brain barrier. The first step in their synthesis is the transfer of cholesterol from the outer to the inner side of the mitochondrial membrane. Degradation of the side chain occurs, resulting in the formation of prognenolone (PREG) and dehydroepiandrosterone. For the conversion of cholesterol to prognenolone cytochrome P450scc is necessary, which is located in the mitochondria of the glands that secrete hormones and glial cells, but also in the white matter of the brain. Besides cholesterol, each neuroactive steroid requires a unique combination of enzymes and other molecules to have its specific activity (Vander, Sherman, & Luciano, 2001). PREG can be converted either to DHEA by cytochrome P450c17, through Δ5 pathway or androgen through Δ4 pathway. DHEA is converted either androgen by the enzyme 3b HSD (3b-hydroxysteroid dehydrogenase), thus the

final product is testosterone, or the more stable sulfide ester of DHEAS (DHEA sulfate), the enzyme sulfotranferase. Progesterone is metabolized to glucocorticoids, mineralocorticoids or neuroactive steroids from a variety of enzymes. The synthesis of glucocorticoids (corticosterone in rodents and cortisol in humans) and mineralocorticoid (aldosterone) requires hydroxylation at position 21, which is mediated by the microsomal enzyme R450c21and then R450c11.

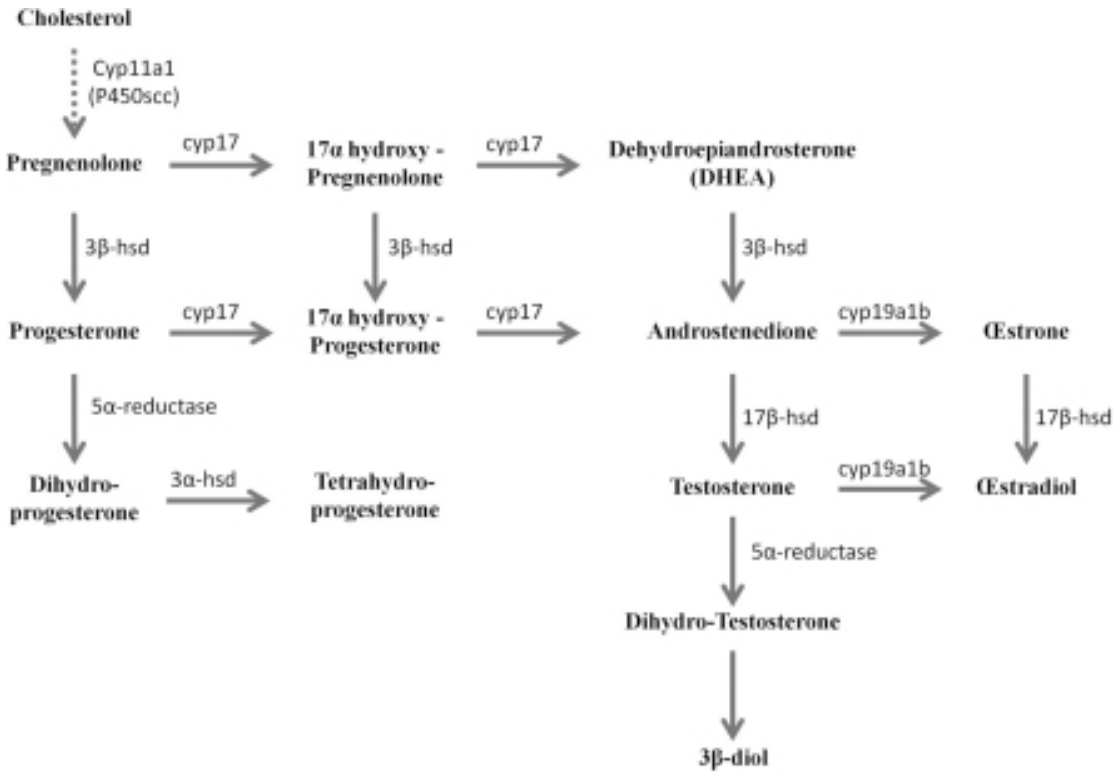


Figure 1h. Biosynthetic pathway of neurosteroids (Diotel *et al.,* 2011)

Effect of neurosteroids

Neurosteroids exert either endocrine or paracrine action in the nervous system. They can affect neurotransmission via interaction with membrane ion channels or through interaction with other neurotransmitters. Neurosteroids are also able to bind to cytoplasmic receptors and enter the nucleus, or bind direct to the nucleus. In this way they control gene expression. DHEA in some cases act as allosteric regulator of neurotransmitter receptors, such as GABA (Herd, Belelli, & Lambert, 2007; Hosie, Wilkins, da Silva, & Smart, 2006; Majewska, Harrison, Schwartz, Barker, & Paul, 1986; Puia et al., 1990), the NMDA (Wu, Gibbs, & Farb, 1991) and the sigma

receptors (Maurice, Junien, & Privat, 1997), while PREG acts as a negative regulator of $GABA_A$ receptors (Majewska, Bisserbe, & Eskay, 1985; Rabow, Russek, & Farb, 1995) and AMPA receptors (Shirakawa, Katsuki, Kume, Kaneko, & Akaike, 2005; Wu & Chen, 1997), while as a positive modulator of NMDA receptors (Bowlby, 1993; Horak, Vlcek, Petrovic, Chodounska, & Vyklicky, 2004). Neurosteroids seem to have a very broad range of potential therapeutic effects in conditions such as epilepsy (Reddy & Rogawski, 2009) and brain injuries (Dubrovsky, 2005; Morrow, 2007), and as mentioned above, the occurrence of neurodegenerative diseases. Such molecules are able to protect a variety of cell types from apoptosis that results due excitoxicity, ischemia, oxidative stress, etc. They contribute to injured tissue repair processes, aging, and have antioxidant and anti-inflammatory properties. Also play an important role in enhancing memory, and experiments in rats have shown that they inhibit the action of drugs causing amnesia.

In recent studies it was indicated that DHEA binds also to GPCRs. DHEA mimics the anti-apoptotic action of the neurotrophic factor NGF. In more details, in cells of pheochromocytoma of the adrenal gland that express functional forms of NGF receptors, TrkA and $p75^{NTR}$, deletion of TrkA expression inhibited the anti-apoptotic effect of NGF and DHEA and reduced the expression of anti-apoptotic protein Bcl-2. In cell lines expressing only the $p75^{NTR}$ receptor, administration of NGF promoted apoptotic cell death, which is inhibited by co-expression of the TrkA receptor. In addition, the absence of NGF in sympathetic and parasympathetic neurons can be compensated by DHEA. DHEA also prevents apoptotic cell death. Therefore neuroactive steroids can act as neurotrophic factors (Lazaridis *et al.,* 2011).

DHEA and DHEA-S are lipophilic steroids synthesized in neurons and glia, and have the highest concentration in the peripheral circulation during the stages of growth and puberty. They are secreted mostly by the adrenal glands, but also in the brain. It has been found that their concentration decreases with age, so they are called the hormone of youth, and have been associated with neurodegenerative diseases such as Alzheimer (Charalampopoulos, Remboutsika, Margioris, & Gravanis , 2008; Kroboth, Salek, Pittenger, Fabian, & Frye, 1999). The enzyme that catalyzes the conversion of DHEA to DHEA-S is hydroxysteroid soulfonotransferase, located in the brain and can readily cross the blood brain barrier (Aldred & Waring, 1999). The reverse process is catalyzed by a sulfatase, therefore these two molecules have the ability to interconverted and are closely intertwined.

These neurosteroids have beneficial properties such as they improve appetite, prevent cancer, improve skin appearance, and in countries like the United States they are given without a prescription. They contribute to pain control, as it has been demonstrated that they are upregulated in the spinal cord during caraggenan-induced inflammation, and potentiate the inhibition of thermal hyperalgesia (Poissbeau *et al.,* 2005). The neuroprotective actions are another area of concern as it has been observed that they protect the brain from ischemia and stroke, oxidative stress, and demyelination (Charalampopoulos *et al.,* 2008b). Also it has been observed that DHEA inhibits pro-inflammatory cytokines like IL-6 and TNF-a through inhibition of NF-kB, and its levels and secretion from adrenals are found decreased during chronic inflammation (Straub *et al.,* 2000). Another study showed that chronic administration of DHEA increased and maintained elevated the basal pain thresholds in neuropathic and control rats. This suggests that androgenic metabolites generated from daily-injected DHEA exerted analgesic effects while DHEA itself, before being metabolized, induced a rapid pro-nociceptive action (MensahNyagan*et al.,* 2009). Yet DHEA and DHEA(s) are dangerous due to the metabolism of DHEA into androgens and estrogens, resulting in hormone-dependent tumors, particularly in individuals with genetic predisposition

The analog of DHEA BNN-27

Neurosteroids and DHEA are promising molecules for the treatment of several neurodegenerative and inflammatory diseases, nevertheless as mentioned earlier the use for a long time is likely to result in pathological conditions such as tumors because of their ability to be metabolized in androgens and estrogens. So it was necessary to create molecules mimicking the beneficial effects of DHEA, lacking, however, its side effects. The laboratory of Theodora Kalogeropoulou worked on the synthesis of such analogs that exhibit changes in C-17 chain of DHEA as shown in Figure 1j, so the metabolism in androgens and estrogens is prevented. The DHEA analog BNN27 is able to protect neural crest cells by suppressing the expression of anti-apoptotic proteins Bcl-2, in conditions of food deprivation (Calogeropoulou *et al.,* 2009). It has the same anti-apoptotic effects with DHEA and NGF. BNN27 binds to TrkA/$p75^{NTR}$, activating both receptors (TrkA phosphorylation, $p75^{NTR}$ dissociation from effector RhoGDI), but it does not bind to ERalpha, ERbeta, AR, TrkB, TrkC. In a model of experimental encephalomyelitis BNN27 managed to reverse myelin holes

in the spinal cord, and increased anti-inflammatory cytokines such as IL-10.

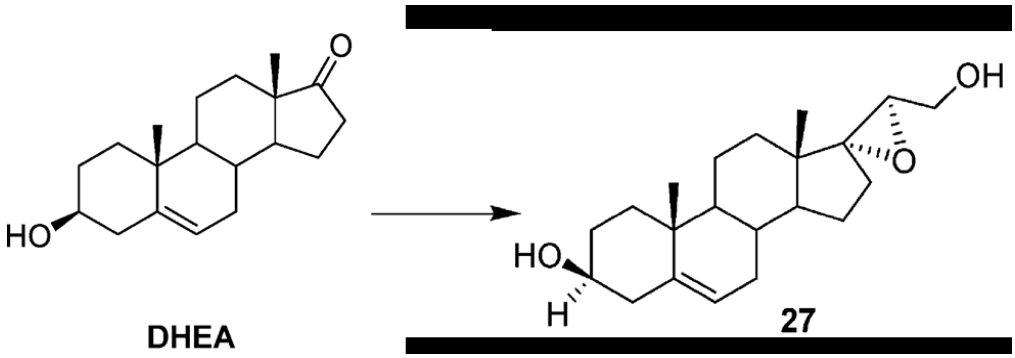


Figure 1j. Structure of DHEA and his analog BNN27

Aim of the study

The information provided above suggests that neurosteroids are increasingly promising molecules for the use of treatment of inflammatory conditions and their complications. Therefore, the aim of the present study was to investigate the role of the new synthetic compound BNN27 in CFA-induced inflammation and hyperalgesia. Specifically, our studies were focused:

1) On the effect of BNN27 on basal and inflammatory pain.
2) On the contribution of BNN27 in the release of pro- and anti-inflammatory cytokines.
3) On the role of NGF in inflammatory pain, in BNN27-treated animals.
4) On the contribution of opioid peptides and receptors in inflammatory pain, following BNN27 administration.

Materials and Methods

2.1 Experimental animals

Experiments were carried out in male and female wild type mice of C57BL6x1291Sv genetic background, 8-12 weeks old. Mice were housed 5-6 per cage according to their gender, 21 days after their birth. They were maintained on a 12 h light/dark schedule with food and water ad libitum. Room temperature was maintained at 22 ± 2°C. All experiments and animal care had been approved from the Committeen of Experimental Animal Protocols of the University of Crete, the Veterinary Department of Crete, and were in accordance with the International Association for the Study of Pain.

2.2 Housing and Acclimation

Mice were housed individually in cages, at least five days before the experiment. This was important to avoid an increase in plasma corticosterone levels derived from stress of being exposed to a new environment. Mice were put for 30 minutes in plantar apparatus (the apparatus used for the evaluation of pain), for five days before the induction of inflammation for acclimation.

2.3 Induction of inflammation and treatments

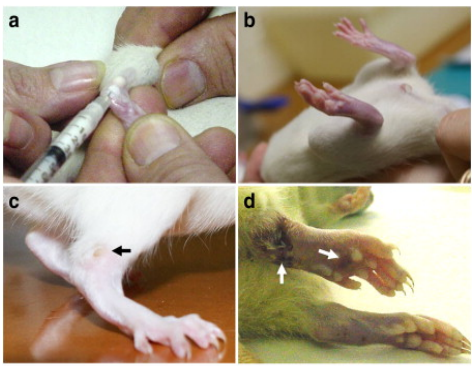


Figure 2.1 Induction of Inflammation with CFA injection

For 4 days separate groups of mice were intraperitoneally (i.p) injected with either 100 mg/kg of the neurosteroid BNN27 diluted in 5% DMSO and water for injection (wfi) in a final volume of 250μl, or with 5% DMSO and wfi in a final volume of 250μl. This group served as the control group. Inflammation was induced with intraplantar (i.pl) injection into the left hind paw of 20μl Complete Freund's Adjuvant (CFA) as seen in Figure 2.1, 24 hours following the last injection of BNN27.

2.4 Nociceptive threshold and paw volume

Thermal nociceptive thresholds were measured using the Hargreaves test (Figure 2.2) at 3, 6, and 24 hours after the last injection of BNN27. These measurements are presented as the basic pain levels. Three measurements were taken from each hind paw, and the average of the three measurements was calculated. The latency (time; s) required to elicit paw withdrawal was measured with an electronic timer after the application of radiant heat to the plantar surface of a hind paw from underneath the glass floor with a high-intensity light bulb. The stimulus intensity was adjusted to 40 IR for female mice and 30 IR for male mice. A cutoff of 20s was set to avoid tissue damage. Twenty-four (24) hours after the last administration of BNN27, mice were injected with CFA as previously described and inflammatory pain levels were measured at 3, 6 and 24 hours after CFA injection. As in the evaluation of basic pain levels, three measurements were taken from each hind paw. The right hind paw which was not injected with CFA served as control.

Figure 2.2 Hargreaves test apparatus

Figure 2.3 Plethysmometer

Paw volume was measured using the plethysmometer (Figure 2.3) at the same time points after the induction of inflammation. The measuring cell consists of two vertical interconnected Perspex tubes; the inflamed paw is dipped in the tube to measure water displacement. A smaller diameter side

tube contains the transducer which measures the conductance between two vertical wire electrodes, proportional to the water level and hence to the displaced volume. The paw volume is shown on the multifunction graphic display in four digits, with 0.01ml resolution. A zero key is provided to zero the meter before each measurement. Two repeated measures were taken from each paw, and their average was calculated to evaluate edema.

2.5 Evaluation of plasma corticosterone levels with RadioImmunoAssay (RIA)

The RadioImmunoAssay technique belongs to the immunoassays where an amount of a specific antibody reacts with corticosterone that is linked to a radioisotope. By adding a larger amount of unlabelled corticosterone, a decreased fraction of radiolabeled hormone binds to the antibody. After separation of bound from free radiolabeled hormone, the amount of radioactivity in one or both fractions is calculated and used to generate a standard curve according to which unknown samples are calculated.

To perform the assay, 5ml of serum were diluted in 1ml steroid solvent. Then, samples of known concentration were diluted for the construction of the standard curve (25, 50, 100, 250, 500 and 1000ng/ml) according to the instructions of the manufacturer. Then, labeled derivative of corticosterone and anti-serum corticosterone were added to all samples except the blind. Samples were vortexed and incubated for 2 hours at room temperature. At the end of the incubation period, precipitation solution was added to all samples. After stirring the material, samples were centrifuged at 1000xg for 40 minutes, at 4^{o}C. The supernatant was removed and the radioactive pellet was counted in a gamma counter. The concentration of corticosterone in each sample was calculated using the calibration curve. The results were expressed in μg/dl. The minimum concentration detectable by this method is 7.7 ng/ml.

2.6 RNA Isolation

Tissues were collected and stored in eppendorfs at -80^{o}C at the end of the experiments. . Five hundred (500) µl of TRIZOL were added to each eppendorf to homogenize the tissues. After the homogenization, samples were centrifuged for 20 minutes, 4^{o}C in 12000 rpm, and the supernatant was placed in a clean eppendorf where 100µl of chloroform were added. Samples and chloroform were stirred for 15 seconds and left on ice for 15 minutes. Samples were centrifuged for 15 minutes, 4^{o}C in 12000 rpm, and the phase containing RNA (upper phase) was transferred in a new epperdorf. Two hundred and fifty (250) µl of isopropanol were added, the mixture was stirred and left for 30 minutes in -80^{o}C for RNA precipitation. After that, samples were centrifuged for 15 minutes; 4^{o}C in 12000 rpm and the supernatant was removed from the tube. The pellet was washed by adding 1ml 75% ice-cold ethanol and centrifuged at 12000 rpm for 5 minutes, 4^{o}C twice. At the end, the pellet was allowed to dry at room temperature and finally dissolved in water for injection, and heated at 65^{o}C for 3minutes. The samples were stored at -80^{o}C until use.

To estimate the quantity and purity of DNA the absorbance at 260nm and 280nm was evaluated, where nucleic acids and proteins absorb respectively. The ratio of the two measurements gives the purity of RNA and should be between 1.5-2. The quantity of RNA is calculated based on the absorption at 260nm.

2.7 DNAse treatment

DNAse is an endonuclease that cleaves DNA non-specifically and gives oligonucleotides with 5'-phosphorylated and 3'-hydroxylated ends. DNAse acts on single stranded and double stranded DNA, chromatin and RNA hybrids. Treatment with DNAse was used for the purification of RNA from genomic DNA. The process included 5 µg RNA, 0.5 µl DNAse, 5 µl of 10x DNAse Buffer I, and ddH_2O in a final volume of 50 µl/reaction. The mixture was incubated for 20 minutes, 37^{o}C, to activate DNAse, and then was incubated for 10 minutes, 75^{o}C to de-activate DNAse.

2.8 Reverse transcription PCR

To enhance, identify and isolate a known sequence of tissue RNA, a method that selectively amplifies specific RNA using polymerase (RTPCR, Reverse Transcription PCR) was used. The PCR reaction follows the reaction of formation complementary DNA (cDNA) using reverse transcriptase. The process is widely used for determining the pattern of expression of a gene.

The reaction started by adding 900ng of total RNA, 1µl Random Hexamers and 0.5µl dNTPs. The mixture was incubated for 5 minutes at 65°C to start the denaturation of RNA and the primers. After that, a second mix containing 2µl 5x Buffer, 0.25µl K RNAse inhibitors and 0.5µl of the enzyme RTase was added to the first mix. The final volume of the mixture was 10µl. The mixture was incubated for 60 minutes at 50^{o}C, where hybridization of the primer and activation of the reverse transcriptase took place. Incubating the samples for 5 minutes at 85^{o}C ended the reaction, and the hybrids were denatured and reverse transcriptase was de-activated. cDNA was either used directly in polymerase chain reaction (PCR) or stored at -20°C.

2.9 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a widely used technique to amplify a DNA sequence. The methods is based on the ability of the chains of double stranded nucleic acids to denaturate and hybridize when incubated at different temperatures. The product is separated and identified after electrophoresis on agarose gel.

The cDNA derived from the RT reaction was incubated in the presence of 200mM of each primer for the desired gene, 200nM of the dNTPs mixture, 1unit of the Platinum Taq Polymerase, a 10x Buffer A containing $MgCl_2$, cDNA and ddH_2O in a final volume of 20µl. The primers that were used for this study, are shown in the table below:

Gene	Sequence of Forward (5'-3') primer	Sequence of Reverse (5'-3') primer	Tm ^{o}C	Product length (bp)	Cycles
mActin	tctctttgatgtcacgcacg	tcagaaggactcctatgtg g	55	500	25

mMOR	acgctcagacgttccattct	tccaaagaggcccactac ac	59	434	40
mKOR	cagctcttggttccccaact g	tgcaaggagcattcaatga catc	59	561	35
mDOR	gtgcaaggctgtgctctcc attg	gtcgggtaggtcaggcgg cagcgccaccg	64	770	30
mPENK	cgacatcaatttcctggcg t	agatccttgcaggtctccc a	60	81	40
mPDYN	tgtgtgcagtgaggattca gg	agaccgtcagggtgagaa aaga	60	138	40
mPOMC	gctgcttcagacctccata gatgtg	cagcgagaggtcgagttt gc	60	121	40
mp75	cctgcctggacagtgttac g	gccaagatggagcaatag aca	60	583	35
mTNF-a	atgagcacagaaagcatg	tacaggcttgtcactcgaa	58	276	40
mNGF	cacccacccagtcttcc	ctcggcattggtctcaaa	58	173	38

2.10 Real-Time PCR

The Real time PCR method is a new PCR method that offers the ability to quantify the results. During this process, the amount of the product is detected by the fluorescence that is emitted by the dye that is linked to it, at each cycle during the reaction and not at the end of the reaction as in conventional PCR. Therefore this method offers several advantages that make it superior over conventional PCR. It is characterized by high sensitivity, accuracy, and repeatability.

For the quantitative Real Time PCR experiments, the dye SYBR Green I was used. The reaction mixture consisted of 1x of 2x Kapa SYBR Green I, 200nM of each primer, 1x of 50x Rox low, 1μl cDNA and ddH_2O in a final volume of 10μl reaction. In the initial stage, the reaction mixture contains the denatured DNA, primers and the dye, where the free molecules fluoresce only pale, giving a very low background signal. This signal can then be removed from the other signals, when they are being analyzed. The hybridization of the primers follows and synthesis of double stranded DNA starts. This forces the free molecules of the dye to bind directly to all duplexes emitting intense fluorescence. After that, an extension period follows when more dye molecules bind to the newly synthesized molecule, leading to an increase in the fluorescence signal. The process is repeated at each cycle after the denaturation step of the two chains of dsDNA.

2.11 Electrophoresis on Agarose gel

For electrophoresis of samples derived from PCR, 12μl of the PCR product, or a marker of known molecular weight were mixed with loading buffer and placed in the wells of an agarose gel. The electrophoresis apparatus was connected to a power supply apparatus that applied voltage to the gel resulting in the separation of DNA. After the separation of the DNA, the gel was exposed to UV radiation, and the DNA became visible due to the addition of ethidium bromide.

In more details, to prepare the gel, 2gr agarose were placed in a conical flask, and 100ml TAE1x were added. The conical flask was shaken gently and placed in the microwave until the complete dissolution of the agarose. The solution should not be boiled for a long time. Then the conical flask was placed in the hood until the solution was cooled to a temperature of about 50^{o}C. At this point, a quantity of ethidium bromide to a final concentration of 500 ng/ml was added. The solution was transferred to a gel tray where specific combs were put to form the wells. The gel was allowed for 30 minutes to solidify, and then it was ready for electrophoresis.

To prepare 1x TAE:

Materials	Final concentration
Tris(pH 8)	10nM
EDTA	10mM
Calcium acetate	5nM
NaCl	10nM

To prepare loading buffer:

Materials	Final concentration
Tris(pH 8)	10nM
EDTA	60mM
Glycerol	60%(v/v)
(OrangeG)	0,15%(w/v)

2.12 Bradford

For the isolation of proteins, tissues were homogenized in 500ml lysis buffer containing PBS and protease inhibitors. The supernatants were collected after centrifugation for 20 minutes, 4^{o}C, 12000 rpm, and stored at -80^{o}C.

Bradford is a colorimetric method, based on the absorption of the dye Coomassie Brilliant Blue G-250, which under acidic conditions its red form is converted to a blue form when bound to the protein. Initially, the red form of the Coomassie gives a free electron in the ionizable group of the protein, which causes cleavage of the protein. After the protein binds to the dye, causes a shift of the maximum absorption of the dye from 365 nm to 595 nm. The increase in absorbance is proportional to the protein that binds to the dye. This results in calculating the total protein of the solution. Evaluation of the amount of protein is feasible by using a standard curve that is made by different quantities of BSA (Bovine Serum Albumin).

2.14 Enzyme-Linked Immunoabsorbent Method (ELISA)

For our study, we used the sandwich ELISA for TNF-a, IL-6, IL-1β and IL-10 (Figure 2.4). This method measures the amount of an antigen between two antibodies. The desired antigen must contain at least two antigenic regions capable of binding to the antibody since at least two antibodies act in this type of ELISA. In this method either monoclonal or polyclonal antibodies can be used to capture and detect antigens. The detection is performed using horseradish peroxidase. The substrate of this enzyme is hydrogen peroxide. The decomposition of hydrogen peroxide is coupled to the oxidation of a hydrogen donor thus inducing a color change during the reaction.

The procedure includes at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid medium (usually one polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via binding with the second antibody for the same antigen, in a "sandwich ELISA"). After immobilization of the antigen, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation. Between each step, the plate is typically washed with a mild detergent solution to remove proteins

and antibodies, which are not specifically bound. After the final wash step, the plate is added the enzyme's substrate to produce a visible signal, which indicates the amount of antigen in the sample. The amount of test antibody was measured by estimating the amount of the colored end product by scanning the optical density of the plate at 450nm.

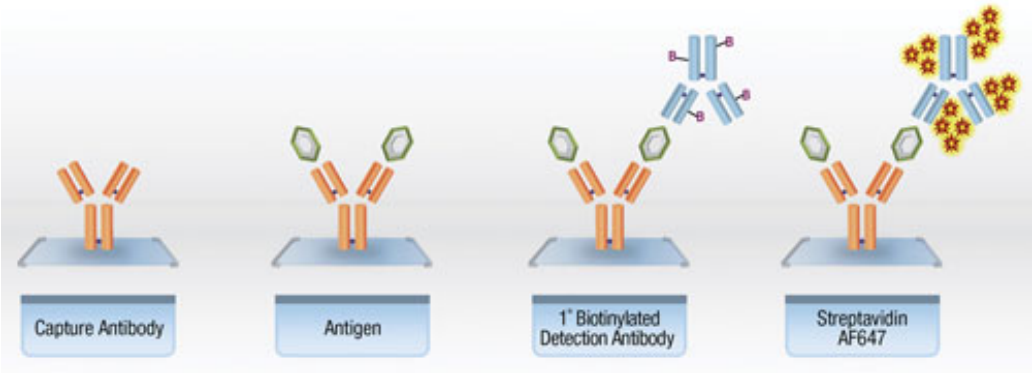


Figure 2.4 Synopsis of sandwich ELISA procedure

Solutions:

• *Wash Buffer (Wash Buffer)*

0.05% (v/v) solution of non-ionic detergent Tween-20 in PBS. The solution was stored at 4°C.

• *Solvent reagents (Reagent Diluent)*

A solution of 1% (w/v) BSA or Assay diluent in PBS. The solution was filtered with a 0.2 micropore diameter and used the day of preparation.

• *Substrate solution (Substrate Solution)*

A solution consisting of equal volumes of hydrogen peroxide (Color Reagent A) and tetramethylbenzidine (Color Reagent B).

• *Stop solution reaction (Stop Solution)*

A solution of 2N sulfuric acid. The solution was stored in a dark container at room temperature.

2.13 Column chromatography

To isolate the intracellular peptide beta-endorphin, the hind paws were homogenized in 500µl of 0.1N HCl. The homogenates were centrifuged for 20 minutes, 4^{o}C, 12000 rpm, and the supernatants were kept at -80^{o}C until use.

The peptide from the homogenates, was concentrated by a C-18 reverse phase column (Sep-Pak, Waters Associates, Milford, MA). Specifically, the supernatants were extracted by activated Sep-Pak cartridges, washed with 20 ml 0.1 N HCI, eluted with 3 ml acetonitrile 80%-0.01% HCI, then dried under vacuum (Speed-Vac) and maintained at -20°C until their final analysis. The activation of the Sep-Pak cartridges was performed with 10ml of acetonitrile and 20ml HCl 0.1N.

2.14 Identification of beta-endorphin

The immunoplate in this kit was pre-coated with secondary antibody and the nonspecific binding sites were blocked. The secondary antibody could bind to the Fc fragment of the primary antibody (peptide antibody) whose Fab fragment would be competitively bound by both biotinylated peptide and peptide standard or targeted peptide in samples. The biotinylated peptide interacted with streptavidin-horseradish peroxidase (SA-HRP) that catalyzed the substrate solution. The intensity of the yellow was directly proportional to the amount of biotinylated peptide-SA-HRP complex but inversely proportional to the amount of the peptide in standard solutions or samples. This was due to the competitive binding of the biotinylated peptide with the standard peptide or samples to the peptide antibody (primary antibody). A standard curve of known concentration could be established accordingly. The unknown concentration in samples could be determined by extrapolation to this standard curve.

The procedure is summarized below:

- 50µl/well of standard, sample, or positive control, 25µl primary antibody and 25µl biotinylated peptide were added
- The mix was incubated for 2 hours at room temperature
- The plate was washed 4 times with 350µl/well of 1x assay buffer
- 100µl/well of SA-HRP solution were added
- The mix was incubated for 1 hour at room temperature

- The plate was washed 4 times with 350μl/well of 1x assay buffer
- 100μl/well of TMB substrate solution were added
- The mix was incubated for 1 hour at room temperature
- The reaction was terminated with 100μl/well of 2N HCL
- The absorbance was read at 450nm

2.15 Tissue preparation for histological studies

To study the differences in the infiltration of different immune cell types in the hind paw of mice treated with BNN27 or not, after CFA inflammation, the tissues were removed and placed in 4% PFA (40ml ddH2O + 4gr PFA → stirring at 55^{o}C + 4 drops of NaOH 4% + ddH2O to a final volume of 50ml. Extra 50ml PB 0.2M were added and the solution was filtered) for 24 hours for stabilization of their structure. Then they were left in 30% sucrose for cryoprotection until they were soak enough and precipitate.

2.16 Tissue Freezing and production of cryostatic sections

For tissue freezing, after the incubation in sucrose, the tissues were covered with OCT compound in dry ice, and stored at -80°C until they were sectioned. To cut the hind paws into sections of 10nm, the cryostat was allowed a few minutes to descend the temperature from -15^{o}C to -25^{o}C. Then the tissue was covered with OCT compound and placed with the desired orientation in the table of the machine.

2.17 Hematoxylin and Eosin staining of hind paws and adrenals

Hematoxylin and Eosin staining is commonly used in medicine and histology. The staining method involves hemateinan oxidation product of hematoxylin, which colors nuclei of cells blue. Counterstaining with eosin that colors eosiniphilic structures such as connective tissue/cytoplasm in other colors like pink or red follows the nuclear staining.

Procedure:

- 2 minutes in 100% ethanol
- 5 dips in ddH_2O
- 30 dips in hematoxylin
- 5 dips in ddH_2O
- 17 dips in eosin
- 5 dips in 75% ethanos
- 5 dips in 80% ethanol
- 5 dips in 95% ethanol
- 5 minutes in xylene
- Covering with endelan and coverslips

2.18 Primary spleen cell culture

To investigate the effect of BNN27 in T-lymphocytes *in vitro*, spleens from control mice and mice treated with BNN27 (both groups were injected with CFA) were isolated.

Procedure:

- Spleens were removed and placed in petri dishes (1 Petri dish/treatment) with 15ml 1x cold PBS..
- Spleens were smashed between the rough surfaces of two slides.
- Spleens were transferred in 50ml falcon where 3ml of Buffer I containing 0.8gr NH_4Cl, 0.084gr $NaHCO_3$ and 0.037gr EDTA in a final volume of 100 ml ddH_2O, were added.
- Spleens with Buffer I were left for 30 minutes in 37 ^oC in waterbath.
- Centrifugation for 25 minutes in room temperature, 1800rpm to remove red blood cells.
- Supernatant was removed, and the pellets were redissolved with Buffer I and PBS.
- Centrifugation for 25 minutes in room temperature, 1800rpm to remove red blood cells.
- Pellets were redissolved in 10ml medium RPMI.
- Centrifugation for 5 minutes in room temperature, 1200 rpm.
- The supernatant was removed and the pellets were redissolved in medium RPMI with 4 μg/ml of the mitogen concanavalin-A, 10% FBS, 1% Penicillin/Streptomycin, and 1mM Sodium Pyruvate.
- after the next day, IL-2 0.5 ng/ml was added to the culture, to stimulate the cells to differentiate as T-lymphocytes.
- The cells were left for about a week to proliferate. In this stage only renewal of their medium took place.

Treatment with BNN27

- The cells were transferred from the flask to 50ml falcon.
- Centrifugation for 5 minutes in room temperature, 1800 rpm.
- The pellet was redissolved in 10ml medium.
- 90μl trypan blue was added in 10μl of cells, and the cells were counted with

hemocytometer.

- Calculation of the number of cells needed to have 250,000 cells/well, and they were placed in 96-well plate.
- Three wells were used for each group (control, control+BNN27, BNN27, BNN27+BNN27)
- The next day, the proper treatment in each group with BNN27 in concentration 10^{-7} in RPMI (the stock was 10^{-2} in DMSO), was performed.
- The cells were collected at 3, 6, 24 hours after the treatment.
- Centrifugation for 10 minutes, 22^{o}C, 1800 rpm.
- The supernatant was kept in -80^{o}C for ELISA.

2.19 Statistical Analysis

The results were analyzed by Graphpad Prism 5.0 using T-test assesses, Microsoft Office Excel and SPSS. Quantification of results from PCR was performed using Tina Scan program. Histological results were analyzed using Image J.

Results

To investigate the role of the neurosteroid analog of DHEA BNN27 in inflammatory pain induced by CFA injection into the hind paw of mice, initially, paw withdrawal latency and paw volume were estimated. To study further this question, the involvement of different pro-inflammatory and anti-inflammatory cytokines and the opioid system were studied at the molecular and biochemical level, using RT-PCR, qPCR, and ELISA, *in vivo* and *in vitro*.

5.1 Evaluation of pain threshold

The first aim was to determine the effect of BNN27 in thermal hyperalgesia, in male and female mice under basal conditions. This goal was achieved using the Hargreaves test and the measurements were taken 3, 6, and 24 hours following the last injection of BNN27 (100mg/kg), as described in Materials and Methods. The results are shown in Figure 5.1. Administration of BNN27 in female mice, for 4 consecutive days, resulted in significantly increased thermal nociceptive threshold 3 and 6 hours after the last administration of BNN27, but not after 24 hours.

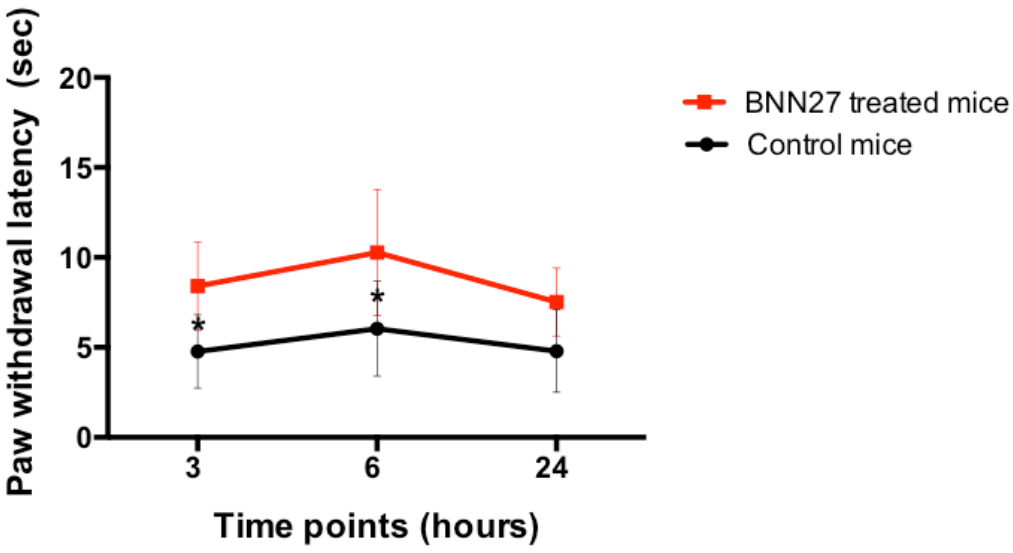


Figure 5.1.**Decreased hyperalgesia in female mice treated with BNN27.** Administration of BNN27 (100mg/kg) reduced basic pain levels as measured by the Hargreaves test 3 and 6 hours after the last treatment. No changes were observed 24 hours after BNN27 injections (Control n=5 BNN27 n=5, $p<0.05$).

In male mice, BNN27 increased significantly paw withdrawal latency at all time points studied, as seen in Figure 5.2

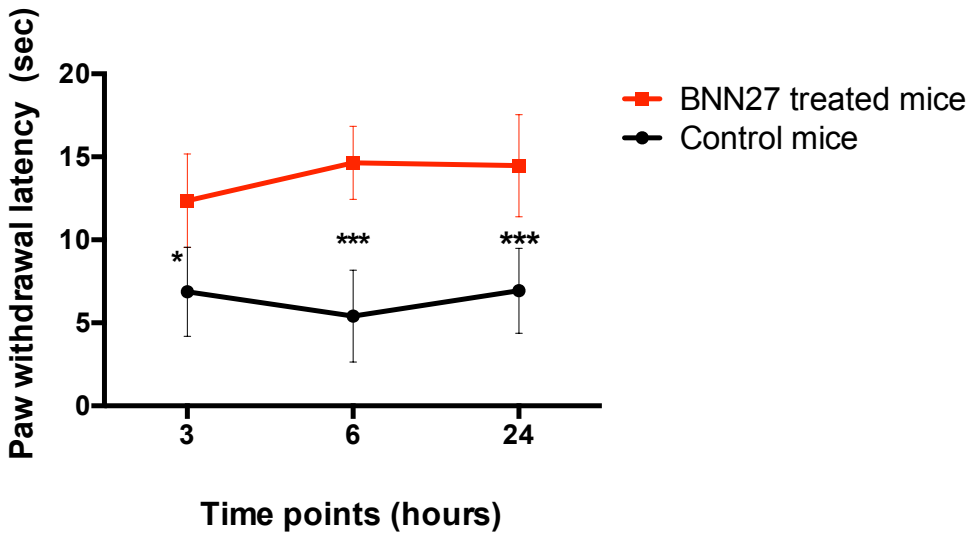


Figure 5.2.**Decreased hyperalgesia in male mice treated with BNN27.** Administration of BNN27 (100mg/kg) reduced basic pain levels as measured by the Hargreaves test 3, 6 and 24 hours after the last treatment (Control n=5 BNN27 n=5, 3 hours $p<0.05$, 6 hours $p<0.001$, 24 hours $p<0.001$).

Following the evaluation of basic pain levels, and 24 hours after the last injection of BNN27, mice were injected with 20 μl CFA into their left hind paw. The inflammatory pain elicited was studied in both sexes (Figure 5.3 and Figure 5.4 for female and male mice, respectively) at the same time points used for the evaluation of basic pain. The results showed that the analog of DHEA exerts analgesic effects during CFA-induced inflammation, in a statistically significant way even 48 hours after the last administration of the compound. In female mice, BNN27 increased paw withdrawal latency at the same levels, at all time points (Figure 5.3), whereas in male mice, the best efficacy of BNN27 was observed 24 hours after the induction of inflammation (Figure 5.4).

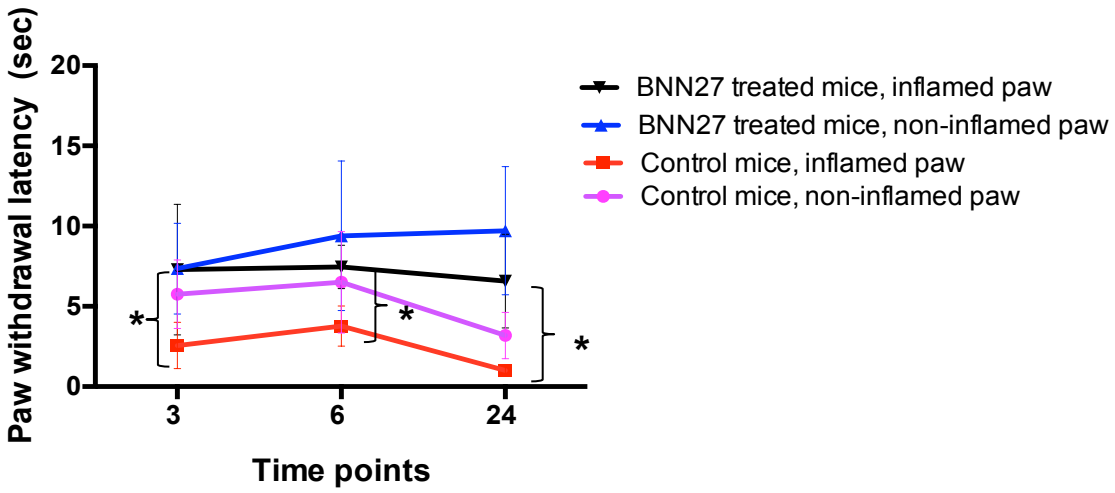


Figure 5.3. **Decreased hyperalgesia in female mice treated with BNN27 after CFA-induced inflammatory pain.** Administration of BNN27 (100mg/kg) reduced inflammatory pain levels as measured by the Hargreaves test at 3, 6 and 24 hours after the induction of inflammation (Control n=5 BNN27 n=5, 3 hours $p<0.05$)

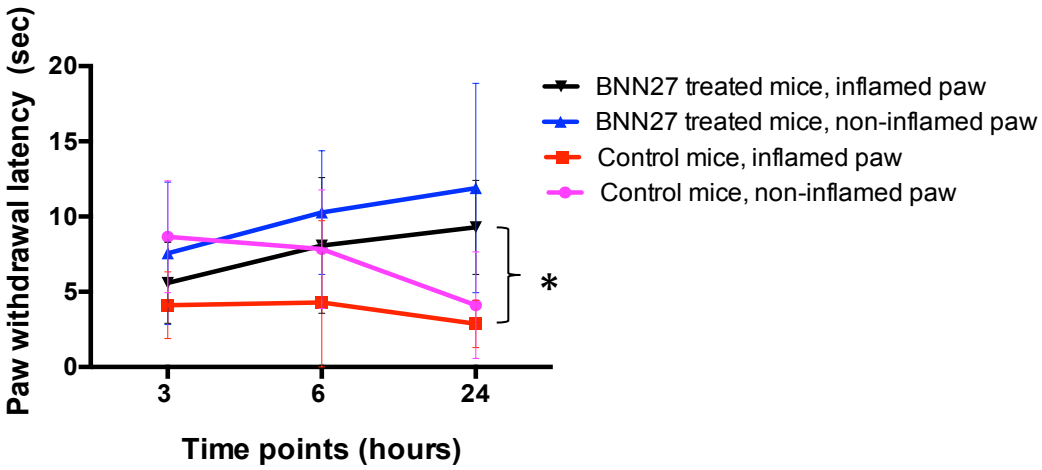


Figure 5.4. **Decreased hyperalgesia in male mice treated with BNN27 after CFA-induced inflammatory pain.** Administration of BNN27 (100mg/kg) reduced inflammatory pain levels as measured by the Hargreaves test at 3, 6 and 24 hours after the induction of inflammation (Control n=5 BNN27 n=5, 24 hours $p<0.05$)

5.2 Corticosterone levels

During CFA induced inflammation, levels of serum corticosterone increase. In our study, BNN27 treatment significantly decreased serum corticosterone levels compared to vehicle treatment (Figure 5.5) without however, affecting adrenal size (Figure 5.6).

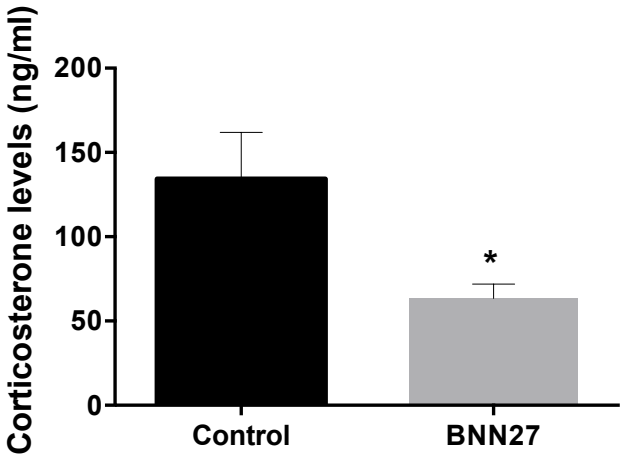


Figure 5.5.**Decreased corticosterone levels after BNN27 administration.** (Control n=9 BNN27 n=8, $p<0.05$, T-test Unpaired)

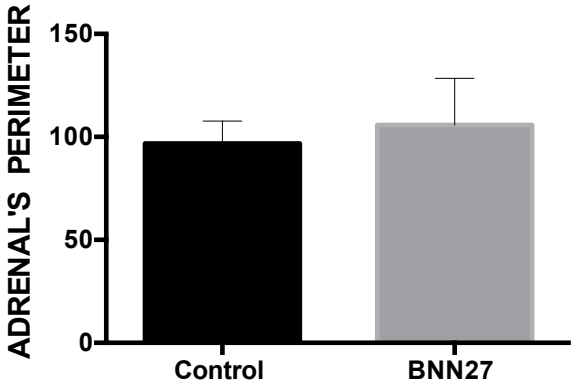
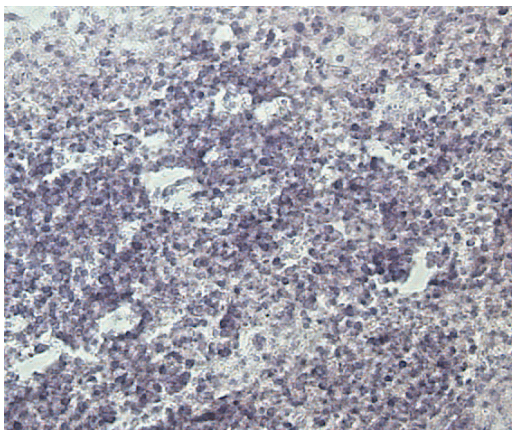


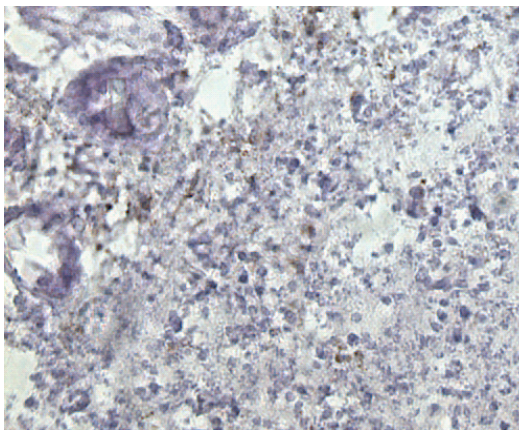
Figure 5.6. **BNN27 did not affect adrenal size.** Adrenal size was measured using Image J software after Hematoxylin/Eosin staining (Control n=10 BNN27 n=10)

5.3 Histological studies in inflamed paw

To observe the infiltration of different inflammatory cell types in the inflamed paw we performed Hematoxylin/Eosin staining on cryostatic sections. Inflamed paw of mice that had been treated with BNN27, seem to have less infiltration of immune cells compared to control mice as shown in the figure below, 24 hours after CFA-induced inflammation.



Inflamed tissue of control mice 24 hours after the induction of inflammation



Inflamed tissue of BNN27-treated mice 24 hours after the induction of inflammation

5.4 Paw volume

Administration of CFA in mice induces edema at the site of the injection (Ren & Dubner, 1990). To investigate if the administration of BNN27 affects paw edema, we measured paw volume using the plethysmometer as mentioned in chapter Materials and Methods. Paw volume was increased, especially 6 and 24 hours following the injection with CFA (Figure 5.7 and 5.8) but to our surprise no changes were observed between mice treated with BNN27 or vehicle. The results were similar in female and male mice (Figure 5.7 and Figure 5.8)

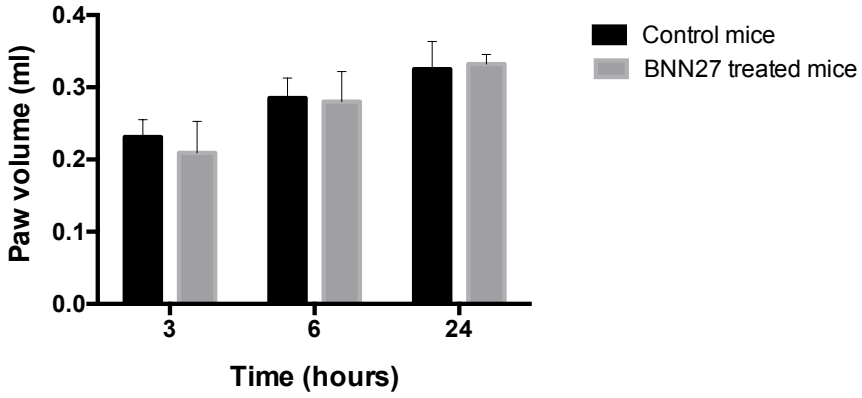


Figure 5.7. **Paw volume following CFA-induced inflammation and treatment with BNN27.** Increased paw volume was observed after the induction of inflammation in female mice. BNN27 did not exert any effect on it. (Control n=5 BNN27 n=5)

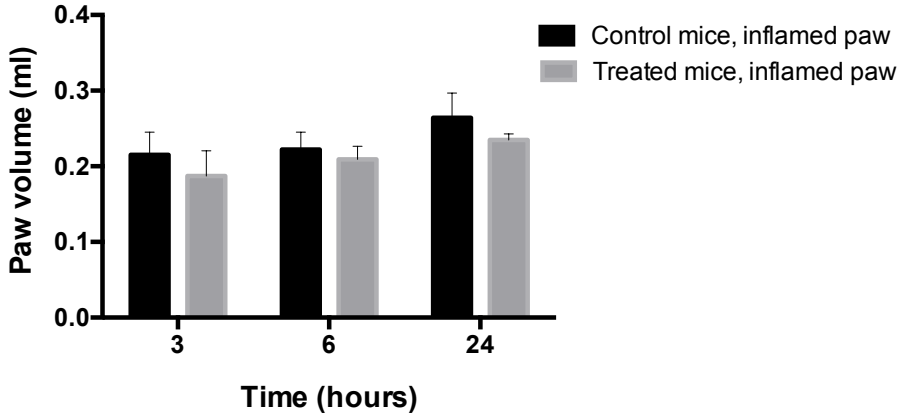


Figure 5.8. **Paw volume following CFA-induced inflammation and treatment with BNN27.** Increased paw volume was observed after the induction of inflammation in male mice. BNN27 did not exert any effect on it. (Control n=10 BNN27 n=10)

5.5 Effect of BNN27 on cytokines at 6 and 24 hours after the induction of inflammation

CFA-induced inflammation provokes the release of many inflammatory mediators such as cytokines, from immune cells and organs topically at the site of inflammation, but also systemically. Here, the role of BNN27 on TNF-a, IL-6, IL-1β and IL-10 production was examined.

IL-6

Administration of BNN27 increased protein levels of IL-6 in inflamed paw at 6 hours following inflammation, while at 24 hours had no effect as shown in Figure 5.9.

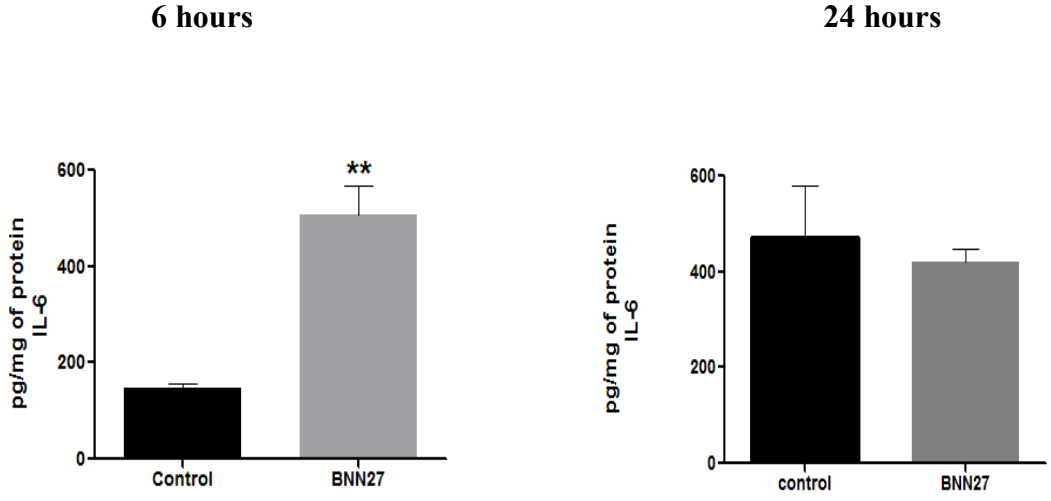


Figure 5.9.**Effect of BNN27 on tissue IL-6.** BNN27 increased protein levels of IL-6, 6 hours following inflammation, while it did not affect it at 24 hours. (6 hours: Control n=3, BNN27 n=3, $p<0.01$, T-test Unpaired, 24 hours: Control n=10 BNN27 n=10, $p>0.05$, T-test Unpaired)

IL-1β

On the other hand, mice treated with BNN27 exhibited similar protein levels of IL-1β in inflamed paw with untreated mice (Figure 5.10).

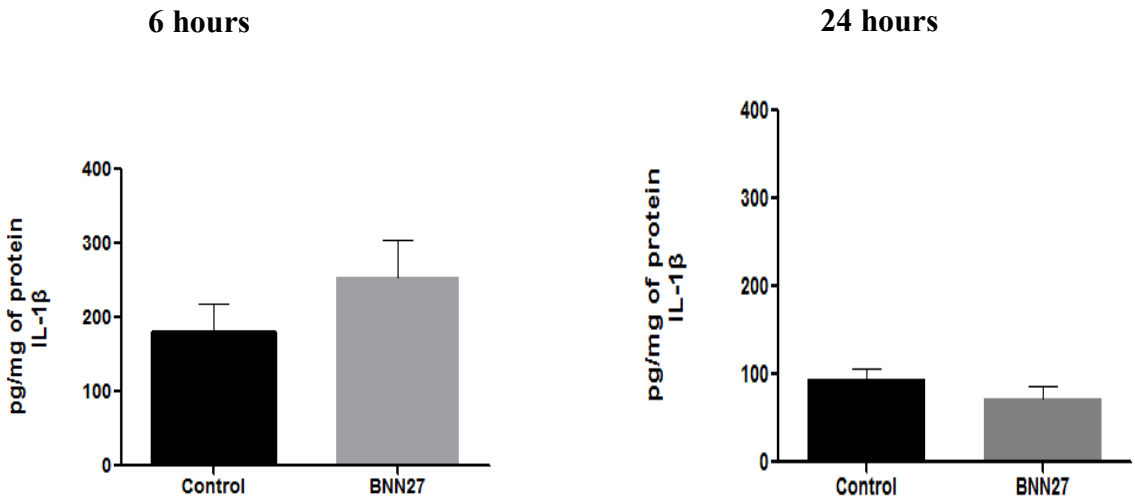


Figure 5.10. **Effect of BNN27 on tissue IL-1β.** BNN27 had no effect on protein levels of IL-1β at 6 and 24 hours following inflammation. (6 hours: Control n=5, BNN27 n=5 $p>0.05$, T-test Unpaired, 24 hours: Control n=10, BNN27 n=10, $p>0.05$, T-test Unpaired)

TNF-a in inflamed paw

TNF-a was also examined in the inflamed paw of mice. Treatment with BNN27 increased protein levels of TNF-a at 6 hours following CFA injection, while at 24 hours following inflammation, BNN27 decreased protein levels of TNF-a (Figure 5.11).

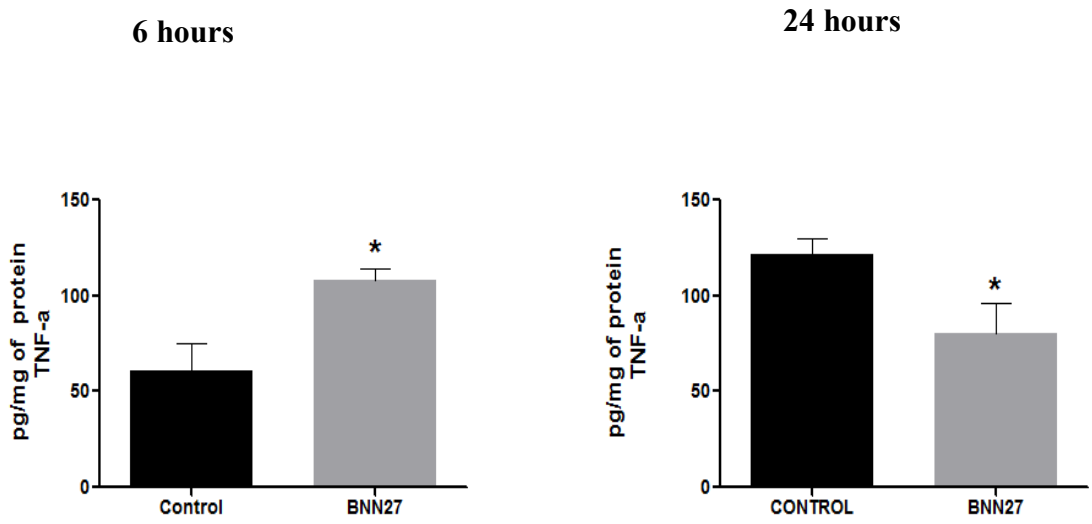


Figure 5.11. **Effect of BNN27 on TNF-a in inflamed paw.** BNN27 increased TNF-a in inflamed paw at 6 hours following inflammation (Control n=4, BNN27 n=4, $p<0.05$, T-test Unpaired). At 24 hours BNN27 decreased protein levels of TNF-a (Control n=5, BNN27 n=4, $p<0.05$, T-test Unpaired)

TNF-a in spleens

Protein levels of TNF-a were also evaluated in spleens, and the results were consistent with the results in inflamed paws. At 6 hours BNN27 increased protein levels of TNF-a in spleens, while at 24 hours BNN27 decreased protein and mRNA levels of TNF-a as shown in Figure 5.12.

Protein levels of TNF-a

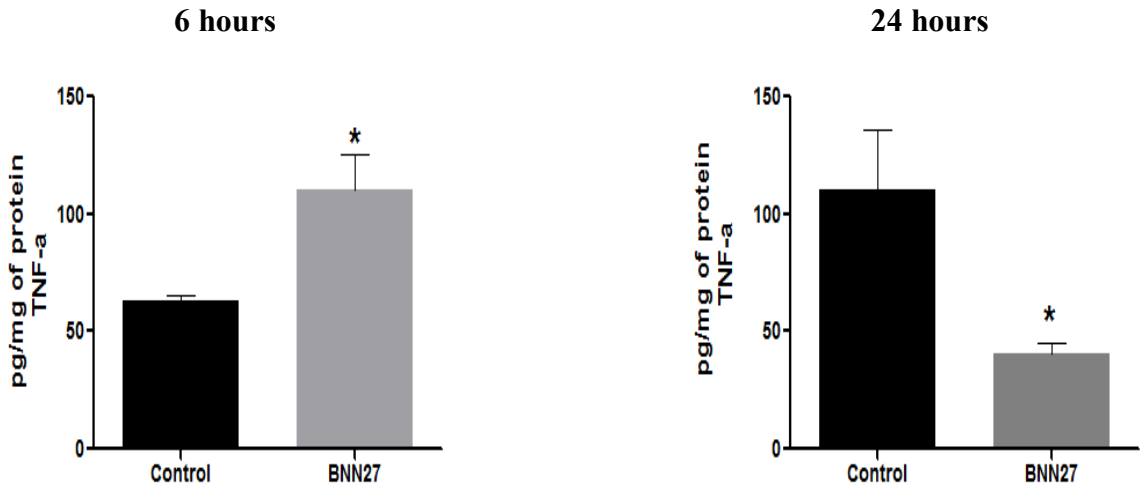


Figure 5.12. **Effect of BNN27 on TNF-a in spleens.** BNN27 increased TNF-a in spleens at 6 hours following inflammation (Control n=4, BNN27 n=4, $p<0.05$, T-test Unpaired). At 24 hours BNN27 decreased protein levels of TNF-a (Control n=3, BNN27 n=5, $p<0.05$, T-test Unpaired)

mRNA levels of TNF-a in spleens 24 hours following inflammation

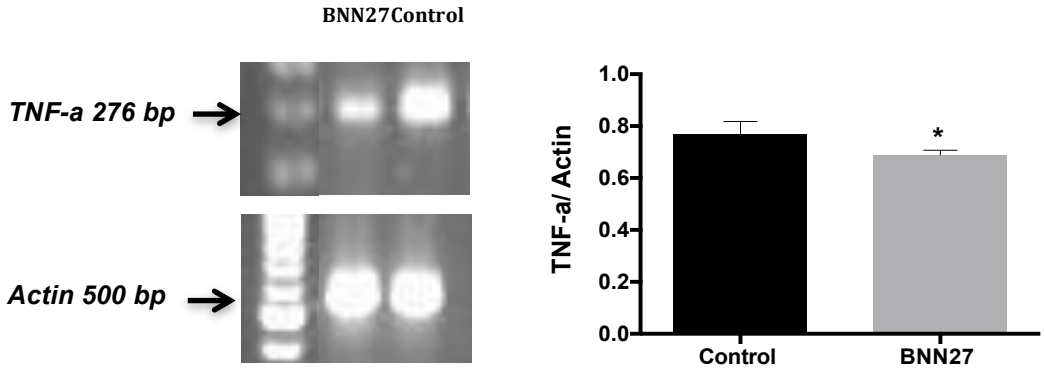


Figure 5.12. **Effect of BNN27 on mRNA levels of TNF-a in spleens.** Administration of BNN27 decreased the transcription of TNF-a gene in spleens 24 hours following the induction of inflammation. (Control n=4, BNN27 n=3, $p<0.05$, T-test Unpaired)

IL-10

BNN27, when administered in mice, increased protein levels of IL-10 in inflamed paw at 6 and 24 hours following the induction of inflammation (Figure 5.13).

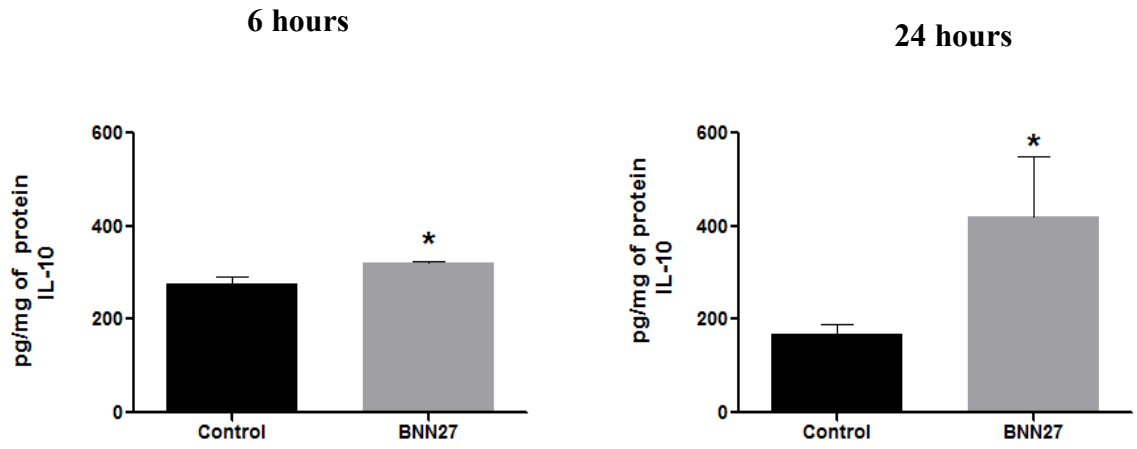


Figure 5.13. **Effect of BNN27 on IL-10.** Administration of BNN27 increased protein levels of IL-10 at all time points examined (6 hours: Control n=3, BNN27 n=3 $p<0.05$, T-test Unpaired, 24 hours: Control n=5, BNN27 n=3, $p<0.05$, T-test Unpaired)

5.6 Effect of BNN27 on NGF at 6 and 24 hours following the induction of inflammation (Inflamed paw)

The neurotrophic factor NGF is synthesized and released from immune cells at the site of inflammation (Woolf *et al.,* 1994; Rueff *et al.,* 1996). NGF and its receptors increase at the hind paw of mice after CFA injections. In DRG of inflamed mice, NGF levels are also increased and cause up-regulation of substance P, the transmitter of pain signal, and other inflammatory mediators (Fbbro *et al.,* 2004; Rueff *et al.,* 1996). Here we examined the mRNA levels of NGF and its receptor $p75^{NTR}$ in DRG and inflamed paw, at 6 and 24 hours following CFA injection. Our results showed that the mRNA of NGF was reduced in DRG obtained from mice treated with BNN27 at all time points. In inflamed paw, BNN27 increased the mRNA levels of NGF at 6 hours following inflammation, while at 24 hours no changes were observed (Figure 5.14 and Figure 5.15). The mRNA of $p75^{NTR}$ remained unchanged in DRG and inflamed paw at all time points examined (data not shown). In addition, BNN27 did not affect the mRNA levels of substance P in inflamed paw and DRG at all time points examined (data not shown).

NGF in inflamed paw

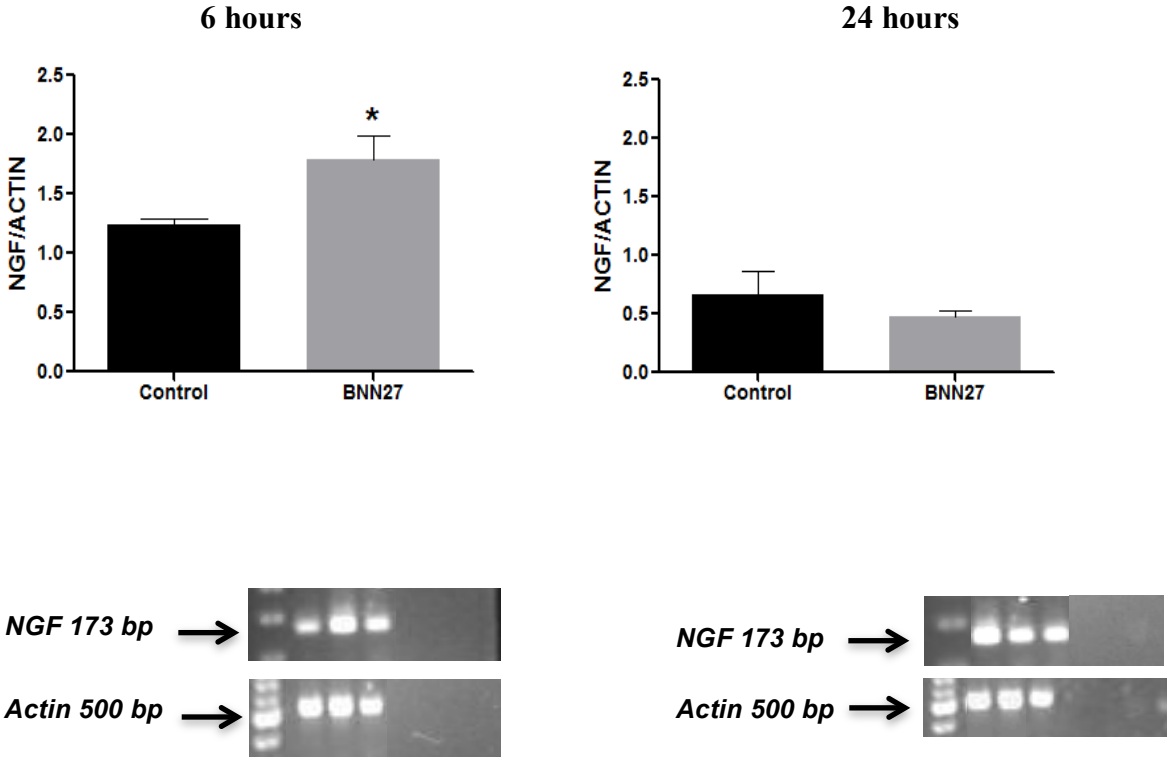


Figure 5.14. **Effect of BNN27 on NGF in inflamed paw.** BNN27 increased mRNA levels of NGF in inflamed paw at 6 hours following inflammation (Control n=4, BNN27 n=4, $p<0.05$ T-test Unpaired). At 24 hours following the induction of inflammation BNN27 did not affect NGF mRNA levels in inflamed paw (Control n=6, BNN27 n=5, $p>0.05$, T-test Unpaired, Order of the samples: 6 hours ladder, Control, BNN27, noRT Brain, noRT, H_2O, 24 hours ladder, Brain, Control, BNN27, noRT Brain, noRT, H_2O)

NGF in DRG

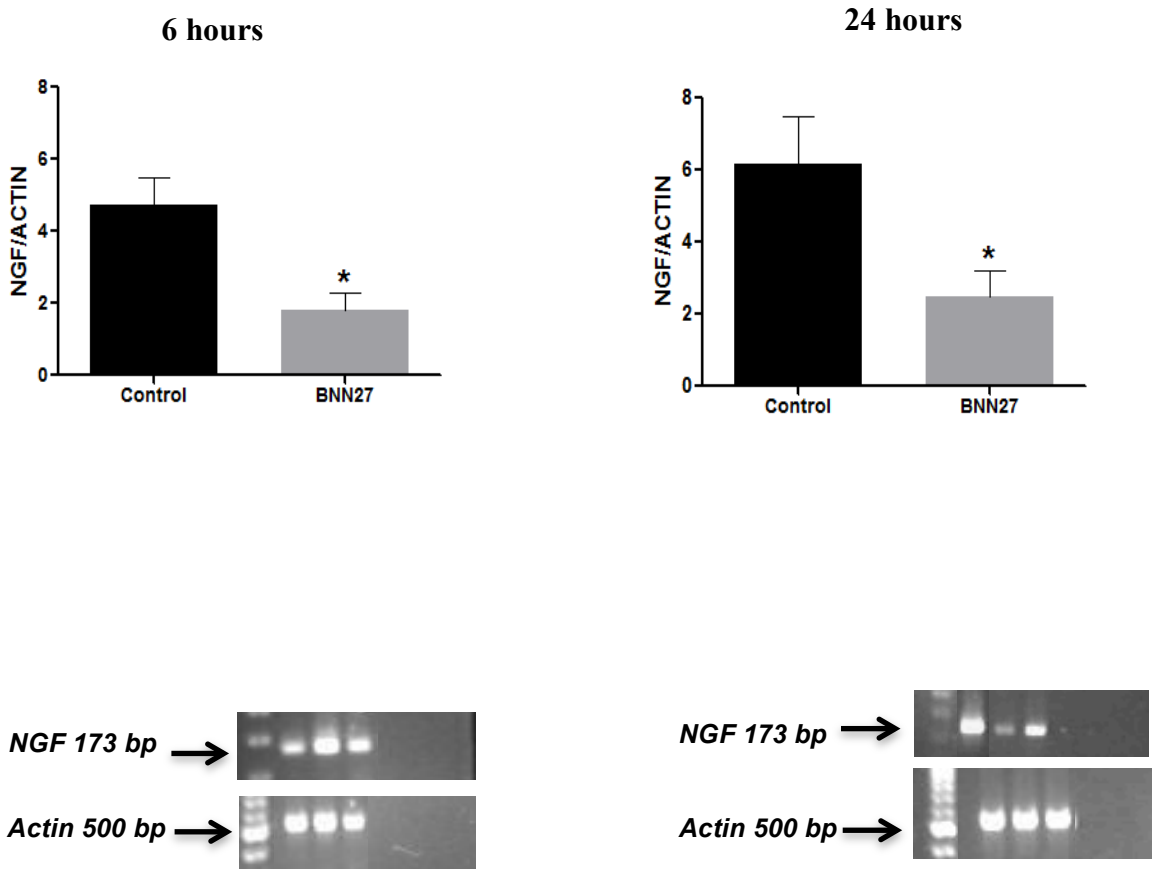


Figure 5.15. **Effect of BNN27 on NGF in DRG.** Administration of BNN27 decreased mRNA levels of NGF in DRG at 6 and 24 hours following the induction of inflammation. (6 hours: Control n=3, BNN27 n=3, $p<0.05$, T-test Unpaired, 24 hours: Control n=5, BNN27 n=6 $p<0.05$ T-test Unpaired, Order of the samples: 6 hours ladder, BNN27, Control, BNN27, noRTBrain, noRT, H_2O, 24 hours ladder, Control, BNN27, Brain, noRTBrain, noRT, H_20)

5.7 Effect of BNN27 on μ-opioid receptor

Based on our data, we hypothesized that the antinociception observed after the administration of BNN27 is mediated by the opioid system. To examine this hypothesis, PCR using primers for opioid receptors MOR, DOR, KOR was performed in DRG and inflamed paw. MOR mRNA was detected in DRG and inflamed paw. BNN27 increased MOR mRNA levels in inflamed paw (Figure 5.16). In DRG, MOR mRNA levels tended to be higher in BNN27 treated animals but they did not reach significance (Figure 5.16). The mRNAs of KOR and DOR were detected in DRG, but not in inflamed paw (data not shown).

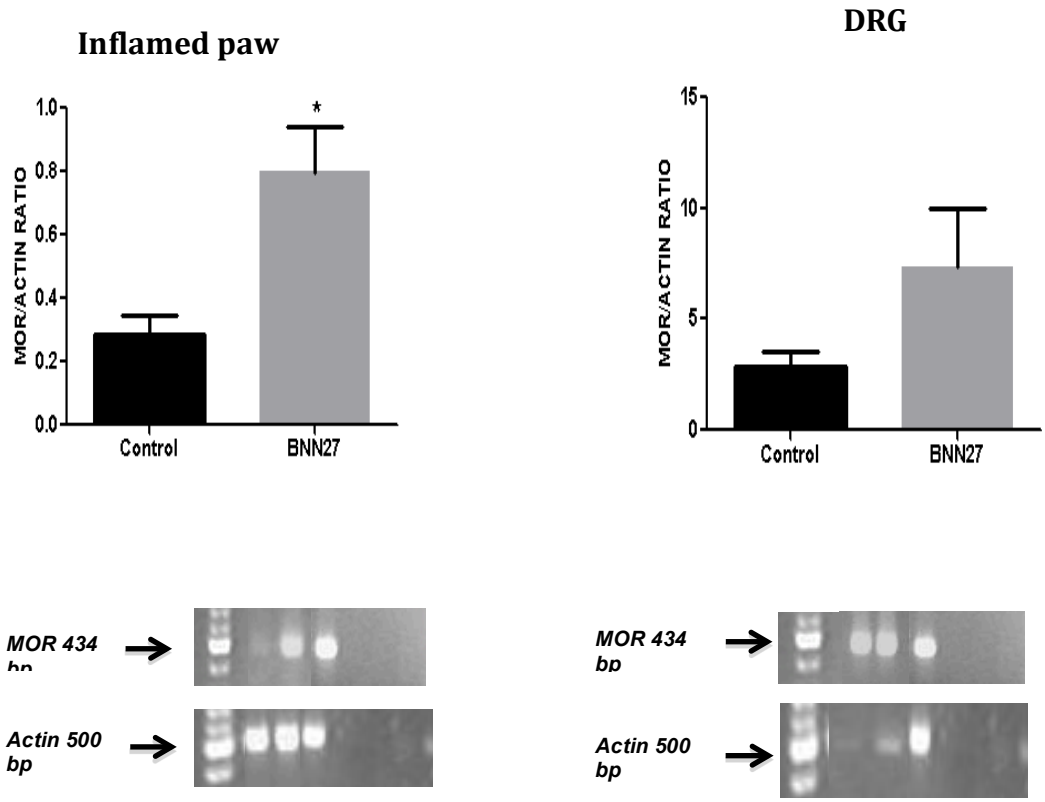


Figure 5.16. **Effect of BNN27 on MOR.** Administration of BNN27 increased MOR mRNA levels at 6 hours following inflammation (Control n=3, BNN27 n=3, $p<0.05$, T-test Unpaired). At 24 hours following inflammation, MOR mRNA levels tended to be higher in in DRG of BNN27 treated mice (Control n=2, BNN27 n=3, $p>0.05$, T-test Unpaired, Order of the samples: Inflamed paw ladder, Control, BNN27, Brain, noRTBrain, noRT, H_2O, DRGs ladder, BNN27, Control, Brain, noRTBrain, noRT, H_2O)

5.8 Effect of BNN27 on POMC mRNA and β-endorphin levels, 6 and 24 hours following the induction of inflammation

Opioid peptides bind to opioid receptors and evoke analgesia (Stein, 1993). RT-PCR using primers for the precursors of β-endorphin and encephalin, pro-opiomelanocortin (POMC) and pro-enkephalin (PENK) respectively, were performed at 6 and 24 hours following the induction of inflammation. It was observed that PENK mRNA was up-regulated in spleens and inflamed paws of mice treated with BNN27, 24 hours after CFA injections (Figure 5.18). Unlike PENK, POMC mRNA was found to be down-regulated in mice treated with BNN27 (Figure 5.17). Pro-dynorphin mRNA was undetectable in inflamed paw. At 6 hours POMC and β-endorphin were found elevated in inflamed paw.

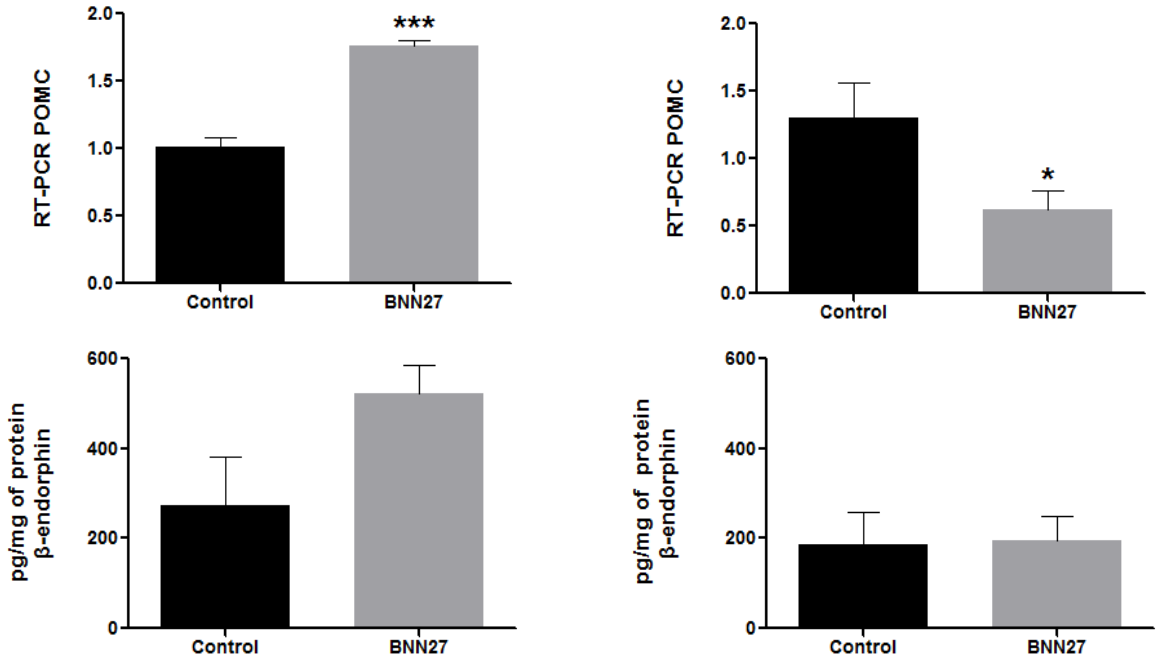


Figure 5.17. **Effect of BNN27 on POMC and β-endorphin in inflamed paw.** Administration of BNN27 increased POMC mRNA (Control n=4, BNN27 n=5 $p<0.001$, T-test Unpaired) and showed a tendency to increase protein levels of β-endorphin (Control n=4, BNN27 n=4, $p>0.05$, T-test Unpaired) at 6 hours following inflammation. At 24 hours BNN27 decreased POMC mRNA (Control n=4, BNN27 n=4, $p>0.05$, T-test Unpaired), while

it did not affect protein levels of β-endorphin. (Control n=4, BNN27 n=4, p>0.05, T-test Unpaired).

PENK mRNA in spleens and inflamed paws

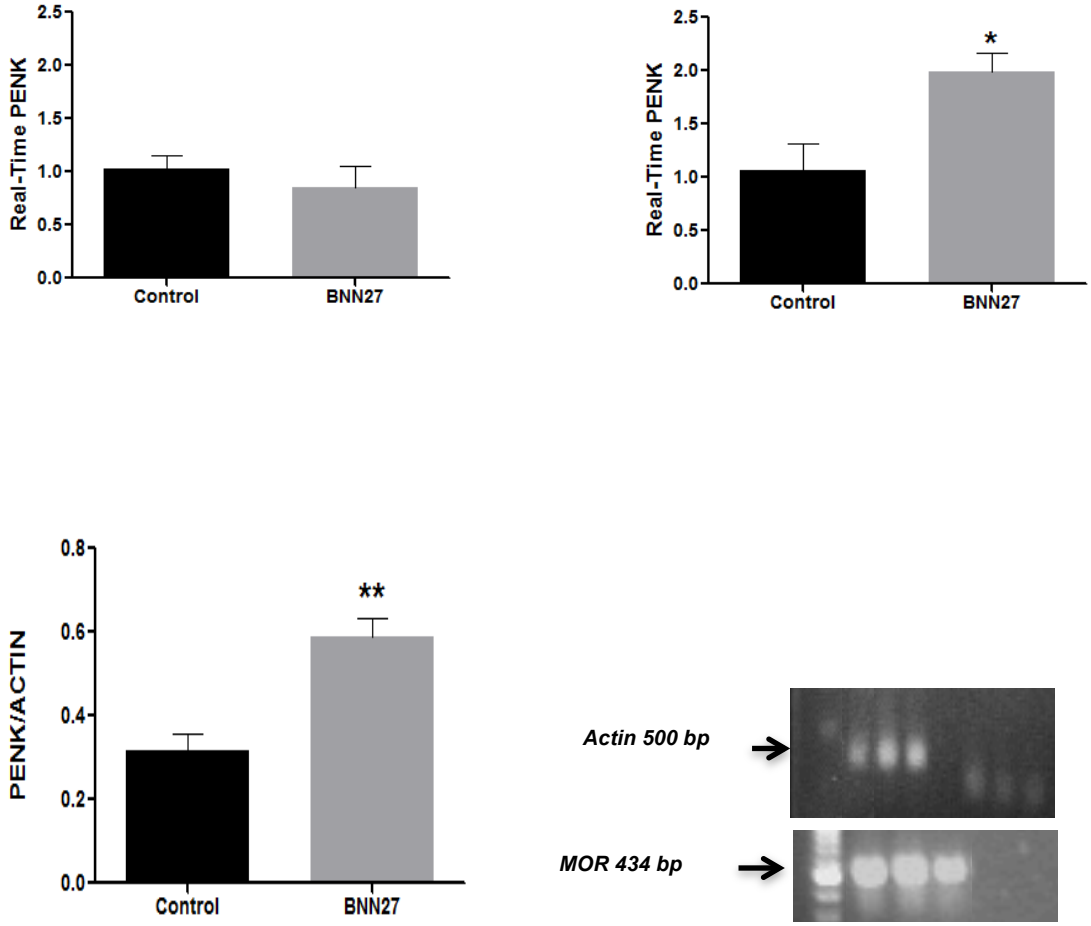


Figure 5.18. **Effect of BNN27 on PENK in spleens and inflamed paws.** Administration of BNN27 increased PENK mRNA in inflamed paws at 24 hours following inflammation (Control n=4, BNN27 n=5, p<0.05, T-test Unpaired). At 6 hours BNN27 did not affect mRNA levels of PENK in inflamed paws. (Control n=4, BNN27 n=4, p>0.05, T-test Unpaired). BNN27 also increased mRNA levels of PENK in spleens 24 hours following inflammation (Control n=5, BNN27 n=5, p<0.01, T-test Unpaired, Order of the samples: Control, BNN27, Brain, noRTBrain, noRT, H_2O)

5.9 Effect of BNN27 on primary spleen cell culture

The effect of BNN27 on cytokine release was studied also *in vitro*, in T-cell cultures from spleens isolated from control and BNN27-treated mice. Cytokines such as TNF-a, IL-6, IL-1β and IFN-γ were measured by ELISA at 3, 6 and 24 hours following the treatment of the cultures with BNN27 (10^{-7}M) or vehicle. As shown below, the maximum effect of BNN27 was measured 3 hours after the treatment (Figure 5.19).

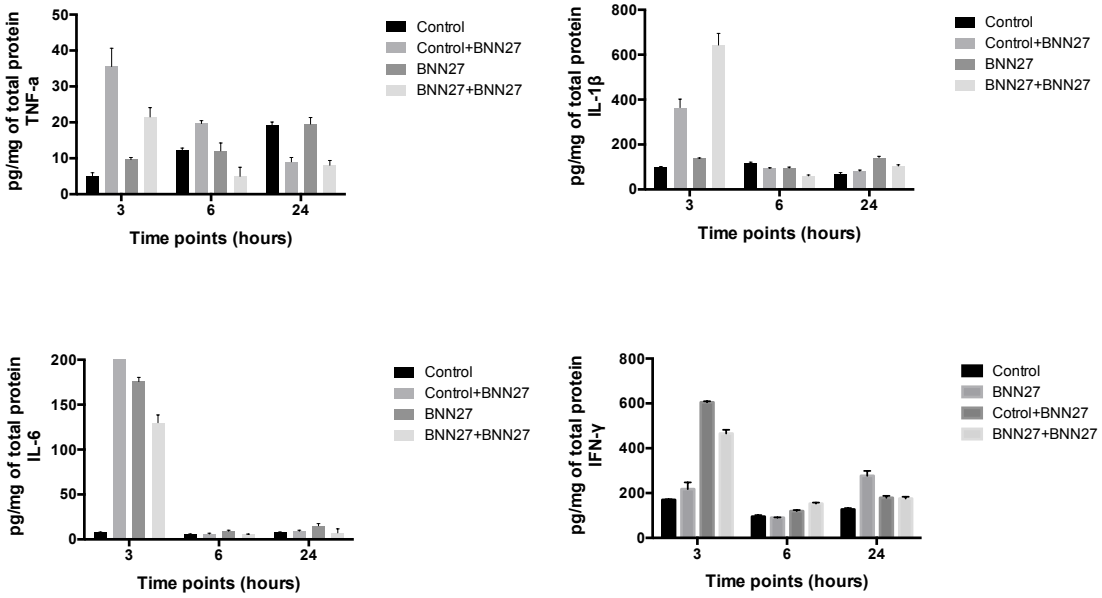


Figure 5.19. **BNN27 increased cytokine secretion *in vitro*.** Treatment of T-cell cultures obtained from spleens with BNN27 resulted in increased cytokine release (TNF-a $p<0,01$, IL-6 $p<0,001$, IL-1β $p<0,01$, IFN-γ $p<0,05$, n=3 in all groups)

Discussion

The main purpose of the present study was to examine the analgesic and anti-inflammatory effects of the new synthetic analog of DHEA, BNN27. Our findings indicate that, in the presence of inflammation that causes also hyperalgesia, this new

molecule is efficient to provide analgesia. The mechanism of action of BNN27 implicates pro-inflammatory and anti-inflammatory cytokines and the opioid system as well, topically at the site of inflammation, but also centrally in dorsal root ganglia (DRG).

We initially evaluated the effect of BNN27 on basic pain levels in mice. Therefore, mice received treatment with BNN27 once per day for four consecutive days and paw withdrawal latency was tested at 3, 6 and 24 hours after the last administration of the compound. Interestingly, BNN27-treated mice had significantly elevated pain threshold as compared with vehicle-treated mice. We then, examined the effect of BNN27 on inflammatory pain. The first step to achieve this goal was the induction of inflammation. Many animal models of inflammatory pain have been developed, with CFA-induced inflammation to be the leading one. Complete Freund's adjuvant, when injected to the paw of rats or mice, evokes cutaneous inflammation within minutes to hours that peaks between 5 to 8 hours (Zhang &Ren, 2011). They also develop edema that peaks 24 hours after the injection, hyperalgesia and allodynia that peaks within 5 hours and lasts for 1 to 2 weeks (Ren&Dubner, 1999; Zhang &Ren, 2011).

In our experiments, all mice were injected with 20μl CFA into their left hind paw. This resulted, as expected, in hyperalgesia and edema as measured by Hargreaves test and plethysmometer, respectively, at 3, 6 and 24 hours after the injection. Treatment with BNN27 for four days prior to CFA injection reversed hyperalgesia caused by CFA at all time points tested, with maximum results at 6 and 24 hours following the induction of inflammation. This is not the first time that neurosteroids are tested for their analgesic effects in experimental pain models. It was shown in previous studies that during carrageenan-induced inflammation in rats, endogenous neurosteroids are released and prevent thermal hyperalgesia via inhibition of γ-aminobutyric acid type A ($GABA_{A)}$ receptors of dorsal horn (Poisbeau*et al.,* 2005). Allopregnanolone, which belongs in the same biosynthetic pathway with DHEA, exhibited analgesic effect in carrageenan-induced hyperalgesia model in rats. Allopregnanolone influenced the excitability of the central nervous system by acting as a positive allosteric modulator of $GABA_A$ receptors (Svenssonet *al.,* 2013). Intrathecal administration of allopregnanolone reversed thermal and mechanical hyperalgesia as measured by Hargreaves and von Frey hair tests, respectively. Increased paw withdrawal latency was also observed in non-inflamed paws. These

results are consistent with the results presented in this study with the neurosteroid BNN27.

During inflammation production of many pro-algesic and analgesic mediators occurs. Inflammation triggers synthesis and release of pro-inflammatory and anti-inflammatory cytokines from resident and other cells and organs of the immune system, neuropeptides such as substance P, prostaglandins, NGF and many other molecules which contribute also to the activation of the immune system (Rittner*et al.,* 2004).

Pro-inflammatory cytokines, such as IL-6, IL-1β and tumor necrosis factor (TNF-a), are produced following CFA injection to initiate the inflammatory response (Zhang *et al.,* 2007; Rittner*et al.,* 2004). The inflammation that is developed by CFA is characterized by the production and release of these cytokines at the inflamed tissue (Rittner*et al.,* 2005; Woolf *et al.,* 1997). Our results confirmed these previous observations, as the mRNA and protein of TNF-a, IL-6 and IL-1β were detected in inflamed paws of mice.

Administration of BNN27 in mice diminished the transcription of TNF-a gene, and its protein in inflamed paws and spleens 24 hours after the induction of inflammation. Inflamed paws of mice were also examined 6 hours after CFA injections, and to our surprise at this time point, BNN27 increased protein levels of TNF-a in inflamed paws. Anti-inflammatory properties of neurosteroids like DHEA have been described previously in LPS-induced inflammation models, where DHEA decreased serum TNF-a (Danenberg*et al.,* 1992). Woolf et al (1997), also demonstrated that 3 hours after CFA injection, TNF-a was elevated, and peaked within 24 hours, the time point that BNN27 diminished its levels in spleens and inflamed paws in our studies. The elevated levels of TNF-a at 6 hours in mice treated with BNN27 are likely due to stimulation of the immune system at this time point to trigger the production and release of anti-inflammatory agents. Similar results were obtained *in vitro* results. Thus, 3 hours after the treatment of T cells with BNN27 we found increased TNF-a, while 24 hours after the treatment with BNN27 TNF-a levels were decreased.

IL-6 and IL-1β are found elevated in many inflammatory conditions including CFA-induced inflammatory states. As in the case of TNF-a, IL-6 and IL-1β when injected intraplantarly induce inflammation, thermal and mechanical hyperalgesia in

rats (Woolf *et al.,* 1997; Opree*et al.,* 2000; Rittner*et al.,* 2005). Although IL-6 is mostly characterized as pro-inflammatory cytokine, many studies argue its anti-inflammatory role too. IL-6 contributes to the transition from innate to acquired immunity. In early stages of inflammation, IL-6 irritates accumulation of neutrophils that secrete proteases in the inflamed tissue. Twenty four hours after this stage, activation of a different pathway by IL-6, provokes the replacement of neutrophils by monocytes and T cells to prevent further tissue damage (Schelle*et al.,* 2011). This is due to inhibition of neutrophil-attracting chemokines, and attraction of monocyte-attracting chemokines. IL-6 also contributes to monocytes differentiation, induces neutrophils apoptosis, while it prevents apoptosis of T cells (Schelle*et al.,* 2011). These properties of IL-6 can explain its elevated levels 6 hours following CFA injection in BNN27 treated mice, and its high levels *in vitro* 3 hours after incubation of spleen T cells with the compound. It is possible that in this early stage of inflammation BNN27 triggers the release of IL-6 to start the infiltration of monocytes and T cells in the inflamed tissue and initiate acquired immunity.

IL-1β peaks 6 hours in CFA inflammation, and then its levels become steady for about a week (Woolf *et al.,* 1997). In our study IL-1β protein levels were elevated during inflammation, but BNN27 did not cause any changes to its level at any time point studied. The *in vitro* studies revealed increased IL-1β 3 hours after the treatment with the neurosteroid. This can be supported by Rittner's group that proposed the analgesic role of IL-1β*in vitro* in leukocytes. In their experiments CRF, noradrenaline and IL-1β bound to their receptors on leukocytes and stimulated the release of opioid peptides, in a model of CFA inflammation (Rittner*et al.,*2005). It is possible that IL-1β has the same effect in T lymphocytes, but more experiments are necessary to investigate the release of opioid peptides and receptors, to confirm this hypothesis.

Later in the inflammation, anti-inflammatory cytokines are released to counteract pro-inflammatory cytokines, by local or migrating cells. Here the anti-inflammatory cytokine IL-10 was studied 24 hours after the induction of inflammation, in inflamed paws. IL-10 activates macrophages and suppresses pro-inflammatory cytokines like TNF-a and IL-6. It is also believed that it contributes to chronic pain, as it appears decreased in blood of patients with chronic pain (Zhang *et al.,* 2007). DHEA influences IL-10 since its administration in mice increased IL-10 levels in serum (Cheng *et al.,* 2000). Similarly, BNN27 increased IL-10 production in hind paws examined in our study, but also in an experimental model of autoimmune

encephalomyelitis in mice as demonstrated by Aggelakopoulou and her colleagues in 2012 (personal communication). During CFA inflammation IFN-γ is found elevated to defense against mycobacteria (Matthys*et al.,* 2000). The *in vitro* experiments have shown increased production of IFN-γ at 3 hours after treatment of T cells with BNN27. Theses results strongly indicate that BNN27 is effective in suppressing pro-inflammatory cytokines, and promoting anti-inflammatory cytokines, enhancing defense of the organism against inflammation.

Nerve Growth Factor is a peptide that belongs to neurotrophins. NGF is crucial for the development and function of neurons in peripheral and central nervous system, but its most important role is in neuronal survival. This neurotrophin exerts its biological function by binding to its high affinity TrKA receptor, and its low affinity $p75^{NTR}$, that are spread widely in the nervous and immune system (Aloe *et al.,* 2012). Its contribution to inflammation and hyperalgesia is also widely accepted. In the skin NGF is produced from keratinocytes, and during tissue trauma and inflammation from residual or immigrating immune cells. NGF exerts its biological action either directly by binding to its receptors either indirectly by promoting the release of other molecules like neuropeptides and neurotransmitters.

Following inflammation, skin levels of NGF are rapidly increased, and in later stages this increase is observed also in DRG. Release of NGF during inflammation is triggered by cytokines such as TNF-a and IL-1β (Rueff*et al.,* 1996). NGF is capable to cause hyperalgesia. Indeed, when it is injected intraperitoneally or intraplantarly to rats, evokes mechanical and thermal hyperalgesia. Mechanical hyperlagesia is measurable within 6-7 hours after the injection, in contrast to thermal hyperlagesia that is provoked within minutes following NGF injections (McMahon, 1996; Rueff*et al.,* 1996). TrKA is the main receptor contributing to the hyperalgesic actions of NGF, as its blockade attenuates the immediate component of thermal hyperalgesia. In our study, we the expression of the mRNA of NGF in inflamed paws of mice 6 and 24 hours after the induction of inflammation. At 6 hours mRNA levels of NGF were detectable in inflamed tissue in both groups (control and BNN27 treated mice). BNN27 increased the mRNA levels of NGF at this time point, although the same mice exhibited increased paw withdrawal latency. It is likely that at this time point the analgesic effect of BNN27 is independent of its pro-inflammatory effect. At 24 hours mRNA levels of NGF were similar in both groups. NGF receptor p75 was examined in inflamed paw and DRG as a study in 2008 supported its contribution in CFA-

induced hyperalgesia. Antibody against p75 inhibited hyperalgesia induced by CFA (Watanabe *et al.,* 2008). In our experiments mRNA of p75 was detectable in DRG and inflames paws, but BNN27 did not cause any changes to its level.

mRNA levels of NGF were also measured in DRG. As mentioned above, during inflammation NGF is increased in DRG possibly to facilitate transmission of nociceptive signals, by increasing levels of the algogenic peptides substance P and CGRP that transmit pain signals from nociceptors (Fbbro*et al.,* 2004; Rueff*et al.,* 1996). It is also believed that increased levels of NGF prevent the inhibitory role of GABA in action potential transmission within DRG. BNN27 treated mice had reduced level of NGF mRNA in DRG at 6 and 24 hours after CFA injection compared with non-treated animals. This may suggest a central mechanism of analgesia mediated by this molecule. We measured also mRNA levels of substance P at the same time points in DRG and inflamed paws, and no differences were detected. Further experiments to investigate protein level of these molecules are needed.

Opioid receptors and their endogenous or exogenous ligands contribute to analgesia. The mRNA of opioid receptors is synthesized in DRG and the protein is transported to the peripheral nerve terminals via axonal transport (Stein, 1995). Their endogenous ligands, opioid peptides (β-endorphin, dynorphin, enkephalin) bind to opioid receptors according to the affinity of each peptide to each receptor. The antinociceptive properties of opioid peptides rely on the fact that they can decrease the excitability of primary afferent neurons by inhibiting the adenylate cyclase. Additionally they increase potassium currents and decrease calcium currents. This cascade of events has inhibitory effects on the firing of neurons and the release of neurotransmitters (Stein, 1995). Therefore, we investigated the contribution of opioid peptides and their receptors during BNN27-induced analgesia in DRG and inflamed paws of mice. The mRNA of all opioid receptors was detected in DRG, and we have noticed an increase in the mRNA of μ-opioid receptor in mice treated with BNN27, but this increase was not significant, probably because of the small number of samples. In inflamed paws, the mRNA of μ-opioid receptor was only detected and BNN27-treated mice exhibited significantly elevated levels of MOR mRNA. Kappa receptor mainly contributes to mechanical hyperalgesia following CFA injection it rats as it has been proposed in previous studies (Auh*et al .,* 2012). Delta and mu receptors are more important in thermal hyperalgesia. It is widely accepted that immune cells like leukocytes synthesize opioid receptors and peptides (Stein, 1993;

Stein, 1995; Rittner*et al.,* 2005; Sitte*et al.,* 2006), so it is likely that the source of μ-opioid receptor in inflamed paws is the immune cells. Further studies are important to investigate the contribution of protein levels of μ-opioid receptor and its axonal transport from DRG neurons.

Several studies demonstrate that immune cells like leukocytes express mRNA for the precursors of opioid peptides (POMC, PDYN, PENK) and the protein. After CFA-induced inflammation, several amounts of β-endorphin and enkephalin have been detected in immune cells that infiltrate the inflamed tissue, while dynorphin was undetectable (Stein *et al.,* 1990). These observations are in accordance with our data presented here, which revealed the presence of POMC and PENK mRNA in inflamed paws of mice. We have found that at 6 hours after inflammation POMC mRNA and the protein β-endorphin were increased in inflamed paws of mice that have received BNN27, while 24 hours after the initiation of the inflammatory response PENK mRNA was increased in the same mice. This indicates that in different stages of inflammation, different molecules contribute to provide analgesia.

In summary, CFA injection produces thermal hyperalgesia and edema in mice, and triggers the production and release of many proinflammatory molecules in the inflamed tissue and DRG. The new synthetic analog of DHEA, BNN27, which lacks its side effects, is capable to produce analgesia by enhancing the release of anti-inflammatory molecules like IL-10 and opioid peptides, and suppressing the release of proinflammatory molecules such as NGF and TNF-a. More studies are necessary to investigate its role and possible mechanism of actions, extensively.

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