CENTRAL NUCLEUS OF THE AMYGDALA IN DESCENDING CONTROL OF PAIN-RELATED BEHAVIOR

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ACADEMIC DISSERTATION

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Dedicated to my chronic pain

"For me, I am driven by two main philosophies, know more today about the world than I knew yesterday. And along the way, lessen the suffering of others."

Neil DeGrasse Tyson

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Abstract

The central nucleus of amygdala (CeA) is known to be involved in pain and nociception, but the mechanisms or its role in descending control of pain-related behavior is poorly understood. The aim of this study was to investigate the involvement of the neuropeptide corticotropin-releasing factor (CRF) and the glutamatergic system of the CeA in pain and nociception in healthy control animals and in an animal model of chronic neuropathic pain induced by spared-nerve injury (SNI). Two aspects of pain were studied: emotional-like pain behavior was assessed by using the aversive place-avoidance paradigm and sensory-discriminative was assessed by determining the mechanical limb-withdrawal threshold and the thermal (heat) limb-withdrawal latency. Moreover, the aims were to determine whether medullospinal serotoninergic pathways and the midbrain periaqueductal grey (PAG), respectively, were involved in relaying pain-modulation induced by the CeA in SNI and healthy control animals. Additionally, hemisphere of the CeA and submodality of pain stimulus were among studied parameters. Surgical procedures and electrophysiological recordings were performed under general anesthesia.

The studies on the role of the CeA in the emotional-like aspect of pain in SNI rats revealed that activation and blocking of the group I metabotropic glutamate receptors (mGluRs) facilitates and inhibits, respectively, the aversive aspect of pain. Furthermore, increase of endogenous CRF as well as blocking glutamatergic N-methyl-D-aspartate (NMDA) receptors in the CeA reduced the aversive aspect of neuropathic pain.

The studies on the sensory-discriminative aspect of pain revealed that an increase of endogenous CRF in the CeA is pronociceptive in both control and SNI rats. CeA injection of a high dose of glutamate had a mechanical antinociceptive effect that was mediated by NMDA receptors in healthy but not SNI rats. A low dose of glutamate had a pronociceptive effect mediated by NMDA receptors in SNI rats. Furthermore, tonic descending pronociception induced by NMDA receptors and the mGluR1 in the CeA contributes to the maintenance of neuropathic hypersensitivity.

The investigation on the role of serotonergic neurons of the rostroventromedial medulla (RVM) in modulation of spinal nociception by amygdaloid glutamate in SNI rats indicated that the RVM is a relay for both descending pro- and antinociceptive effects from the CeA.

The investigation on the role of the PAG in the descending control of nociception induced by glutamate in the CeA of healthy rats indicated that the PAG is a relay in the descending control of nociception induced by amygdaloid glutamate.

Furthermore, the right-hemispheric lateralization of the pronociceptive effect by amygdaloid CRF in controls was lost in SNI rats. However, descending antinociception induced by the glutamatergic system of the CeA showed no hemispheric lateralization in healthy controls; a high dose of glutamate in both the left and right CeA induced equal attenuations of mechanical and thermal nociception, which effects were, respectively, NMDA-dependent and NDMA-independent.

1. List of original publications

I. Bourbia N., Ansah OB., Pertovaara A., Corticotropin-releasing factor in the rat amygdala differentially influences sensory-discriminative and emotional-like pain response in peripheral neuropathy, J Pain. 2010;11:1461-71.

II. Ansah OB., Bourbia N., Goncalves L., Almeida A., Pertovaara A., Influence of amygdaloid glutamatergic receptors on sensory and emotional pain-related behavior in the neuropathic rat, Behav Brain Res. 2010;209:174-8.

III. Bourbia N., Sagalajev B., Pertovaara A., Descending effect on spinal nociception by amygdaloid glutamate varies with the submodality of noxious test stimulation, Neurosci Lett. 2014;570:26–31

IV. Sagalajev B*, Bourbia N*, Beloushko E, Wei H, Pertovaara A, Bidirectional amygdaloid control of neuropathic hypersensitivity mediated by descending serotonergic pathways acting on spinal 5-HT_3 and 5-HT_{1A} receptors., Behav Brain Res. 2015;282:14-24.

V. Bourbia N., Pertovaara A., Involvement of the periaqueductal gray in the descending antinociceptive effect induced by the central nucleus of amygdala. Unpublished manuscript.

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2. Abbreviations

AB: accessory basal nucleus

AMY: Amygdala

ANOVA: Analysis of variance

BA: basal nucleus of amygdala

BLA: Basolateral complex of amygdala

CeA: Central nucleus of amygdala

CeC: Capsular subdivision of the CeA

CeL: Lateral subdivision of the CeA

CeM: Medial subdivision of the CeA

Co: Cortical nucleus of amygdala

CRF: Corticotropin-releasing factor

CRF-BP: Corticotropin-releasing factor binding protein

DEG/ENaC-channel: Degenerin/Epithelial sodium channel

ITC: Intercalated nuclei

L: Lateral nucleus of amygdala

LC: Locus coeruleus

MeA: Medial nucleus of amygdala

mGluR: Metabotropic glutamate receptor

Mrgprd: Mas-related G-protein coupled receptor member D

NMDA: N-methyl D-aspartate

PAG: Periaqueductal gray

RVM: Rostral ventromedial medulla

SNI: Spared-nerve injury

TRP-channel: Transient receptor potential channel

TRPV1: Transient receptor potential vanilloid 1

3. Introduction

Pain is “An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”, according to definition by the International Association for the Study of Pain (IASP). Pain is an important alarm to inform that the homeostasis of the body is in danger by the presence of a noxious stimulus or an injury. Pain becomes pathological when it persists without any tissue damage, or after the pathology has healed, or the pathology related to the pain becomes chronic (as often is the case with neuropathic or arthritis pain). Pain is considered as chronic after it has persisted for three months or longer, although according to some clinicians and researches the definition chronic pain should be used only when pain has persisted for six months. There are two main kinds of pain which can be further subdivided according to the localization and etiology of the pain: nociceptive pain and neuropathic pain. Nociceptive pain is characterized by pain resulting from the activation of the nociception system by a noxious (chemical, thermal or mechanical) stimulus. Neuropathic pain is pain resulting from a damage of the nervous system. It can be peripheral or central according the localization of the damage in the peripheral or central nervous system, respectively[1].

Pain is a complex experience which includes 6 components: sensory-discriminative component which tells about localization and intensity of pain; emotional component which refers for instance to unpleasantness and suffering[2,3]; motor component which includes the motor reflexes; autonomous component[4,5] which refers to autonomous responses like respiratory and blood pressure changes; cognitive component which involves memory of previous pain experience, distraction/catastrophizing, and various behavioral and psychological[3,6] responses that depend on social environment and personality.

The prevalence of neuropathic pain is between 6.9% and 10%[7]. Neuropathic pain affects negatively the patient’s life, it represents an important economic burden[8], and its treatment is challenging.

The amygdala is known to be involved in emotions[9–11], anxiety[12] and pain[13–15], and it is also among key targets when studying neuropathic pain and various aspects of it. While amygdala has been intensively studied, the contribution of amygdala to processing and regulation of neuropathic pain is poorly known. The central nucleus of amygdala (CeA) receives nociceptive information from spino-parabrachial projections[16]. CeA has been involved in different types of analgesia (conditional hypoalgesia, morphine antinociception)[17–20]; anxiety-like aspects of pain[14,21] and neuroplasticity[22].

The CeA has, at least, two types of GABAergic neurons co-releasing either enkephalin or corticotropin releasing factor (CRF)[23]. Animal studies have shown that CRF is involved in sensory-discriminative[24] and emotional aspects of pain[21,25,26] through metabotropic receptors CRF-1 and CRF-2[27]. Furthermore, the glutamatergic system of the CeA is also involved in pain processing[28–30] and its affective aspect[31].

4. Literature review

4.1.History of pain theory

History of medicine goes back at least to Greek antiquity and Hippocrates (460–380 BCE). In the Hippocratic treatise *Breathes*, the relationship between anesthesia and analgesia is discussed for the first time. Hippocrates wrote: "*At this time the patients are unconscious ["anaesthetoi"] of everything, deaf to what is spoken, blind to what is happening and insensible to pain ["analgetoi"]*". The most frequent words used for pain in the *Hippocratic Collection* were *Algos*, *Algema*, *Odyne* and *Ponos* (modern Greek word for pain). *Algos, algema* and *ponos* are used to describe more general type of pain, possibly chronic, involving the whole body (for *algos word)* or a limb/organ (for *algema* and *ponos* word) while *odyne* refers to pain which is sharp, acute and localized in specific area of the body[32].

In Greek antiquity, while Aristotle (384-322 BCE) postulated that sensations including pain arise from heart, Anaxagoras (510-428 BCE) and Pythagoras (570–495 BCE) were among the first philosophers who proposed that the sensations and thought are located inside the brain.

During the Roman Empire, the Greek physician Galen of Pergamon (129-200/216 CE) also known under the Latin name Claudius Galenus and considered as a father of the pharmacology, proposed that feelings arise from the brain. Galenus considered that only injuries cause pain. Additionally, Galenus postulated that three conditions are required for pain perception: "the organ to receive impression, a connecting passageway and an organisational centre"[33]. Meanwhile in China, the physicist Hua Tuo (145–208 CE) used a concoction based on cannabis in wine named "mafeisan" as an anesthetic/analgesic during surgery, probably making him the first physician to develop an analgesic and anesthetic compound in the history of medicine[34].

During Middle Ages, the Persian Muslim philosopher, writer and physician Ibn Sina, also known with the Latin name Avicenna (980–1037 CE), studied extensively pain and wrote *The Canon of Medicine* where he described that pain is an independent sensation dissociated from touch and temperature[35], it is caused by a temperament change (physical condition change) of an organ, it can be chronic and pain perception is processed by the brain. In this book, he also described 15 types of pain according to its cause using the Arabic words Waja' (hurt) and Alam (pain) : "These were: itching (exposure to irritating substance or salt); coarse (coarse substance); pricking (something stretches membranes); compressing; stretching (bloat or muscle or nerve stretch); disintegrating (a substance disintegrating inside the muscle and membranes); breaking (bone change); soft (muscle change); penetrating (a thick substance or bloat trapped in colon); stabbing, *Massli* (a substance trapped inside an organ); numbing (extreme cold or obstruction of vessels); pulsating (a tumour or swelling close to arteries); heavy (a tumour or a swelling in lungs, kidney or spleen); tiredness; and bitter (ulcers) (The Canon, pp. 54, 55)"[33].

In the 17th century, the French philosopher René Descartes (1596-1650), father of the modern philosophy, wrote *Discours de la méthode pour bien conduire sa raison, et chercher la vérité dans les sciences* (the French original work from 1637 was translated into English as Discourse on the Method of Rightly Conducting One's Reason and of Seeking Truth in the Sciences). In this work, Descartes pointed out the skepticism, doubt, reasoning and his wishes to push forward sciences, technology and medicine research. He also wrote the treatise L'Homme, which contained a hypothetical anatomy drawing symbolizing the noxious information transmission from peripheral system to spinal cord and then to the pineal organ in the brain, considered at this time as the pain perception seat.

Beginning from the 19th century, thanks to animal experimentation and improved scientific methods, pain researches were able to develop new concepts and theories leading to four theories for explaining mechanisms underlying pain sensations: specificity theory, intensity theory, pattern theory and gate control theory[34,35]:

- The specificity theory was developed by Moritz Schiff (1823-1896). According to this theory, specialized organs (nociceptors) are activated by a noxious stimulus and encode intensity of noxious stimulation by an increase in activity of nociceptive nerve fibers. The theory proposed by Schiff was based on previous studies by Bell and Magendie showing the sensory fibers are specifically in the posterior spinal nerve root, known as the Bell-Magendie law. This law was confirmed by Johannes Peter Müller (1801-1858) who developed the "law of specific nerve energies". Additionally, Moritz Schiff confirmed that temperature and nociceptive pathways are different from other sensory pathways like from that of touch.
- The intensity theory was introduced by Wilhelm Erb (1840-1921) who rejected the idea of specialized peripheral nociceptive nerves and stipulated that the intensity of the stimulus tells about the nature of the information (i.e.; low-intensity stimulation evokes a non-nociceptive and high-intensity stimulation a nociceptive signal).

The works of Alfred Goldscheider (1858–1935), Magnus Blix (1849–1904) and Max von Frey (1852–1932) lead to the discovery of nociceptive-specific primary sensory nerves. Furthermore, in 1906, Charles Scott Sherrington (1857-1952) introduced the concept of nociception.

In the 20th century, the development of electrophysiological recordings of nerve activity helped Joseph Erlanger and Herbert Spencer Gasser to characterize the primary sensory neuron according to its conduction velocity and diameter in 1924. The studies of Georges H. Bishop, Peter Heinbecker, Yngve Zotterman and their collaborators during the 30's allowed associating peripheral Aδ and C fibers with pain signaling.

- The pattern theory introduced by John P. Nafe in 1929 postulated that the composition of the activity pattern in a population of fibers determines the quality of sensation.
- The gate control theory developed by Ronald Melzack and Patrick D. Wall[36] in 1965 postulated that there is a gate system in the spinal dorsal horn. The postulated gate system is composed of an inhibitory interneuron which inhibits or facilitates

nociception depending on the type of the primary afferent fiber activity. This spinal gate is modulated by descending pathways. If activity in non-nociceptive nerve fibers (Aß fibers) prevails, the inhibitory interneuron is activated, closing the gate by reducing the afferent input from nociceptive nerve fibers to spinal projection neurons (pain-relay neurons). This leads to analgesic effect. If activity of nociceptive nerve fibers (C fibers) prevails, the inhibitory interneuron is disinhibited, the gate is opened, and the spinal pain-relay neurons can be freely activated by nociceptive nerve fibers.

Between 1967 and 1969, the studies of Paul R. Burgess, Edward R. Perl and their collaborators characterized nociceptors and provided evidence for myelinated Aδ, unmyelinated C nociceptive nerve fibers[37,38] and nociceptive-specific neurons in lamina I of the spinal dorsal horn[39].

During the 20th century, combinations of different experimental techniques (electrophysiology, immunohistochemistry, behavior, anatomy, etc.) allowed a better understanding of pain and nociception at molecular and cellular levels; i.e., from the receptor level to the integrative level [e.g., the descending periaqueductal gray (PAG) – rostroventromedial medulla (RVM) - spinal cord pathway, or involvement of the amygdala in different aspects of pain].

The current knowledge on nociception and pain will be briefly described during the next chapters. The emphasis is on neuropathic pain and experimental animal models used in the study of neuropathic pain. Of pain-related structures, the emphasis is on the amygdala and the role of the PAG – RVM system in mediating the amygdaloid corticotropin-releasing factor (CRF) and glutamate receptor-induced descending effects on nociception.

4.2.Nociception

The nociceptor is a sensory receptor specialized on transducing noxious (harmful) stimuli into electrical signals. Noxious stimuli activating nociceptors can be mechanical, thermal or chemical. Mechanical, chemical and thermal noxious stimulation are different submodalities of nociception. Noxious heat and some chemical stimulus (like capsaicin) are detected by TRPV1 or other TRP-channels family members that are a family of ion channel receptors expressed on terminals of nociceptive nerve fibers and that transduce noxious stimuli into electrical signals. Mechanical noxious stimuli are detected by DEG/ENaC-channel family or Mrgprd, a sensory neuron-specific G protein-coupled receptor[40,41].

Primary afferent nociceptive nerve fibers carrying the nociceptive signals to laminae I and II of the spinal dorsal horn consist of myelinated Aδ fibers (divided in two groups: Aδ mechanosensitive and Aδ mechanothermal nerve fibers) and unmyelinated polymodal C fibers. Aδ fibers are responsible for the sharp “first pain”. C fibers which have a slower conduction velocity are responsible for the diffuse and long-lasting “second pain”. Deep or burning pain sensations are typically induced by C fibers.

One class of primary nociceptive neurons is silent under normal circumstances but can be activated by inflammatory mediators like bradykinins, prostaglandins and histamine released during tissue damage. Thus, tissue damage and inflammation can sensitize the silent nociceptor and "wake it up" [42,43].

4.3.Ascending pain pathways

After arrival to the spinal dorsal horn, the nociceptive signal is carried rostrally via ascending tracts (see below) to elicit pain sensation in the brain. However, the nociceptive signal arriving from the periphery is not relayed as such but in the spinal dorsal horn, the nociceptive signal is subject to modulation by a number of endogenous mechanisms, such as the gate control (see above) and descending pathways (see below).

The nociceptive signal arriving the spinal dorsal horn is not only involved in eliciting pain sensation, but it is also involved in evoking the nociceptive withdrawal reflex, a motor reflex which allows avoiding further damage from noxious stimuli. The nociceptive withdrawal reflex circuitry involves a nociceptive spinal dorsal horn neuron, multiple excitatory interneurons, and α-motoneurons in the spinal ventral horn that innervate flexor muscles (withdrawal reflex). In parallel, the nociceptive signal activates an inhibitory circuitry that inhibits α-motoneurons innervating extensor muscles (reciprocal innervation) allowing to induce the withdrawal reflex.

Peptidergic primary afferent nociceptive nerve fibers (among which TRPV1 expressing ones are a subset[44]) and non-peptidergic primary afferent nociceptive nerve fibers (among which Mrgprd expressing ones are a subset[45]) follow specific parallel pain pathways[46]. Peptidergic nociceptive nerve fibers may project to the lamina I of the spinal dorsal horn to activate neurons of the spinothalamic and spinomesencephalic tracts while non-peptidergic nociceptive nerve fibers may project to the lamina II neurons that are connected to lamina V neurons with ascending projections via the spinohypothalamic, spinoamygdalar and spinostriatal tracts. Finally, both peptidergic and non-peptidergic nerve fibers may converge to spinal dorsal horn neurons that have ascending projections to the hypothalamus and amygdala.

There are several ascending tracts from the second-order afferent neurons of the spinal dorsal horn to higher brain centers.

4.3.1. Ascending spinothalamic and trigeminothalamic tract

The ascending projections of second-order afferent neurons receiving nociceptive information cross at the spinal cord level. In the case of the spinothalamic tract carrying information from the body, the projections of spinal pain-relay neurons ascend in the contralateral side to the ventral posterior lateral nucleus of the thalamus while the

nociceptive information from the face ascends in the trigeminothalamic tract to the ventral posterior medial nucleus of the thalamus. Both the spinothalamic and the trigeminothalamic tract have synaptic contacts with third-order thalamic neurons which project to higher brain centers, particularly to the somatosensory cortex (Fig.1).

4.3.2. Ascending spinomesencephalic and spinoparabrachial tract

In the spinomesencephalic tract, the second-order afferent neurons ascend to the mesencephalic reticular formation and the periaqueductal gray matter (PAG). In the spinoparabrachial tract, the second-order afferent neurons project from the spinal dorsal horn to the parabrachial nucleus (Fig. 1).

4.3.3. Ascending spinoreticular tract

The second-order afferent neurons of the spinoreticular tract ascend from the spinal cord level both to the reticular formation and the medial thalamus.

4.3.4. Other ascending pathways

Among other nociceptive pathways ascending directly from the spinal dorsal horn to supraspinal structures are the spinohypothalamic and spinoamygdalar tract through which the ascending second-order neurons project to the hypothalamus and the amygdala, respectively. The spino-parabrachial-amygdaloid pathway involved a third order of afferent neurons. The second order is similar to the spinoparabrachial tract followed by the projection of the third-order afferent neurons from the parabrachial nucleus to the amygdala (Fig. 1).

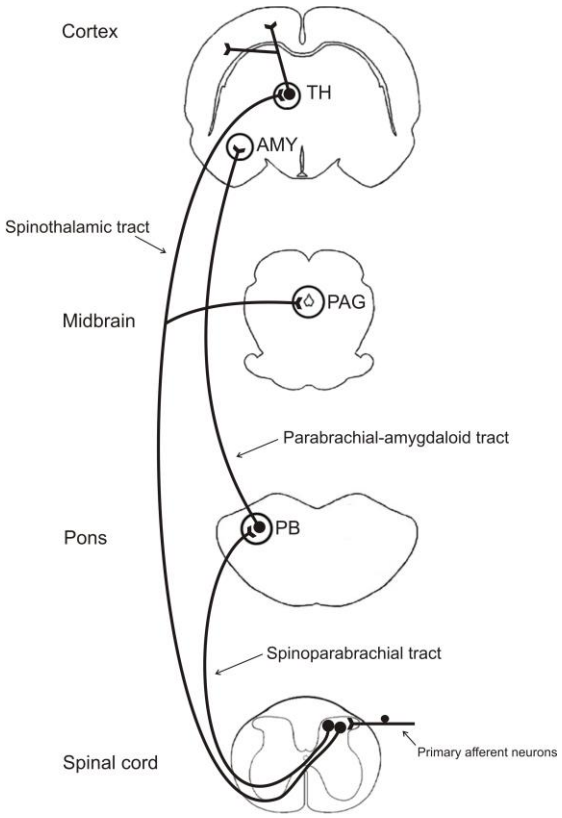


Fig. 1: Schematic representation of the spinothalamic and the spino-parabrachial-amygdaloid ascending pathways. TH = thalamus, AMY = amygdala, PAG = periaqueductal grey, PB = parabrachial nucleus.

4.4.Descending pain modulating pathways

Descending pain modulating pathways allow modulation of nociceptive signals and autonomic responses to a noxious stimulus already at the spinal cord level (Fig. 2).

Some of the descending pathways originating in supraspinal structures (e.g., brainstem structures, hypothalamus) have direct projections to the spinal dorsal horn where they can induce antinociceptive or pronociceptive effects[47].

There are also descending pathways that have indirect, polysynaptic projections to the spinal dorsal horn. The best-known indirect descending pain modulating pathway is the PAG–RVM-spinal dorsal horn system[48] (see chapter on the PAG-RVM system). Related to this is the amygdala-PAG pathway that has been shown to modulate pain behavior as shown e.g. by antinociception induced by stimulation of the central nucleus of the amygdala (CeA) and that may at least partly be mediated by the opioid system of the PAG[49–51] (Fig. 2).

Descending pain modulating pathways can be classified based on their neurotransmitters.

4.4.1. Descending noradrenergic pathways

In the central nervous system, noradrenergic cell groups are classified as A1–A7. The spinal dorsal horn receives noradrenergic innervation from the A5 (in ventrolateral pons), A6 (also known as the locus coeruleus in the pontomesencephalic junction) and A7 (in lateral part of the pons) cell groups[52]. Electric stimulation of A5, A6 or A7 elicits spinal release of noradrenaline and analgesia that can be reversed by intrathecal administration of α_2-adrenoceptor antagonists at the spinal cord level[47,53].

4.4.2. Descending dopaminergic pathways

Various dopaminergic central nervous sites play a role in pain modulation[54]. The hypothalamic A11 nucleus is the main source of dopaminergic innervation of the spinal cord. Electric stimulation of the A11 or spinal administration of dopaminergic compounds have been shown to induce an analgesic effect that is mediated by dopamine D2-like receptors at the level of the spinal dorsal horn[55]. Moreover, dopamine has been shown to suppress pain in the striatum[56,57].

4.4.3. Descending serotonergic pathways

Brainstem-spinal serotoninergic projections may facilitate or inhibit pain depending on the subtype of the spinal serotonin receptor and the pain state (acute versus chronic pain)[47,58]. In pathological pain states, like that induced by nerve injury, descending facilitatory effect of the serotoninergic system predominates[59]. Moreover, the release of serotonin in the injured or inflamed tissue contributes to the peripheral sensitization of the nerve[60].

4.4.4. Other descending pathways

Other descending pathways include those with histamine, vasopressin or oxytocin as the main pain modulating neurotransmitter. Furthermore, several other transmitters participate in descending pain modulation. Among them are GABA, glutamate, opioids, acetylcholine and CCK.

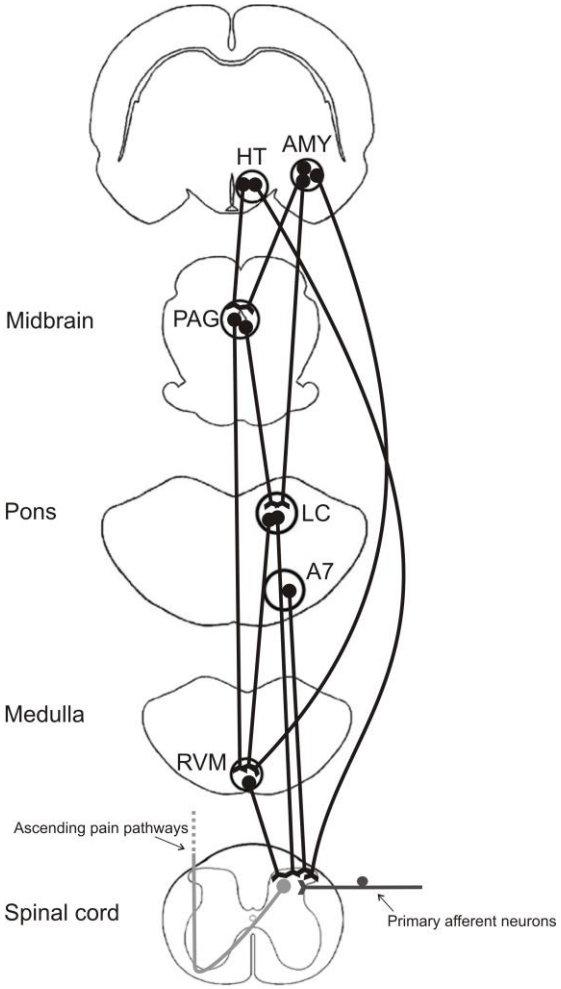


Fig. 2: Schematic representation of the main descending pain pathways. HT = hypothalamus, AMY = amygdala, PAG = periaqueductal grey, LC = Locus Coeruleus, RVM = rostral ventral medulla.

4.5.Pain matrix

Among pain-related cortical areas are the somatosensory cortex, anterior cingulate cortex, prefrontal cortex and insula [61]. Together, these areas contributing to pain processing were first called “neuromatrix” by Ronald Melzack[62], then named “pain matrix” by Irene Tracey and Emily Johns[63].

4.6.Neuropathic pain

Neuropathic pain is a chronic pain disorder induced by a lesion or a disease of the central or peripheral nervous system, named respectively central or peripheral neuropathic pain. While the definition of neuropathic pain by IASP was considered too vague (“Pain caused by

a lesion or disease of the somatosensory nervous system."), Misha-Miroslav Backonja defined neuropathic pain as: "Neuropathic pain is in this case defined as pain occurring in the area or body part associated with neurological disease or injury. This type of pain manifests not only with positive sensory phenomena, such as pain, dysesthesia, and different types of hyperalgesia, but also with negative sensory phenomena and negative and positive motor symptoms and signs."[64]. The prevalence of neuropathic pain is between 6.9% and 10%, it affects negatively the patient's life and represents an important economic burden[7,8].

Neuropathic pain disorder includes symptoms like mechanical and thermal allodynia and hyperalgesia, spontaneous (or ongoing) pain, and other ongoing unpleasant sensations[65]. While the mechanisms underlying neuropathic pain are unclear, there is evidence of the involvement of sensitization at supraspinal, spinal and peripheral levels.

Peripheral sensitization after nerve injury involves changes in the expression of membrane receptors like the voltage-gated sodium channel $Na_V1.8$. It is mainly expressed on primary sensory afferent neurons where it contributes to persistent pain[66]. An increase in the expression of sensory neuron-specific cation channel Transient Receptor Potential Vanilloid 1 (TRPV1)[67], and upregulation of calcitonin gene-related peptide mRNA in intact dorsal root ganglion neurons[68] are other mechanisms. Furthermore, electrophysiological studies have shown that nerve injury may induce ectopic afferent discharge particularly in rapidly conducting A fibers that was associated with tactile allodynia[69]. Spontaneous activity has been demonstrated also in nociceptive C-fibers of an intact nerve adjacent to injury[70,71]. Together, various peripheral sensitization mechanisms (changes in the expression and localization of receptors, hyperactivity from injured and uninjured fibers, release of cytokines by immune cells at the site of the nerve injury[72]) contribute to the maintenance of neuropathic pain.

Central sensitization is an increase in the response of central pain-relay neurons following nerve injury. In the spinal dorsal horn, a phenomenon called "windup" contributes to amplification of nociceptive signals. In windup, repetitive stimulation of nociceptive C-fiber afferents induces an increase in the response of spinal dorsal horn neurons. Windup has been demonstrated also in human subjects[73], independent of hyperalgesia[74,75]. Amplification caused by the windup phenomenon contributes to increased excitability of nociceptive spinal dorsal horn neurons. However, windup is neither sufficient nor necessary for central sensitization and hyperalgesia[76,77]. Nerve injury induces various molecular changes within the spinal dorsal horn that reduce the control of nociception induced by synthetic as well as endogenous antinociceptive compounds[78,79]. For example, after spinal cord injury the response of spinal dorsal horn neurons is enhanced[80]. This is associated with a loss of GABAergic inhibition[81] that is likely to contribute to mechanical allodynia[80] and maintenance of chronic pain.

At the supraspinal level, central sensitization involves modifications in ascending pathways (facilitation of nociceptive information, see example above) and in descending pathways (impairment of the balance between descending facilitation and inhibition of nociception in favor to facilitation). These injury-induced changes in ascending and descending pathways

are likely to contribute to the maintenance of neuropathic pain. Impairment in the antinociceptive efficacy of the opioids in the thalamus, PAG and anterior cingulate cortex is one of consequences of these injury-induced central changes[82,83]. Similarly, descending serotoninergic pathways sensitize the pronociceptive TRPV1 channels on central terminals of primary afferent nerve fibers within the spinal dorsal horn[84]. Furthermore, not only neurons but also glial cells participate in the enhancement of descending facilitation in neuropathic pain conditions[85].

Together, peripheral and central sensitization mechanisms contribute to the maintenance of neuropathic pain.

4.7.Animal models in pain research

Animal models provide an important tool to understand the mechanisms of pain and to develop new treatments. The study of pain and analgesia mechanisms in humans is limited to brain imaging, sampling of blood and peripheral tissues (e.g., skin biopsies), genotyping, and various other noninvasive methods. Animal models allow studying neurochemistry, physiology and anatomy of pain and nociception in controlled groups using standardized methods. In animal models, it is possible to investigate even within the same animals chronic pain at different hierarchies levels (molecular, cellular, network and behavioral levels). On tip of human studies, experimental animal studies are important to understand the complexity of pain and to develop effective treatments. The value of the results from animal studies in the translation of findings to clinical applications in humans has been questioned. Several drugs proven potent in rodent models failed in clinical phase 2 or 3 for being non-effective or for inducing important adverse effects which could not be predicted from pre-clinical studies. Anyhow, many pain treatments developed in animal studies have been successfully translated to clinical therapy. Among the recent successes are ziconotide (approved by FDA) for severe chronic pain[86] and tanezumab, a monoclonal antibody selectively targeting nerve growth factor, for treatment of osteoarthritic pain[87].

Rodents are the most commonly used experimental animals in pain research even though Gigliuto and collaborators[88] suggest using pigs because of their similarities to humans. While pigs can be more accurate in predicting human pharmacokinetics than rodents, their use is limited due to economic reasons. By nature, pigs need more space and food than rodents which contributes to their higher costs in terms of care of the animals (food, cage, etc.) and rent for the space.

Nociception tests and animal models of chronic pain are key elements in experimental pain research[89] Tests to assess emotional aspects of pain were developed only recently although emotional aspects of pain have a major impact on quality of life in patients suffering of chronic pain.

Nociception tests provide an acute method for assessing the avoidance threshold/latency to a noxious stimulus. For instance, tail flick, hot plate and plantar tests allow the assessment

of withdrawal latency of the tail or paw to a hot stimulus. Application of calibrated von Frey monofilaments representing different stimulus forces allows assessing the withdrawal threshold to a mechanical stimulus. Formalin test allows assessing sustained pain behavior induced by an intradermal injection of the chemical stimulus formalin[89].

The place-avoidance paradigm of LaBuda and Fuchs[90] was developed to assess the aversive aspects of pain in animal models of arthritic and neuropathic pain. In this paradigm, animals are placed in a two-chamber box with one of the chambers being dark and the other one exposed to light. Rats are free to move from between the chambers. In the dark chamber, supposed to be preferred by rats, the injured paw (hypersensitive paw) is exposed to noxious mechanical stimulation while in the light chamber, supposed to be anxiogenic for rats, the non-injured paw is exposed to noxious mechanical stimulation. The rats are supposed to compare the unpleasantness evoked by light with that evoked by stimulation of the injured-paw in the dark chamber that as such is more pleasant for the rat than the light chamber; if unpleasantness evoked by stimulation of the injured paw predominates, the rat is expected to prefer the light-exposed area. Among other tests developed for assessing pain affect are those based on vocalization or facial recognition[89].

Neuropathic pain models aim to reproduce human clinical conditions in which an injury of the nervous system causes neuropathic pain. Several experimental models of peripheral nerve injury have been developed. The spared nerve injury (SNI) model is one of the most commonly used ones. It consists of a ligation and an axotomy of two of the three terminal branches of the sciatic nerve. This leads to a long-lasting mechanical allodynia that corresponds to the nerve cut-induced neuropathic pain in human patients[91]. In case the rats develop autotomy (self-mutilation) behavior in the injured limb, it is considered the end-point and the rats are immediately euthanized. In the SNI model, only the area innervated by the sural nerve (the spared branch of the sciatic nerve) remains sensitive giving a restricted foot area for application of mechanical test stimuli. Due to denervation of the plantar skin, thermal plantar test cannot be performed in the SNI model.

A perfect behavioral test of nociception or an experimental animal model of pain should respond to these 5 points: specificity, sensitivity, validity, reliability and reproducibility[92]. Unfortunately, no test of nociception meets all of these criteria. For instance, the test of mechanical nociception, the mechanically-evoked flexor reflex, can be induced by a non-nociceptive stimulus. Furthermore, noxious stimuli have a high intensity and, therefore, it is difficult to exclude co-activation of non-nociceptive nerve fiber when a noxious stimulus is applied. If the pharmacological agent studied in the behavioral test affects the motor system, it provides a confounding factor for interpretation of the results in terms of nociception then it can be considered that the validity of the model is challenged. For these reasons, the animal model and the nociceptive test should be chosen carefully according the specific question of interest. Furthermore, various additional tests can be required to differentiate whether the observed changes are due to a selective action on the nociceptive system. For example, the rotarod test can be used to exclude an impairment of the motor coordination by the tested drugs.

Finally, studying experimental chronic pain that would exactly mimic chronic pain in the clinic would require long-lasting chronic pain models which is ethically debatable.

4.8.Amygdaloid complex

4.8.1. Anatomy

Amygdala (a Greek word meaning almond), also called amygdala complex, is a set of several nuclei: 1) the basolateral (BLA) group includes the lateral (L), basal (BA) and the accessory basal nucleus (AB) also known as the basomedial nucleus, 2) the central nucleus (CeA) is composed of the capsular (CeC), lateral (CeL) and medial (CeM) subdivisions, 3) the cortical nucleus, 4) the medial nucleus., 5) other amygdaloid nuclei includes the anterior amygdala area, the amygdalo-hippocampal area, and the intercalated nuclei (ITC) (Fig. 3)[93,94].

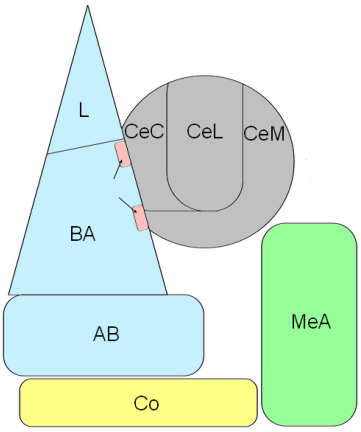


Fig. 3: Schematic representation of the main amygdala nuclei: BLA group (blue), central nucleus (grey), cortical nucleus (Co, yellow), medial nucleus (MeA, green) and the intercalated nuclei (ITC, pink).

The subdivision of amygdala is still subject to debate but it is no more considered as a distinct structural and functional unit. On the contrary, amygdala is considered to be part of different systems depending on the architecture, neuroanatomy, embryonic development, chemistry and functional role of each nucleus. According to Swanson and Petrovich[95], the CeA is striatal, the basolateral and lateral nuclei are extensions of the temporal and frontal lobes, the cortical and medial nuclei belong to the olfactory system, and the rest form the association part of the olfactory system. Also Heimer has proposed the concept of the extended amygdala[96]. It is divided into two parts: i) Medial extendend amygdala which is a continuum from the medial nucleus of amygdala through the lateral sublenticular extended amygdala to the medial bed nucleus of the stria terminalis; ii) Central extended amygdala which is a continuum of the central amygdala to the lateral bed nucleus of the stria terminalis by the surrounding ventral pallidum. Heimer considers the basolateral amygdaloid complex (including basal and lateral nuclei) a cortical structure[97].

4.8.2. Input

Amygdala receives input from two main sources: Cortical and thalamic inputs provide sensory and memory-related information; Hypothalamic and brainstem inputs provide information related to behavior and the autonomic system. Sensory inputs to amygdala originate mainly from cortical structures but also from thalamic nuclei (such as the medial geniculate and posterior internuclear nuclei) and the parabrachial nucleus, which provides nociceptive inputs[98]. Polymodal inputs arise mainly from the prefrontal cortex, perirhinal cortex and hippocampus to provide information related to memory, behavior and reward. Furthermore, hypothalamus and brainstem (midbrain, pons, medulla) inputs target mostly the CeA[93].

4.8.3. Output

Cortical and basolateral nucleus of amygdala send projections to cortical sensory areas. There are reciprocal projections between the frontal cortex/perirhinal area and the basolateral amygdala which arise from basolateral glutamatergic pyramidal-like neurons. There is also a reciprocal projection between the olfactory cortex and the cortical nucleus of amygdala. Projections from medial and central nuclei of amygdala form the extended amygdala (see above: anatomy of amygdala). Furthermore, CeA projects to hypothalamus, periaqueductal gray, parabrachial nucleus and nucleus of the solitary tract to control autonomic responses and nociception[50,99]. Moreover, central extended amygdala has several projections to monoaminergic and cholinergic neuron groups which innervate the forebrain and the memory system of the temporal lobe[93]. The CeA projections are inhibitory[100,101] by GABAergic neurons[102].

4.8.4. Central nucleus of amygdala (CeA)

The CeA is subdivided into three parts: medial, lateral and capsular. The laterocapsular division is considered as the nociceptive part of the amygdala[103]. Contrary to cortical-like neurons of the basolateral amygdala, those of the CeA are striatal-like and pallidal-like neurons[104] which contain several types of neuropeptides including substance P, vasoactive intestinal peptide, neurotensin, galanin, somatostatin, corticotropin-releasing factor (CRF) and enkephalin.

The CeA is known to be involved in emotions, autonomic responses, conditioned fear, social behavior, learning, memory, and reward[105]. The CeA is involved in different pathologies including anxiety[106], depression, schizophrenia, epilepsy, addiction and drug dependence[105,107,108].

The CeA is a major nucleus involved in nociception and pain. It influences descending modulation of pain[18,109–111], it is involved in emotional and sensory aspects of pain[94] and in behavioral and emotional responses to noxious stimulation. The CeA is considered to play a key role in persistent pain[14,112], probably contributing to the maintenance of pathophysiological pain in neuropathic and arthritic conditions and to depression-anxiety comorbidity[113,114].

4.9. Periaqueductal gray (PAG) – Rostral ventromedial medulla (RVM) system

The PAG is a midbrain structure surrounding the cerebral aqueduct. The RVM is a medullary structure which contains the nucleus raphe magnus and the adjacent reticular formation. Based on the response to noxious peripheral stimulation, PAG and RVM neurons can be subdivided into three types of cells: neutral-, ON- and OFF-Cells. The role of neutral cells is poorly known while PAG and RVM OFF-cells and ON-cells are considered to be involved in nociception since they, respectively, reduce or increase their activity during a noxious stimulus[115,116].

The PAG exerts a major role in the descending inhibition pathway relayed through the RVM[48] to the spinal dorsal horn. Both PAG and RVM receive direct projections from the spinal cord[117,118]. PAG has reciprocal connections with several brain structures including the cerebral cortex, hypothalamus and amygdala.

The PAG-RVM pathway is the best known descending pathway controlling nociception: PAG has direct excitatory and inhibitory projections to ON-, OFF- and neutral-cells of the RVM[119], all of which project to spinal cord[118,120]. Electric stimulation of the PAG or RVM induces analgesia in rats and cats[121–125]. PAG stimulation has proved analgesic even in humans[106]. The PAG-RVM antinociception is at least partly mediated by opioids[124] and partly by non-opioids like the cannabinoid system[126,127]. Furthermore, the spinal antinociceptive effect induced by PAG activation is blocked by inhibition of the RVM[128,129]. Interestingly, opioidergic descending antinociceptive effect induced by amygdala is mediated by PAG and RVM[49–51,130].

It is noteworthy that RVM stimulation has a biphasic (facilitatory and inhibitory) effect on spinal nociception[131–133]. While the PAG-RVM pathway is important for pain inhibition, it is also involved in the maintenance of chronic pain. Indeed, RVM[134–138] and PAG[139–142] participate together in the maintenance of persistent pain.

4.10. Corticotropin-releasing factor (CRF)

CRF, also known as corticotropin-releasing hormone, is a 41 amino-acids peptide which acts through two G-protein receptors CRF-R1 and CRF-R2, with higher affinity to the first one[27].

The free level of endogenous CRF is modulated by the CRF-Binding Protein (CRF-BP) which also keeps a pool of CRF available[143,144].This pool of CRF can be displaced by the high affinity CRF-BP ligand inhibitor, CRF_{6-33}[145].

CRF is widely spread over the central nervous system, with two distinct areas: the hypothalamic–pituitary–adrenal (HPA) axis, and the extrahypothalamic center which covers several nuclei including the amygdala. These two CRF areas are not separated but can influence each other. For instance, chronic overexpression of amygdaloid CRF induces hyperactivation of the HPA axis, causes anxiety and changes in gene expression[146].

In the CeA, CRF can be found, in order of quantity, in the lateral, medial and ventral subdivisions[105], where the presynaptic neurons contain CRF-R2 while the postsynaptic neurons contain both CRF-R1 and CRF-R2[147].

CRF of the CeA has several roles in behavior and physiology. It modulates GABAergic[148] and glutamatergic systems[149]. CRF is involved in addiction, withdrawal and drug-seeking[107,150], emotional processing, stress and anxiety[106,151], fear[152] and mental disorders[153].

CRF of the CeA is known to be involved in pain and nociception. Neuropathy induces an increase of amygdaloid CRF[154] and both CRF-R1 and CRF-R2 have differential effects on nociception processing, pain behavior and plasticity in arthritis[21,25].

4.11. Glutamatergic system

Glutamate is the major excitatory amino acid neurotransmitter in the mammalian central nervous system. Glutamate is highly regulated to avoid its excitotoxicity. The glutamate binds to ionotropic (AMPA, kainate, NMDA) and metabotropic receptors (mGluRs) (Table 1).

AMPA and kainate receptors are present in presynaptic and postsynaptic neurons. They are permeable to K^+ and Na^+ and Ca^{2+} depending on the subunit composition of the receptors[155], when glutamate or their specific agonist binds to them. AMPA and kainate receptors contribute to synaptic plasticity[156,157], and they can activate each other[158]. The calcium-permeable AMPA receptors are involved in pain and nociception by regulating depression-like behaviors in rat model of chronic neuropathic pain[159] and by regulating paired-pulse depression in nociceptive sensory synapses of rat's dorsal root ganglion and dorsal horn neurons[160].

NMDA receptor has the highest affinity to glutamate. NMDA receptor subunits include NR1, NR2A-D and NR3A-B[161]. At a resting potential, NMDA receptor is blocked by extracellular Mg^{2+} and Zn^{2+}. Removal of this block is voltage-dependent and activation of the NMDA receptor requires co-activation of glutamate and glycine to allow cationic ($Na^+$and Ca^{2+}) influx and (K^+) outflux. Activation of NMDA receptors is known to be involved in synaptic plasticity, memory and learning, and their neuronal correlates, long-term potentiation (LTP) and long-term depression (LTD)[162–164].

The metabotropic receptors are divided into three groups: Group I includes mGluR1 and mGluR5, Group II mGluR2 and mGluR3, and Group III mGluR4, mGluR6, mGluR7 and mGluR8. Metabotropic receptors are G-protein-coupled receptors which activate downstream mechanism such as phospholipase C and diacylgycerol (Group I receptors), or suppress downstream mechanisms such as intracellular cyclic adenosine monophosphate (cAMP; Group II and Group III receptors). In presynaptic neurons, mGluRs reduce GABAergic and glutamatergic transmission[165] while in postsynaptic neurons they modulate the activity of ionotropic channels[166]. Altogether, mGluRs participate in modulation of synaptic neurotransmission and neuronal excitability.

The CeA contains metabotropic and ionotropic glutamate receptors[93]. Interestingly, the subunit composition of NMDA receptors in the CeA does not change during development from immature to adult synapses, in contrast to NMDA receptors in pyramidal neurons of BLA synapses[167].

Results on the NMDA-dependency of LTP inside the CeA have been controversial. Kiritoshi et al. (2010) have shown that NMDA is involved in LTP in the CeL[168]. In line with this, Cheng et al. (2011) described an NMDA-dependent LTP in the CeC[169]. However, López de Armentia et al. (2007) reported that in the CeL receiving nociceptive inputs from the parabrachial nucleus LTP is NMDA-independent, whereas LTD is NMDA-dependent[170].

Both ionotropic and metabotropic glutamate receptors of the CeA play a role in pain processing. Interestingly, LTP is not mediated by NMDA receptors in neuropathic as it is in arthritic conditions[171]. Furthermore, in arthritic animals blockade of non-NMDA and NMDA receptors reduces neuronal activity in the CeA[172]. Paradoxically, both activation and blockade of NMDA receptors in the CeA of healthy controls has reduced pain affect induced by acute noxious stimulation[31,173]. Based on these findings, it seems that the glutamatergic system of the CeA has pleiotropic actions on the pain-nociception system that vary depending on the receptor subtype and pain state. In line with this, the mGluRs of the CeA have been shown to have different actions on nociception and pain affect depending on the subtype of the mGluR and the pathophysiological condition (healthy control, neuropathy, or arthritis)[29,109,174,175].

	Ionotropic			Metabotropic		
Receptors	NMDA	AMPA	kainate	Group I	Group II	Group III
Protein subunit	NR1 NR2A NR2B NR2C NR2D NR3A NR3B	$GluR_1$ $GluR_2$ $GluR_3$ $GluR_4$	$GluR_5$ $GluR_6$ $GluR_7$ KA1 KA2	$mGluR_1$ $mGluR_5$	$mGluR_2$ $mGluR_3$	$mGluR_4$ $mGluR_6$ $mGluR_7$ $mGluR_8$

Table 1: Classification of ionotropic and metabotropic glutamate receptors.

5. Aims of the study

The general aim of the study was to investigate the involvement of the central nucleus of amygdala (CeA) on pain and nociception in healthy control animals and in an animal model of chronic neuropathic pain. Two aspects of pain were studied: sensory-discriminative and emotional-like pain behavior. Additional study parameters were the lateralization of pain processing and the dependence of the results on the submodality of noxious test stimulation. The specific aims were:

- To analyze the effect of CRF and glutamatergic system of CeA on emotional-like aspect of pain in healthy controls and animals with peripheral neuropathy.
- To investigate the effect of CRF and glutamatergic system of CeA on sensory-discriminative aspect of pain in healthy controls and animals with peripheral neuropathy.
- To determine whether the CeA control of spinal hypersensitivity in animals with peripheral neuropathy is mediated by the descending serotoninergic pathway.
- To decipher whether the PAG is involved and necessary to the antinociception induced by CeA glutamate activation in healthy animals
- To observe whether lateralization of pain processing takes place in healthy and SNI animals.

6. Materials & Methods

6.1. Animals and Ethical permits

All experiments were performed using adult male Hannover-Wistar rats (Harlan, Horst, Netherlands; weight: 250–350 g). The rats were house in a 12-h light-dark cycle with food and water access *ad libitum*. They were housed in groups of 3 to 4 rats per cage except after intracerebral insertion of cannula, they were in single cages. The animals were sacrificed immediately after the experiments or if a human end-point was reached by an overdose of sodium pentobarbital.

The methods had been approved by the Animal Experimental Board of Finland and the experiments were performed according to the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC; Studies I & II) or the guidelines of European Communities Council Directive of 22nd September 2010 (2010/63/EU; Studies III, IV & V). All efforts were made to limit distress and to use only the number of animals necessary to produce reliable scientific data.

6.2. Drugs

CRF-BP ligand inhibitor human/rat CRF_{6-33}, the nonselective CRF receptor antagonist a-helical CRF_{9-41}, Glutamate (L-glutamic acid monosodium salt), NMDA receptor antagonist (+)-MK-801 maleate, $mGluR_{1/5}$ agonist DHPG (S)-3,5-dihydroxyphenylglycine, $mGluR_5$ agonist CHPG (RS)-2-chloro-5-hydroxy-phenylglycine, $mGluR_5$ antagonist MPEP 6-methyl-2-(phenylethynyl)pyridine, 5-HT_{1A} receptor antagonist WAY-100635, 5-HT_3 receptor antagonist ondansetron, 5-HT_{1A} receptor agonist 8-OH-DPAT, dopamine D2 receptor antagonist raclopride, the opioid receptor antagonist naloxone hydrochloride and dimethylsulfoxyde (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

The $mGluR_1$ antagonist CPCCOEt 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester was purchased from Tocris (Bristol, UK).

The α_2-adrenoceptor antagonist atipamezole was obtained from OrionPharma (Turku, Finland).

Sodium pentobarbital and physiological saline were purchased from OrionPharma, (Espoo, Finland)

Buprenorphine was purchased from Reckett & Colman (Hull, England)

Drugs were dissolved in saline, except for CPCCOEt that was dissolved in DMSO.

6.3.Anesthesia

Anesthesia was produced by intraperitoneal administration of 50 mg/kg of sodium pentobarbital and to prevent postoperative pain, animals were treated with intramuscular or subcutaneous administrations of 0.01 mg/kg of buprenorphine in the area of surgery after sni-sham and stereotaxic surgery. During the operations, the level of anesthesia was monitored by assessments of muscle tone, size of the pupils, limb withdrawal threshold to a noxious pinch of the paw, and ear reflex to an ear pinch. Supplemental doses of sodium pentobarbital were given if necessary.

During electrophysiological recordings or prolonged operations, the anesthesia was continued by administering sodium pentobarbital at the dose of 15–20 mg/kg/h or more if needed.

6.4.Stereotaxic surgery, cannula, microelectrode and catheter insertion and microinjection

6.4.1. Cannula insertion and recording electrode placement

Intracerebral fixation of cannula was done under anesthesia. The rat was lying on a warmed blanket to keep the rat's body temperature within physiologic range and the rat's head was fixed to a stereotaxic apparatus. Subcutaneous injection of 0.01 mg/kg of buprenorphine was done before scalp incision. Exposure of the skull allowed drilling holes above the desired stereotaxic coordinates according the atlas of Paxinos and Watson[176] to insert a 26-gauge guide cannula (C315G, PlasticsOne, Roanoke, VA, USA). Two extra holes were drilled to insert dental screws into the skull allowing the fixation of the cannula to the skull with dental cement. The tip of the guide cannula was positioned 2 mm above the desired injection site.

During acute electrophysiological recordings, the recording electrodes were inserted into the desired brain site through a hole in the skull with a micromanipulator.

6.4.2. Intrathecal catheter (i.t.) insertion

The anesthetized rat was lying on the table and the lumbar level of the spinal cord[177] was exposed to insert the catheter (Intramedic PE-10, Becton, Dickinson and Company, Sparks, MD, USA). I.t. catheter was installed in the same operation as nerve injury at least two weeks before actual drug testing. After recovery from anesthesia, only rats without motor impairment and responding with bilateral hind limb paralysis to i.t. administration of

lidocaine (4 %, 7–10 µl followed by 10 µl of saline for flushing) were studied further. For i.t. injections, a 50-µl Hamilton syringe (Hamilton Company, Bonaduz, Switzerland) was used.

6.4.3. Drug injection sites in the brain

The targets for intracerebral drug injections were:

- The left or right CeA, in the capsule lateral of central nucleus of the amygdala (CeC): 2.1 mm posterior from the bregma, 4.3 mm lateral from the midline, and 7.8 mm ventral from the dura mater.
- The rostral ventromedial medulla (RVM): 11 mm posterior from the bregma, in the midline, and 10.5 mm ventral from the dura mater.
- The right PAG: 7 mm posterior from the bregma, 0.8 mm lateral from the midline, and 5 mm ventral from the dura mater.

The control drug injection site was in the right internal capsule: 2.1 mm posterior from bregma, 3.6 mm lateral from the midline, and 5.0 mm ventral from the dura mater.

The target for i.t. drug injections was the lumbar level of the spinal cord.

6.4.4. Intracerebral microinjections

Drugs or saline were microinjected into the brain through a 33-gauge stainless steel injection cannula (C315I, PlasticsOne) connected to a 10-µl Hamilton syringe (Hamilton Company) by polyethylene tubing (Intramedic PE-10, Becton, Dickinson and Company). The injection cannula protruded 2 mm below the tip of the 26-gauge guide cannula (C315G, PlasticsOne).

The volume of intracerebral injections was 0.5 µl. To monitor the injection, a small air bubble was formed prior to drug or saline aspiration inside the catheter connecting the injection cannula with the syringe. Then, during the slow injection the movement of the bubble was watched to confirm the drug injection. Finally, injection needle was retained within the cannula for an additional 20 seconds after drug infusion to prevent backflow of the drug into the injection cannula.

The average spread of an intracerebral injections of dyes at the volume of 0.5µl is 1.04 mm[178]. Therefore in this thesis, we can expect a similar spread for the intracerebral injections, thereby covering also other subnuclei of the amygdala. Because of the proximity between the CeA and the BLA or the ITC, we could not exclude their contributions. However, since the CeA is the main output of the amygdala to the nociceptive descending controls[94], it may be argued that the CeA was involved in this thesis.

6.5. Peripheral neuropathy model induced by spared-nerve injury (SNI)

The spared-nerve injury is a model for peripheral nerve injury induced by ligation and axotomy of two of the three terminal branches of the sciatic nerve as described by Decosterd and Woolf [91].

After the rat was anesthetized, it received intramuscular 0.01 mg/kg of buprenorphine to prevent pain and was shaved in the left limb area for surgery, and the limb skin was opened with a scalpel; an incision was made in the muscle to allow the access to the sciatic nerve. Common peroneal nerve and tibial nerve were separated from the sural nerve with a glass stick, then they were ligated together and an axotomy was performed with a pair of scissors, without touching the sural nerve. Finally, the muscle and the skin were sutured. The rat was under surveillance until it recovered from anesthesia. During the following days the animal was carefully monitored to ensure that the healing of the wound was complete. Only SNI rats showing hyperalgesia to a calibrated monofilament producing a force of < 2g were selected for the experiments.

The operation for sham animals followed the same procedure as that in the SNI group, except for the ligation and axotomy of the two sciatic nerve branches.

The SNI surgery in the rat reproduces the symptoms of a peripheral neuropathy in humans, with long-lasting allodynia and hyperalgesia. The spared sural nerve innervates the lateral part of the foot pad. This induces a limitation when using nociceptive tests. While assessing withdrawal reflex of the limb to a calibrated mechanical stimulation of the sural nerve area (see monofilament chapter) is still feasible, the assessment of withdrawal latency to a noxious heat stimulus that with conventionally used devices is applied to the plantar skin cannot be done.

6.6. Habituation

All rats were habituated to the animal room, handling and the experimental conditions during 3 consecutive days before experiments started. This included habituation to a transparent box of the plantar test device, the test device used for assessing responses to mechanical stimulation, the place-avoidance test device, and handling with a gentle restraint to be performed when injecting drugs into the brain.

6.7. Behavioral assessment

6.7.1. Mechanical nociception

Withdrawal reflex threshold to a mechanical noxious stimulus was done by applying a calibrated series of monofilaments (North Coast Medical, Inc., Morgan Hill, CA, USA) producing a force from 0.008 to 300g (I, III and V), or from 1 g to 15 g (II and IV). During testing, the monofilament was applied to the foot pad while the rat was standing on a grid, free to move inside a transparent box. In I to V, mechanical pain sensitivity was determined by assessing responses to 5 repeated application of each stimulus force; an increase in the response rate was considered to represent an increase in hypersensitivity. The studied stimulus forces varied between 1-15 g (II) and 1-300 g (III and V), while in IV only the response to a force of 1.4 g was determined. In I, the stimulus forces varied from 0.008 g to 300 g and the index of sensitivity was the threshold that was defined as the minimum force required for evoking 5 consecutive limb-withdrawals.

6.7.2. Thermal nociception

Withdrawal reflex latency to a noxious heat stimulus was done by applying radiant heat from the testing device (Plantar test model 7370, Ugo Basile, Varese, Italy) to the plantar skin of a rat that was free to move inside a transparent box. The latency in seconds to the limb withdrawal was automatically assessed by the equipment. To avoid heat-induced injury, the cut-off point was set at 15s.

6.7.3. Aversive place-avoidance paradigm

To assess the aversive aspect of pain, the place-avoidance test was performed as described by LaBuda and Fuchs [90]. The rat was placed inside a box that was on top of a metal grid. Half of the box was transparent and exposed to a light source whereas the other half was painted in black and was dark inside. Rats were free to move on the grid from the light area to the dark area, or vice versa. In the dark area, the rats received mechanical stimulation of the injured paw with a monofilament at a force of 300 g, whereas in the light area the rats received mechanical stimulation of the uninjured paw. The duration of testing was 30 min. The aversive place-avoidance test assesses the aversive aspect of pain by measuring the time spent in the dark versus light area of the box. The light exposed area as such is considered anxiogenic and the dark area is normally preferred by rats. In the experimental setup, the rat needs to compare the unpleasantness induced by light *per se* with the unpleasantness induced by stimulation of the injured paw in the dark. The more the animal spends time in the light, the more aversive the pain induced by mechanical stimulation (occurring in the dark) is considered to be.

6.7.4. Course of the studies: behavioral pain assessment following drug injections into the CeA (I, II, III, IV, V), RVM (IV) or i.t. (IV)

Table 2 contains a resume of experimental parameters (time course, cannula placement, types of behavioral test, drugs and its injection period) of each study. Day of surgery is considered as time 0 in the timeline.

	Surgery	Place of cannula insertion	Time (week post-surgery)	Behavioral test	Time (week post-surgery)	Drugs	Injection period
Study I	Sham SNI	R or L CeA or R Internal capsule	1	Mechanical nociception Aversive avoidance behavior	2-4	Saline, CRF_{6-33} (0.01, 0.03, 0.1, 0.3 µg) CRF_{9-41} 1 µg 1 min before CRF_{6-33} 0.03µg	Drugs were administered 15 min before testing mechanical sensitivity and place-avoidance.
Study II	SNI	R and L CeA	<1 or 7	Mechanical nociception Aversive avoidance behavior	1 or 8	Saline, DMSO, DHPG (1.83µg/CeA), MPEP (11,50µg/CeA), MK-801 (1µg/CeA), CPCCOEt (5, 10, 20 µg/CeA)	Drugs were administered 5min before the place-avoidance test and 20min before testing mechanical sensitivity.
Study III	Unop	R or L CeA or R Ic	1	Thermal and mechanical nociception Rotarod test	2-4	Saline, glutamate (32-100µg), MK-801 2µg followed 5min later by glutamate 100µg, MK-801 2µg alone	Pain behavior was assessed 1, 10, 20, 40 and 60 min after drug administration as well as before it.

Study IV	SNI SNI + i.t. catheter	R CeA or R CeA and RVM or R Ic	1	Mechanical nociception	1-2	CeA inj: Saline, glutamate (9 and 100µg), MK-801 (1µg) alone or prior to glutamate. CeA + i.t. inj: Saline, WAY-100635 (3µg), ondansetron (5µg), atipamezole (5µg), raclopride (1µg) or naloxone (1µg) before CeA glutamate inj. MK-801 (1µg) CeA + RVM inj: Saline, 8-OH-DPAT (0.125µg) before glutamate (9 and 100µg)	Mechanical hypersensitivity was assessed in the injured hind limb before the treatment and 5, 15, 30 and 60 min after the treatment.
Study V	Unop	R or L CeA and PAG	1	Mechanical nociception	2-4	CeA injection alone: Saline, glutamate (100µg), MK-801 2µg followed 5min later by glutamate 100µg, MK-801 2µg alone. CeA + PAG injection: Saline, Lido 4% alone or 5min before each CeA injection.	Drugs were administered 1min before behavior tests.

Table 2: Behavioral pain assessment: Time course of each study. Unop = unoperated ; R = right ; L = left ; inj = injection ; CeA = central nucleus of amygdala ; Ic = internal capsule.

6.8.Muscular coordination assessment

Motor coordination and balance of the rats was assessed in the Rotarod test (Ugo Basile). The rats were placed on a drum the revolving speed of which was increasing 2 revolutions per minute. The maximum revolution speed at which the rats were able to stay on the drum was determined one minute after saline or drug administrations.

6.9.Electrophysiological recordings

Single unit recordings of PAG neurons were performed in anesthetized rats fixed in a stereotaxic frame. The recording electrode was placed into the PAG (Bregma = - 7 mm, Lateral = +/- 0.8 mm, Dorsoventral = 4.6-6.2 mm) and the cannula for drug injections was placed into the CeA.

Single neuron activity was recorded extracellularly with lacquer-coated tungsten electrodes (impedance 5–7 MΩ at 1 kHz). The signal was amplified and filtered using standard techniques. Data sampling was performed with a computer connected to a CED Micro 1401 interface and using Spike 2 software (Cambridge Electronic Design, Cambridge, UK).

Only PAG neurons giving an excitatory response to a noxious pinch of the tail were studied. Based on their excitatory response, these neurons were called ON-like cells. The discharge frequency of the ON-like cell was determined by recording first its spontaneous activity, then the responses to brushing the skin (an innocuous stimulus) and noxious pinching of the tail for 5 s with a hemostatic clamp were determined. This testing procedure was repeated at various time points after administration of drugs/vehicle into the CeA.

In the analysis of the stimulus-evoked responses, the baseline discharge frequency recorded before the test stimulation or drug application was subtracted from the discharge frequencies determined during and after stimulation; i.e. positive values represent excitatory responses evoked by peripheral stimulation, and negative values represent inhibitory responses.

6.9.1. Course of the studies: electrophysiological study on PAG ON-Cell activity following glutamate injection into the CeA (V)

To determine whether injection of glutamate into the CeA influences the neuronal activity of PAG ON-cells, extracellular single unit recordings were performed in anesthetized rats fixed to a stereotaxic frame according to the atlas of Paxinos and Watson[176]. The surgical procedures for installation of the injection cannula into the right or left CeA and a microelectrode into the PAG are described above in the section on Stereotaxic surgery. While the level of anesthesia may significantly influence the neuronal responses, anesthesia

was induced and maintained in the identical manner in all experimental conditions to minimize the confounding effect of anesthesia on potential differences in results between test conditions.

Neurons were classified according their response to a noxious pinch of the tail with a hemostatic clamp. Only neurons with an excitatory response during the pinch (classified as On-like cells) and a response to glutamate injection were used for recording experiments.

After characterization of the recorded cell, its spontaneous activity was first assessed for 2 min. Then the effect of 5s of non-noxious mechanical stimulation was assessed by applying a brush to the back skin followed by 5s of noxious mechanical stimulation assessed by applying hemostatic clamp to the tail. Then the spontaneous activity was again assessed for 3 min. After this, left or right CeA was treated with saline, glutamate, MK-801, or a combination of MK-801 followed 2 min later by glutamate. After drug injection, an innocuous brush and a noxious pinch stimulus was applied at time points 1 min and 5 min after drug injection. Spontaneous activity was recorded continuously and the spontaneous discharge recorded for one min before mechanical test stimulations was used to assess the effect of drug treatment on spontaneous discharge rate of the neurons.

6.10. Summary of time course of each study

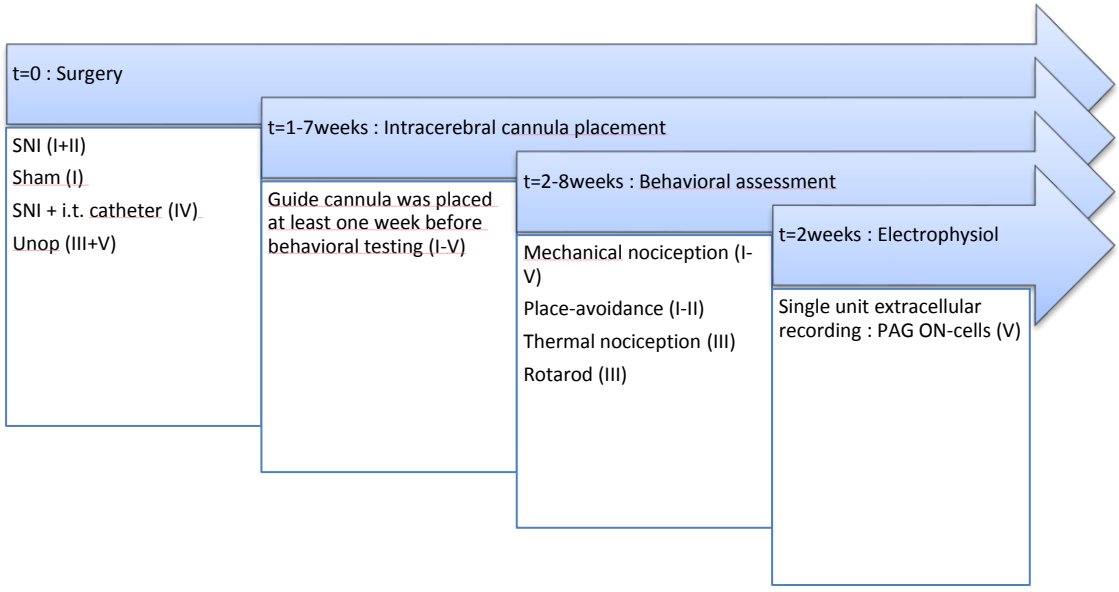


Fig. 4: Summary of the time course of the studies.

6.11. Histology

At the end of each experiment, rats were sacrificed by an overdose of pentobarbital and the brain was removed and immersed in 4% paraformaldehyde. Coronal sections of the brain

were cut to verify the site of injection according to the atlas of Paxinos and Watson[170]. Nissl's staining was used when determining the injection sites in the CeA.

6.12. Statistical analysis

Statistical analysis was done with Prism 5 for Windows software (GraphPad Software Inc., La Jolla, CA, USA). Data are presented as mean ± S.E.M. (I to V) and as median and interquartile range (I). Parametric data were analyzed using one- or two-way ANOVA, with repeated measures or mixed design when appropriate. Post hoc testing of parametric data was performed using t-test with a Bonferroni correction for multiple comparisons or Tukey's test. When comparing only two groups of parametric data, paired or unpaired t-test was used. Non-parametric data were analyzed using Friedman's test or Kruskal-Wallis test followed by Dunn's test. When comparing two groups of non-parametric data, Wilcoxon's signed rank test or Mann-Whitney U test was used. $P < 0.05$ was considered to represent a significant difference.

7. Results

7.1. Modulation of emotional-like aspect of pain by CeA

7.1.1. Reduction of emotional-like aspect of pain by endogenous CRF in the CeA of neuropathic animals (I)

In the aversive place-avoidance test, the SNI group spent less time in the dark (where their injured paw was stimulated) than the sham group as indicated by comparison of saline-treated sham and SNI animals (main effect of SNI: $F_{1,22}$ = 134.96, P < 0.0001). Furthermore, administration of CRF_{6-33} (CRF-BP antagonist that liberates free CRF) at the dose of 0.01, 0.03 and 0.1µg in the CeA of SNI rats reduced of time spent in the dark area ($F_{3,34}$ = 13.32, P < 0.0001) with a significant difference between the left versus right CeA injections ($F_{1,34}$ = 5.63, P < 0.03). Right CeA injection of CRF_{6-33} induced a dose-related increase in the time spent in the dark at all of the three doses while left CeA injection of CRF_{6-33} increased in the time spent in the dark at the doses of 0.01 and 0.03µg. The CRF_{6-33} effect was reversed by pretreatment of the right CeA of the SNI group with the CRF receptor antagonist CRF_{9-41} (1µg) that itself had no effect (Table 3).

In the sham group, administration of CRF_{6-33} in the right or left CeA at doses ranging from 0.01 to 0.1 mg failed to produce a change in time spent in the dark (main effect of drug dose: $F_{3,34}$ = 1.62) (Table 3). Similarly, administration of CRF_{6-33} in the control site (right internal capsule) in the sham or SNI group did not affect aversive place-conditioning.

The increase of the time spent in the dark induced by right CeA injection of CRF_{6-33} in SNI was significant 15 min after the injection to the end of the experiment (30 min after injection) ($F_{1,54}$ = 23.5, P < 0.0001).

7.1.2. Bidirectional modulation of emotional-like aspect of pain by CeA glutamatergic system in rat neuropathic pain model (II)

In the aversive place-avoidance test, bilateral CeA administration of the $mGluR_{1/5}$ agonist DHPG (3.66µg/animal) reduced the time spend in the dark whereas the $mGluR_1$ antagonist CPCCOEt (10 and 20µg/animal) ($F_{2,10}$ = 7.4, P < 0.02), the $mGluR_5$ antagonist MPEP (23µg/animal) and the NMDA-R antagonist MK-801 (2µg/animal) significantly increased time spend in the dark in neuropathic rats.

Unilateral administration of CPCCOEt also increased the time spend in the dark in a dose-related fashion (5-10µg/side) ($F_{2,37}$ = 8.5, P < 0.001). Increase of the time spent in the dark area was of the same magnitude following unilateral injection of CPCCOEt ipsi- as contralateral to nerve injury ($F_{1,37}$ = 0.23) (Table 3). Saline and DMSO alone failed to produce any change in the aversive-place avoidance behavior of neuropathic animals.

CeA treatment	SHAM		SNI		
	Right CeA	Left CeA	Right CeA	Left CeA	Bilateral CeA
CRF_{6-33} (0.01µg)	=	=	↓	↓	
CRF_{6-33} (0.03µg)	=	=	↓	↓	
CRF_{6-33} (0.1µg)	=	=	=	↓	
CFR_{9-41} (1µg) + CRF_{6-33} (0.03µg)			=		
CFR_{9-41} (1µg)			=		
CPCCOEt (5 and 10µg)			↓	↓	↓
MPEP (23µg)					↓
MK-801 (2µg)					↓
DHPG (3.66µg)					↑

Table 3: Results summary of the CeA's role in the modulation of emotional-like aspect of pain. ↑ = facilitation of emotional-like pain; ↓ = inhibition of the emotional-like pain; = = no significance compared to control injection (saline); empty case = no data available.

7.2.Modulation of sensory-discriminative aspect of pain by CeA

7.2.1. Pronociceptive effect of endogenous CRF in CeA of neuropathic or sham control rat (I)

In the sham group, administration of CRF_{6-33} at doses 0.01 to 0.3µg in the right CeA decreased the limb-withdrawal threshold both in the sham-operated left limb (KW = 10.8, P < 0.03) and the unoperated right limb (KW = 20.59, P < 0.0005; Fig. 2B) with a strongest effect at the dose of 0.03µg. In contrast, administration of CRF_{6-33} in the left CeA as well as in the control injection site (internal capsule) failed to induce any significant effect on the withdrawal threshold in sham-operated (KW = 0.78; Mann-Whitney U = 6.0) or unoperated animals (KW = 2.1; Mann Whitney U = 6.0).

In the SNI group, administration of CRF_{6-33} at dose 0.01 to 0.3µg in the right CeA decreased significantly the limb withdrawal threshold of the operated left limb (KW = 10.99, P < 0.03) while its effect was not quit significant on threshold of the unoperated right limb (KW = 8.69,

P = 0.07). Administration of CRF_{6-33} at doses 0.01 to 0.3µg in the left CeA failed to produce effect on the left operated limb whereas CRF_{6-33} at the dose of 0.03 mg significantly reduced the threshold in the unoperated right limb (KW = 12.0, P < 0.02). Injection of CRF_{6-33} (0.03µg) in the control site failed to produce effect on the left operated limb (Mann-Whitney U = 4.5) or the right unoperated limb (Mann-Whitney U = 5.5).

Pretreatment of the right CeA of the sham and SNI animals with CRF_{9-41} (1 µg) prevented the effect of the CRF_{6-33} (0.03µg) on the right unoperated limb (SNI KW = 8.76, P < 0.04) whereas it failed to reverse the effect of the CRF_{6-33} (0.03µg) on the left operated limb. Administration of CRF_{9-41} alone in the right CeA of the sham and SNI animals failed to induce effect on the withdrawal threshold of the uninjured right or injured left limb (Table 4).

The decrease of the unoperated right limb withdrawal threshold by left CeA injection of CRF_{6-33} in SNI animals was most prominent 10 to 15 min after the injection of the drug (KW = 11.5, P < 0.05).

7.2.2. Bidirectional modulation of sensory-discriminative aspect of pain by CeA glutamatergic system in healthy and neuropathic rats (II-III-IV)

7.2.2.1. Antinociceptive effect of glutamate via its NMDA receptor in healthy animals (III)

In healthy animals, unilateral injection of glutamate at the dose of 100µg, but not 32µg, into the right CeA increased the mechanically-evoked withdrawal threshold in the contralateral (Fr = 11.14, P = 0.0001) but not the ipsilateral limb (Fr = 0.14, P = 0.95). Similarly, when the glutamate injection was done in the left CeA, the withdrawal threshold was increased in the contralateral limb (KW = 19.9, P = 0.0002). Pretreatment of the right CeA with MK-801 (2µg), an NMDA-R antagonist, reversed the elevation of the withdrawal threshold of the contralateral limb induced by 100µg of glutamate in the right CeA while MK-801 alone did not. Moreover, glutamate injection into the left CeA induced a fast mechanical antinociceptive effect that lasted at least for 20 min (Fr = 16.3, P = 0.006).

Unilateral injection of glutamate at the dose of 32µg and 100µg into the right CeA induced a dose-dependent increase of the heat-induced withdrawal latency of both the ipsilateral and contralateral limb ($F_{2,10}$= 4.13, P = 0.05). The magnitude of heat antinociception was not different from that when the injections were done in the left as right CeA (main effect of the treated hemisphere: $F_{1,11}$= 0.04), although the antinociceptive effect was greater in the contralateral limb ($F_{1,11}$= 4.84, P = 0.05), independent of the treated hemisphere (inter-action between the treated hemisphere and the test side: $F_{1,11}$= 0.23). Furthermore, a pretreatment of the right CeA with MK-801 at the dose of 2µg failed to influence the amygdaloid glutamate-induced heat antinociception in the contralateral limb. Neither 2µg of MK-801 alone in the right CeA altered heat antinociception (Table 4). Glutamate in the left CeA induced a significant thermal antinociceptive effect one min after the injection. The

thermal pain sensitivity was back to normal by 10 min after the injection (main effect of time ($F_{5,15}$= 4.9, P = 0.007).

7.2.2.2. Nociceptive role of amygdaloid group I mGlu and NMDA receptors in animals with a peripheral nerve injury (II)

In SNI animals, bilateral administration of MK-801, an NMDA-R antagonist (2µg/animal), attenuated significantly the limb withdrawal response induced by mechanical stimulation. Additionally, bilateral and unilateral administration of CPCCOEt, an $mGluR_1$ antagonist, produced a dose-related (10–20µg/animal) decrease in the limb withdrawal response ($F_{2,10}$ = 31.2, P < 0.0001). The antinociceptive effect of unilateral administration of CPCCOEt was stronger when injected in the CeA contra- than ipsilateral to nerve injury ($F_{1,37}$ = 7.55, P < 0.01), independent of the dose ($F_{2,37}$ = 2.65).

Bilateral administration of the $mGluR_5$ antagonist MPEP (23µg/animal), the $mGluR_{1/5}$ agonist DHPG (3.66µg/animal), saline or DMSO (vehicle) alone into the CeA of neuropathic animals failed to influence the mechanically induced limb withdrawal response (Table 4).

7.2.2.3. Bidirectional effect of glutamate via its NMDA receptors in the CeA of animals with a peripheral nerve injury (IV)

Glutamate injection into the right CeA had a bidirectional effect on the mechanical hypersensitivity of the injured left limb (main effect of drug: $F_{2,75}$ = 26.90, P < 0.0001; interaction between drug and time: $F_{8,75}$ = 3.12, P = 0.0043). The dose of 9µg increased the hypersensitivity, with a peak effect at 5 min but dose of 100µg reduced the hypersensitivity, with a peak effect at 15 min (Fig. 5).

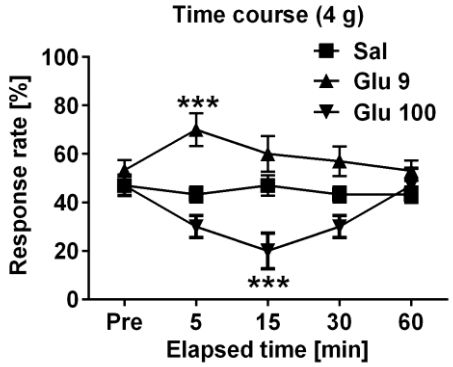


Fig. 5: Time course of the effect of glutamate 9µg and 100µg injections inside the right CeA on the nerve-injured limb withdrawal threshold induced by mechanical stimulus (monofilament of 4 g). Error bars represent S.E.M. ***p<0.005.

Pretreatment of the CeA with the NMDA receptor antagonist MK-801 (0.1µg) completely reversed the increase of mechanical hypersensitivity induced by CeA injection of a low dose of glutamate (9µg) but failed to attenuate the reduction of hypersensitivity induced by CeA injection of a high dose of glutamate (100µg).

CeA injection of MK-801 alone induced a dose-related effect (doses: 0.1, 0.3 and 1µg) on the mechanical hypersensitivity ($F_{3,26} = 8.80$, $P = 0.0003$) (Table 4).

CeA treatment	HEALTHY + SHAM		SNI		
	Right CeA	Left CeA	Right CeA	Left CeA	Bilateral CeA
CRF_{6-33}	$\uparrow^{R}$	=	$\uparrow^{L}$	$\uparrow^{R}$	
CRF_{9-41} + CRF_{6-33}	$=^{R}$		=		
CRF_{9-41}	$=^{R}$		=		
Glutamate (9µg)			$\uparrow^{L}$		
MK-801 (0.1µg) + Glutamate (9µg)			$=^{L}$		
Glutamate (100µg)	$\downarrow^{L}$	$\downarrow^{R}$	$\downarrow^{L}$		
MK-801 (0.1µg) + Glutamate (100µg)			$\downarrow^{L}$		
MK-801 (2µg) + Glutamate (100µg)	$=^{L}$	$=^{R}$			
MK-801 (0.3µg and 1µg)			$\downarrow^{L}$		
MK-801 (2µg)	=	=			$\downarrow^{L}$
CPCCOEt (20µg)			$\downarrow^{L}$	$\downarrow^{L}$	$\downarrow^{L}$
MPEP (23µg)					$=^{L}$
DHPG (3.66µg)					$=^{L}$

Table 4: Results summary of the CeA's role in the modulation of sensory-discriminative aspect of pain, as revealed by limb withdrawal response to mechanical stimulation. ↑ = facilitation of mechanical hypersensitivity; ↓ = inhibition of mechanical hypersensitivity; = = no significance compared to control injection (saline); empty case = no data available; ^{L}= Left paw; ^{R}=right paw.

7.3. Pathway mediating descending modulation of nociception induced by CeA in healthy and neuropathic animals (IV and V)

7.3.1. Involvement of descending serotonergic pathways in the bidirectional effect on nociception induced by glutamate in the CeA of neuropathic animals (IV)

I.t. injection of the 5-HT_{1A} receptor antagonist WAY-100635 at 3µg completely reversed the antihypersensitivity effect induced by a high dose of glutamate (100µg) in the right CeA. In contrast, i.t. injections of an α_2-adrenoceptor antagonist atipamezole (5µg), a dopamine D_2 antagonist raclopride (1µg), or an opioid receptor antagonist naloxone (1µg) were not effective. All four antagonists alone did nothing (Fig. 6A).

I.t. injection of a 5-HT_3 receptor antagonist ondansetron at a dose of 5µg completely reversed the increase of hypersensitivity induced by a low dose of glutamate (9µg) in the right CeA. Ondansetron alone (5µg) failed to induce effect on mechanical hypersensitivity (Fig. 6B).

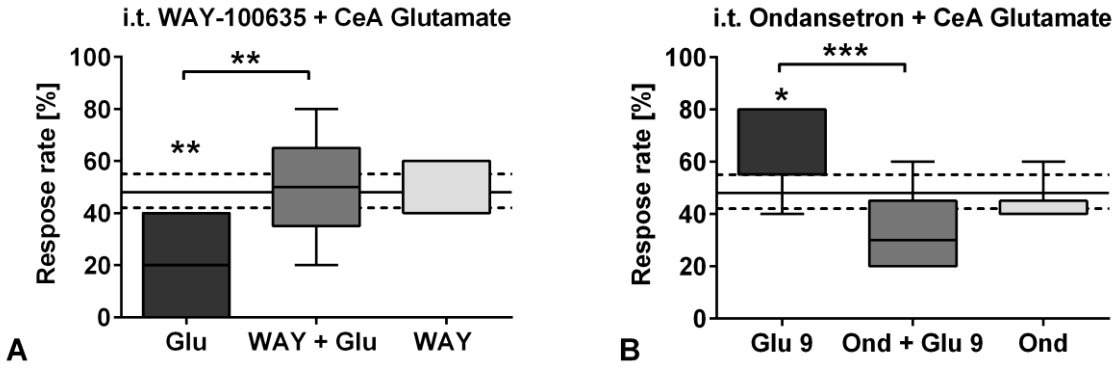


Fig. 6: A. Effect of blocking the spinal 5-HT_{1a} receptors with WAY-100635 (WAY) at 3µg on the reduction of the mechanical hypersensitivity mediated by CeA injection of glutamate (GLU) at 100µg. B. Effect of blocking the spinal 5-HT_3 receptors with ondansetron (Ond) at 5µg on the facilitation of mechanical hypersensitive mediated by CeA injection of glutamate at 9µg. Error bars represent S.E.M. In both graphs, the horizontal lines represent the mean response and its 95% confidence limits in saline-treated animals. *p<0.05, **p<0.01, ***p<0.005.

RVM injection of a 5-HT_{1A} receptor agonist 8-OH-DPAT (0.125µg) reversed both the increase of the hypersensitivity induced by a low dose of glutamate (9µg) in the CeA injection and the decrease of the hypersensitivity induced by a high dose of glutamate (100µg) in the CeA. 8-OH-DPAT (0.125µg) alone failed to produce effect on hypersensitivity.

7.3.2. Involvement of periaqueductal gray in spinal antinociception induced by glutamate in the CeA of healthy animals (V)

Injection of glutamate (100µg) into the right CeA, following pretreatment of PAG with saline, increased the mechanical withdrawal threshold in the contralateral limb (Wilcoxon's test: n = 8, W = -36, P = 0.01) but failed to produce effect in the ipsilateral limb (Wilcoxon's test: n = 8, W = -2, P = 0.89) when compared to the effect of saline injection into the right CeA in animals pretreated with saline inside PAG.

Pretreatment of the PAG with lidocaine (4%) prevented the increase of the mechanically evoked withdrawal threshold in the contralateral limb induced by glutamate (100µg) in the CeA (Fr = 14.86, P < 0.0001). *Post hoc* test showed no difference between pretreatment of the PAG with lidocaine (4%) followed by saline injection in the CeA and pretreatment of the PAG with lidocaine (4%) followed by glutamate (100µg) in the CeA. None of the three drugs administration conditions influenced the ipsilateral limb withdrawal threshold (Fr = 3.39, P = 0.15) (Fig. 7).

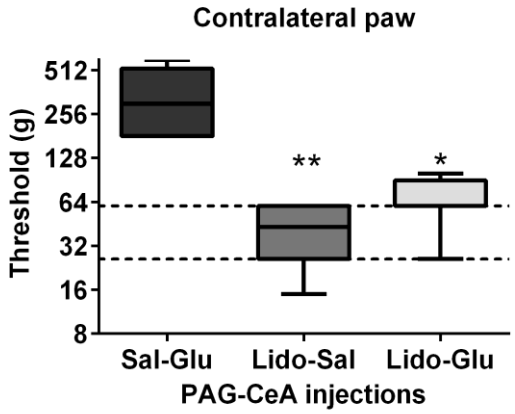


Fig. 7: Effect of pretreatment of the PAG with lidocaine (4%) on the mechanical antinociception mediated by glutamate (100µg) inside the CeA. Error bars represent S.E.M. Horizontal dash lines represent the interquartile range of the median threshold in the corresponding saline-treated animals. Sal = saline, Lido = lidocaine, Glu = glutamate. *p<0.05, **p<0.01.

None of the following four drugs administration conditions influenced the ipsilateral limb withdrawal threshold (Fr = 2.59, P = 0.51) nor the contralateral limb withdrawal threshold (Fr = 6.33, P = 0.08): pretreatment of the PAG with saline followed by injection of MK-801 (2µg) alone, pretreatment of the PAG with saline followed by injection of MK-801 (2µg) prior glutamate (100µg), pretreatment of the PAG with lidocaine (4%) followed by injection of MK-801 (2µg) alone, or pretreatment of the PAG with lidocaine (4%) followed by injection of MK-801 (2µg) prior to glutamate (100µg).

7.3.3. Glutamate in the CeA increases spontaneous discharge of PAG On-cells in healthy animals (V)

Glutamate (100µg) injection into the right CeA increased the spontaneous activity of ON-like cells in the right PAG (time as main factor: $F_{2,26} = 16.85$, P = 0.0001). *Post-hoc* tests showed that the peak increase of activity occurred during the first minute. The discharge rate of ON-like PAG cells varied significantly with the drug treatment condition (drug treatment of CeA as main factor: $F_{2,17} = 8.94$, P = 0.006). Post hoc tests indicated that pretreatment of the right CeA with MK-801 (2µg) prevented the increase of spontaneous activity in PAG ON-like cells following glutamate (100µg) injection into the CeA. MK-801 alone failed to influence the discharge rate of the PAG ON-like cells compared to saline injection ($t_5 = 2.09$, P = 0.09) or compared to spontaneous activity before the CeA injection ($t_5 = 0.52$, P = 0.62) (Fig. 8).

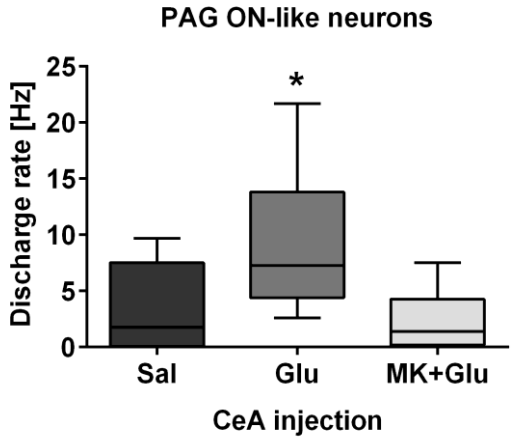


Fig. 8: Effect of CeA injection of glutamate (100µg) alone or pretreated with NMDA-receptors antagonist MK-801 (2µg) on the discharge rate of the PAG ON-Cells. Sal = saline, Glu = glutamate, MK = MK-801. *p<0.05.

8. Discussion

This thesis has pointed the involvement of the glutamate and CRF of CeA in pain processing in healthy and rats with peripheral neuropathy. The studies have emphasized the role of the CeA in emotional-like and sensory-discriminative aspect of pain and they have highlighted the serotoninergic descending pain mechanisms and the role of the PAG in pain inhibition mediated by glutamate of CeA.

8.1.Role of the CeA in the emotional-like aspect of pain (I and II)

The aversive place-avoidance test[90] is designed to highlight the emotional-like aspect of pain by forcing the rat to choose between the light (= anxiogenic) area associated with mechanical stimulation of the non-operated (= normosensitive) limb and the dark (non-anxiogenic) area associated with mechanical stimulation of the operated (hypersensitive) limb.

Our results suggest that the CeA plays a role in the modulation of the emotional-like aspect of pain in a rat model of peripheral neuropathy. This is indicated by the finding that the release of endogenous CRF induced by blocking the CRF-BP as well as blocking amygdaloid group I mGluRs with a selective antagonist reduced the emotional-like aspect of pain.

The free endogenous CRF differentially influenced sensory-discriminative and emotional-like pain responses. The group I mGluR antagonist attenuated emotional-like pain behavior at a lower dose than limb withdrawal response, an index of sensory-like pain. These results suggest that both CRF and group I mGluRs in the CeA modulate the emotional-like pain processing. Interestingly, a recent study showed that blockade of NMDA and non-NMDA receptors in the CeA suppressed the affective but not the sensory-discriminative aspect of pain[179].

Previously, the group I mGluR in the CeA has been shown to be involved in pain-like behavior[30,175]. Also CRF in the CeA is associated with control of pain behavior[21,180] and emotional responses to painful stimulation[181,182]. In earlier studies on the amygdaloid CRF, focus was on pharmacological manipulation of CRFR1 and CRFR2 with synthetic compounds. In this thesis, we demonstrated that blocking CRF-BP, that released free endogenous CRF in the CeA, modulates emotional-like pain behavior. The neuronal mechanisms of this action still remain to be studied. According to recent studies, the PAG plays a role in negative emotions related to pain[183], and in the suppression of emotional-like aspect of pain induced by activation of amygdaloid NMDA receptors[173]. Furthermore, CRF-containing neurons of the CeA project to the serotoninergic neurons in the ventrolateral PAG where they may inhibit emotional responses, such as panic-like behavior[184]. It is hypothesized that the PAG is involved in modulation of emotional-like behavior by CRF and glutamate in the CeA. Furthermore, it has been reported that spinal ligation-induced

neuropathy decreases GABAergic inhibition in the CeA. This contributes to the development of anxiety-like behavior associated with neuropathic pain[185].

8.2. Roles of CRF and glutamate in the CeA in descending control of sensory-discriminative aspect of pain (I - IV)

8.2.1. CRF (study I)

Inhibiting the CRF-BP with CRF_{6-33} is supposed to induce a release of endogenous CRF that decreases the limb-withdrawal threshold to mechanical stimulation in sham and SNI animals. This interpretation is supported by the finding that the non-selective CRF receptor antagonist CRF_{9-41} attenuated the mechanical hypersensitivity effect induced by CRF_{6-33}. Our study shows that the effective dose of CRF_{6-33} was 0.03µg while a higher dose was weaker. In line with this study, administration of a low dose of CRF into the CeA has been associated with facilitation of nociception via CRF-R1 and that of a high dose with inhibition of nociception via CRF-R2[25].

8.2.2. Glutamatergic system (II - IV)

In rats with peripheral neuropathy, amygdaloid glutamate had a bidirectional effect on spinal nociception. A low dose of glutamate in the CeA facilitated mechanical hypersensitivity. This action was prevented by blocking the amygdaloid NMDA receptors. In contrast, a high dose of glutamate in the CeA reduced mechanical hypersensitivity. This descending antinociceptive action was not prevented by blocking of amygdaloid NMDA receptors. Also blockade of $mGluR_1$ and NMDA receptors in the CeA of neuropathic animals had a pronociceptive effect as indicated by facilitation of the limb-withdrawal response to mechanical stimulation. In healthy animals, blocking NMDA receptors in the CeA failed to produce an effect on spinal nociception whereas a high dose of glutamate in the CeA of healthy controls had a mechanical antinociceptive effect. This latter effect was reversed by pretreatment of the CeA with an NMDA receptor antagonist. Altogether, these results suggest that in the CeA of neuropathic animals, a tonic NMDA receptor-mediated glutamatergic facilitation of descending pronociception contributes to hypersensitivity. In line with this, it has been previously shown that amygdaloid group I mGluRs enhance nociception in peripheral neuropathy[109] or arthritis [172]. Moreover, in accordance with our results, glutamate injections into the CeA as well as electric stimulation of the CeA have been shown to induce an antinociceptive effect in healthy animals[50,186]. Furthermore, results of study II suggest that the NMDA receptor in the CeA contributes predominantly to the mechanical antinociceptive effect since a NMDA receptor antagonist MK-801 reversed the mechanical but not thermal antinociceptive effect induced by glutamate in the CeA of healthy controls.

Interestingly, earlier electrophysiological results in peripheral neuropathy indicate that the synaptic plasticity of ascending nociceptive transmission from the parabrachial nucleus to the CeA is NMDA-independent[187] whereas in inflammatory pain it is NMDA-dependent[188]. Furthermore, GABAergic inhibition plays a role in the control of neuronal excitability in the CeA; a decrease of the GABAergic inhibition has been shown in the spinal nerve ligation model of neuropathy, which contributes to the development of neuropathic pain [185]. It is hypothesized that in neuropathic conditions the tonic facilitation of hypersensitivity mediated by the amygdaloid NMDA-receptor could be associated with disinhibition of the amygdaloid GABAergic system.

8.3.A dual involvement of descending serotoninergic pathways in the control of spinal hypersensitivity by the CeA in animals with peripheral neuropathy (IV)

Activation of 5-HT_{1A} receptors in the RVM reversed both the pronociceptive and antinociceptive effects induced by glutamatergic system of CeA. Since 5-HT_{1A} receptors in the RVM are presumably autoreceptors, the activation of which inhibits 5-HT neurons, these findings suggest that serotonergic neurons of the RVM relay both facilitatory and inhibitory effects on spinal nociception (Fig. 2).

The mechanisms underlying the bidirectional effects induced by a low and high dose of glutamate in the CeA and the exact roles of RVM serotonergic neurons and spinal serotonin receptors in relaying the bidirectional effect remain open. It is possible that the low and high dose of glutamate activated different glutamate receptors and neuronal populations in the CeA and subsequently activating of different neuronal populations in the RVM. Indeed, 3 types of serotonergic neurons projecting to the spinal cord have been characterized in the RVM[189]. Alternatively, a gradual increase of the activity in serotonergic raphe-spinal neurons may have shifted the net spinal effect from the 5-HT_3-mediated pronociception to the 5-HT_{1A}-mediated antinociception. Interestingly, an earlier study[131] showed that either electrical or chemical activation of the RVM induces biphasic modulation of spinal nociception in the same dose-related fashion as glutamate in the CeA (study I) namely a low dose of glutamate facilitated spinal nociception whereas a high dose suppressed spinal nociception but in the CeA and RVM.

Blocking the spinal 5-HT_3 reversed the increase of hypersensitivity and blocking the spinal 5-HT_{1A} receptors reversed the decrease of hypersensitivity induced by amygdaloid administration of a low or a high dose of glutamate, respectively. These results are in line with previous findings indicating that the spinal 5-HT_3 receptors have a pronociceptive effect in chronic pain conditions[190], including neuropathy[191,192], while the spinal 5-HT_{1A} receptors have an antinociceptive effect in pathological pain conditions[193,194].

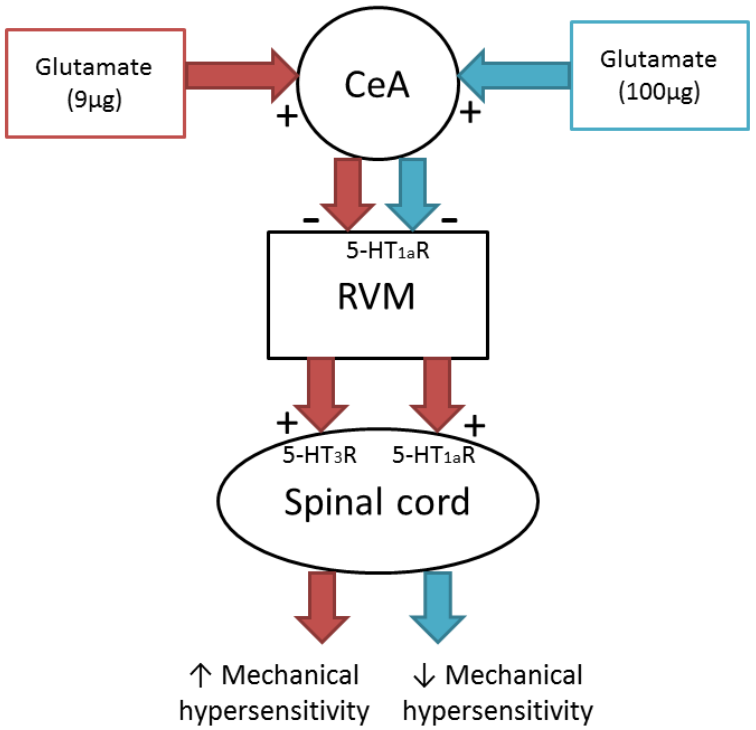


Fig. 2: Involvement of the serotoninergic descending pathways in the bidirectional modulation of the hypersensitivity induced by glutamate injection in the CeA in rats with peripheral neuropathy. + = facilitation, - = inhibition, ↑ = increase, ↓ = decrease.

8.4.Involvement of the PAG in the descending antinociceptive effect induced by glutamate in the CeA of healthy animals (V)

Local lidocaine anesthesia of the PAG blocked the descending antinociceptive effect induced by glutamate in the CeA. This indicates that the antinociceptive effect was relayed through the PAG (Fig. 3). The CeA sends projections to PAG[195,196] which is known to be involved in descending control of pain[197,198]. This is at least partly mediated by the opioidergic system[49,50]. Interestingly, a recent study showed involvement of the PAG in suppression of pain affect mediated by NMDA receptors in the CeA while spinal nociception was not affected by NMDA receptors[173]. However, studies II, III, IV and V showed modulation of spinal nociception induced by NMDA receptors in the CeA. Study V showed that the PAG is involved in descending modulation of nociception induced by the CeA. Differences in experimental conditions, such as doses of the studied compounds, might explain the difference in effects of NMDA receptors on pain and nociception between these studies.

Study V adds electrophysiological information related to the role of the PAG ON-cells that were previously characterized by Heinricher and collaborators[115]. A high dose of glutamate in the right CeA increased in the spontaneous firing rate of PAG ON-Cells. This effect was prevented by pretreatment of the CeA with an NMDA receptor antagonist. Moreover, blocking of the PAG with lidocaine 4% prevented the development of spinal

antinociception induced by glutamate in the CeA. These findings are in line with the hypothesis that PAG ON-Cells have a role in relaying antinociceptive action from the CeA to the spinal dorsal horn. When comparing electrophysiological and behavioral study, it should be taken into account that in the former the rats were anesthetized whereas in the latter they were conscious. Anesthesia may have influenced synaptic transmission from the CeA to the PAG and thereby can modify neuronal responses of PAG cells to glutamate treatment of the CeA as has been shown e.g. in the substantia nigra pars reticulata[199].

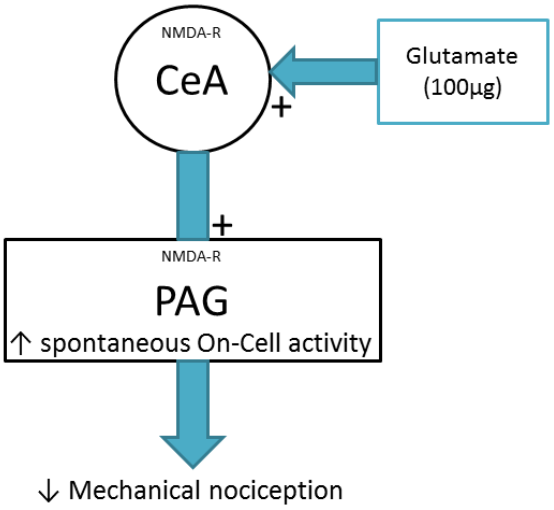


Fig. 3: Involvement of the PAG in descending mechanical antinociception induced by glutamate (100µg) in the CeA of healthy animals. + = facilitation, ↑ = increase, ↓ = decrease.

8.5.Hemispheric lateralization (I, III, V)

Hemispheric lateralization of nociception processing has been reported in electrophysiological[200,201] and molecular[30,202] studies in experimental animals whereas a functional imaging study failed to find this[203]. In neuroimaging of healthy human subjects, a context-dependent deactivation, rather than activation of the amygdala that varies with the hemisphere, has been associated with painful stimulation[204]. This thesis showed a loss of hemispheric lateralization in the effect of amygdaloid CRF on nociception following peripheral nerve injury (I) whereas no hemispheric lateralization was observed in the descending control of nociception by the glutamatergic system of the CeA in healthy animals (III and V). This finding is in line with recent results showing equivalent effects by the glutamatergic system of both left as are right CeA on the affective aspect of pain in healthy animals[31]. It remains to be studied which conditions and underlying mechanisms determine whether the CeA processing of nociception is lateralized or not.

8.6.Perspectives

My thesis has shown some of the neurotransmitter mechanisms that are involved particularly in descending control of pain by the CeA of rats with an experimental model of peripheral neuropathy as well as in healthy controls. However several questions remain unsolved. While the thesis focused on the roles of amygdaloid glutamate and CRF in the maintenance of pain sensitivity in neuropathic and control conditions, the potential role of these amygdaloid neurotransmitters in the development of chronic pain still needs to be understood.

The mechanism underlying the dissociation of the CeA-induced control of the sensory-discriminative and emotional-like aspects of pain is an interesting topic for future studies. Understanding the mechanism underlying this dissociation may provide a possibility for the development of selective therapy acting only on the emotional-aspect of pain that can be the most disturbing symptom from the patient's point of view. Interestingly, a recent study has shown that nasal application of neuropeptide S has a dissociative effect on affective-emotional and sensory aspects of pain through amygdala in animals with experimental arthritis. Neuropeptide S inhibited vocalizations induced by manipulations of the arthritic joint while it failed to influence hind limb withdrawal thresholds[205]. However, to develop this field of research further, there is a need for relevant behavioral tests assessing affective-emotional aspects of pain in experimental animal models of chronic pain.

This thesis has described a descending pain control pathway from the CeA to the spinal cord involving the medullospinal serotonergic pathways acting on the pronociceptive 5-HT_3 and antinociceptive 5-HT_{1A} receptors in the spinal dorsal horn. Antidepressant drugs (tricyclic antidepressants, selective serotonin reuptake inhibitors, and serotonin-norepinephrine reuptake inhibitors) are recommended as the first line treatment for neuropathic pain. However, antidepressants' efficacy varies with the type of the drug and they are not exempted from side-effects[206]. Furthermore, a recent study in a rat model of arthritic pain has shown that the 5-HT_{2C} receptor in the basolateral nucleus of the amygdala reduces the efficacy of the SSRIs to inhibit emotional-like aspect of pain[11]. Thus, understanding of the involvement of the serotoninergic descending pain pathway originating in the amygdala as well as the serotonergic medullospinal pathway driven by the CeA is of importance when attempting to develop more specific and effective pharmaceutical therapies against affective-emotional pain in various pathophysiological conditions.

Furthermore, this thesis has shown that depending on the dose, the effect of glutamate injected into the CeA may vary from pro- to antinociception. Amygdaloid mechanisms explaining these opposite effects are not yet known. Further studies are required to assess which glutamatergic receptors in the CeA induce pronociceptive and which ones antinociceptive effects, and which efferent pathways mediate these opposite effects within and outside the amygdala.

Synthetic CRF-R1 antagonists have been tested in clinical trials for treatment of depression, but so far tests have always failed due to inefficacy or adverse effects [207]. If safety issues

allow, it would be tempting to test whether synthetic CRF-R1 antagonists attenuate chronic pain in human patients. The involvement of the CRF-R1 has been demonstrated in experimental animal models of chronic pain[25,180]. Concerning amygdaloid CRF, this thesis has shown a differential action of the endogenous CRF on sensory-discriminative versus emotional-like pain as revealed by assessments of the withdrawal threshold and aversive place-conditioning in the CeA of animals with peripheral neuropathy, respectively, following block of the amygdaloid CRF-BP. More studies are still needed to understand the complex actions and interactions of the amygdaloid CRF system in pain.

9. Conclusions

The thesis has investigated the role of the CeA in nociception and pain. The results can be summarized as follows:

- CeA plays a role in the modulation of the emotional-like aspect of pain in rats with peripheral neuropathy. Activation and blocking of the group I mGluRs facilitates and inhibits, respectively, the aversive aspect of neuropathic pain. Furthermore, increase of endogenous CRF as well as blocking NMDA receptors in the CeA reduced the aversive aspect of neuropathic pain.
- CeA plays a role in the descending modulation of the sensory-discriminative aspect of pain both in healthy and SNI rats. The CeA can facilitate or reduce sensory component of pain depending on the type and the dose of the amygdaloid neurotransmitter. Blocking the amygdaloid CRF-BP in the CeA has a pronociceptive effect in both control and SNI rats. Chemical activation of the CeA with a high dose of glutamate has a mechanical antinociceptive effect in both healthy and SNI rats. Mechanical antinociception induced by a high dose of glutamate in the CeA is mediated by amygdaloid NMDA receptors in healthy but not in SNI rats. A low dose of glutamate has a pronociceptive effect in SNI rats. The descending pronociception induced by a low dose of glutamate in SNI animals is mediated by amygdaloid NMDA receptors. Furthermore, tonic descending pronociception induced by NMDA receptors and $mGluR_1Rs$ in the CeA is present in SNI but not healthy controls and contributes to the maintenance of neuropathic hypersensitivity.
- Serotonergic neurons of the RVM relay both descending pronociceptive and antinociceptive effects from the CeA to the spinal dorsal horn. Pronociception induced by a low dose of glutamate in the CeA is mediated by medullospinal serotonergic pathways acting on the spinal $5\text{-}HT_3$ receptor whereas antinociception induced by a high dose of glutamate in the CeA is mediated by medullospinal serotonergic pathways acting on the spinal $5\text{-}HT_{1A}$ receptor.
- The PAG is a relay in the descending control of nociception induced by glutamate in the CeA of healthy animals: The descending antinociceptive effect induced by a high dose of glutamate acting on NMDA receptors in the CeA is associated with increased discharge rate of PAG neurons that have ON-like response properties (i.e., they are activated by noxious peripheral stimulation). Blocking the PAG with lidocaine prevents the descending antinociceptive effect induced by glutamate acting on NMDA receptors in the CeA.
- The loss of hemispheric lateralization in the pronociceptive effect of the amygdaloid CRF takes place in peripheral neuropathy while sham-operated control groups conserve a right-hemisphere lateralization in the pronociceptive effect of amygdaloid CRF. However, descending antinociception induced by the glutamatergic system of the CeA shows no hemispheric lateralization in healthy control rats; a high dose of glutamate in both the left and right CeA induces attenuation of mechanical and thermal nociception which effects are, respectively, NMDA-dependent and NDMA-independent.

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"*Odi panem quid meliora*. Ça ne veut rien dire mais je trouve que ça boucle bien."
Roi Loth, *Kaamelott*, Livre V

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Corticotropin-Releasing Factor in the Rat Amygdala Differentially Influences Sensory-Discriminative and Emotional-like Pain Response in Peripheral Neuropathy

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Abstract: The central nucleus of the amygdala (CeA) is involved in processing and regulation of pain. We determined whether amygdaloid corticotropin-releasing factor (CRF) contributes to pain modulation in the neuropathic rat. Emotional aspect of pain was assessed by an aversive place-conditioning test and sensory aspect of pain by determining monofilament-induced limb-withdrawal threshold. CRF_{6-33} (an inhibitor of CRF-binding protein) or CRF_{9-41}, a nonselective CRF receptor antagonist, was microinjected to the left or right CeA or a control site in rats with spared nerve injury (SNI) or sham operation of the left hind limb. In SNI animals, CRF_{6-33} in the left or right CeA, but not in a control site, attenuated emotional painlike behavior and increased sensory pain. In sham controls, CRF_{6-33} in the right but not left CeA increased sensory aspect of pain, without influence on place-avoidance behavior. The effects induced by CRF_{6-33} were reversed by CRF_{9-41}. The results indicate that endogenous CRF in the CeA, through action on CRF receptors, may differentially influence emotional and sensory aspects of pain in neuropathy. While the right CeA had a dominant role in modulation of pain-related responses in sham controls, left as well as right CeA contributed to pain modulation in neuropathic animals.

***Perspective:** An increase in free endogenous corticotropin-releasing factor in the central nucleus of the amygdala was accompanied by increased cutaneous hypersensitivity and decreased emotional painlike behavior in neuropathic animals. This finding indicates that CRF in the amygdala may have differential effects on sensory and emotional aspects of neuropathic pain.*

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***Key words:** Affective-motivational pain, amygdale, corticotropin-releasing factor binding protein, neuropathic hypersensitivity, descending pain control, sensory-discriminative pain.*

The amygdala (AMY), particularly its lateral capsular subdivision (CeLC) in the central nucleus (CeA), is considered to play an important role in processing of emotional aspects of pain, but it may also modulate sensory-discriminative aspects of pain, particularly through its efferent connections to structures involved in descending control of pain.[6,12,29-31] Recent studies indicate that corticotropin-releasing factor (CRF) and its CRF1 and CRF2 receptors are involved in generating emotional-affective pain responses.[11,18]

Chronic pain induced by peripheral neuropathy has been associated with negative emotions, anxiety, depression,[20,25] and even anatomical[14] and functional[16] changes of the amygdala. Moreover, peripheral nerve injury has induced changes in descending pain control originating or relaying in the amygdala.[2,39] While there is evidence indicating that the amygdaloid CRF has a key role in mediating anxiety-like and pain-related responses in arthritic animals[11,17,18,20] and that the expression of CRF mRNA is increased in the AMY of nerve-injured animals,[38] the contribution of the amygdaloid CRF to neuropathic pain is still not known.

In addition to CRF, CRF-binding protein (CRF-BP) has been localized to the CeA.[35] CRF-BP modulates CRF actions by binding free CRF with high affinity.[5] The regulation of the amygdaloid CRF-BP is not well understood. In vitro studies have shown that CRF-BP is modulated by several mechanisms involving, for example, cytokines, CRF, and glucocorticoids.[21,27] CRF-BP

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appears to have a crucial role in controlling the action of amygdaloid CRF on its receptors. Intracerebral administration of CRF_{6-33}, a CRF-BP ligand inhibitor, provides a tool for experimental modulation of the concentration of free CRF within the AMY and, consequently, the activation level of amygdaloid CRF receptors by the endogenous ligand;[5,36] ie, CRF_{6-33} in the AMY increases free CRF and thus induces activation of amygdaloid CRF receptors.

The purpose of this study was to assess the role of amygdaloid CRF in control of neuropathic pain by microinjecting CRF_{6-33} or CRF_{9-41}, a nonselective CRF receptor antagonist, into the CeA of nerve-injured or sham-operated control animals. An aversive place-conditioning test was used to assess affective-emotional aspects of pain,[23] whereas the hind limb-withdrawal threshold evoked by mechanical stimulation of the skin was used to assess sensory aspect of pain. Moreover, since recent studies indicate hemispheric lateralization in processing of inflammatory pain by the CeA,[8,19] we assessed whether the pain-modulatory effect by amygdaloid administration of CRF_{6-33} varies with the hemisphere in animals with a peripheral neuropathy.

Methods

Animals

The experiments were performed in adult, male Hannover-Wistar rats weighing 180 to 206 g at the beginning of the experiment (Harlan, Horst, the Netherlands). The Experimental Animal Ethics Committee of the Provincial Government of Southern Finland (Hämeenlinna, Finland) approved methods, and the experiments were performed according to the guidelines of European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to limit distress and to use only the number of animals necessary to produce reliable scientific data. Rats were housed in a 12-hour light/dark cycle with free access to food and water.

Techniques for Producing Neuropathy

An experimental model of peripheral neuropathy was induced by a spared nerve injury (SNI).[10] The unilateral axotomy and ligation of the tibial and common peroneal nerves on the left side was performed under pentobarbitone anesthesia (50 mg/kg ip) as described in detail earlier.[10,13] Briefly, the skin of the lateral surface of the thigh was incised and a section made directly through the biceps femoris muscle exposing the sciatic nerve and its 3 terminal branches. Following ligation and removing 2 to 4 mm of the distal nerve stumps of the tibial and common peroneal nerves, muscle and skin were closed in 2 layers. In sham-operated animals, the surgical procedure was identical, except that the tibial and common peroneal nerves were not ligated or sectioned. After the surgery, the animals were allowed to recover before the actual testing that was performed 1 week after the operation.

Assessment of the Limb-Withdrawal Threshold

Development of neuropathic hypersensitivity was verified behaviorally in animals habituated to the experimental conditions 1 to 2 hours daily for 2 to 3 days. For assessment of tactile allodynia, the hind limb-withdrawal threshold was determined bilaterally by stimulating the sural nerve areas in the hind paws with monofilaments. The calibrated series of monofilaments used in this study produced forces ranging from .008 to 60 g (North Coast Medical, Inc. Morgan Hill, CA). The monofilaments were applied to the lateral foot pad with increasing force until the rat withdrew its hind limb. The lowest force producing a withdrawal response was considered the threshold. The threshold for each hind paw of each rat was based on 3 separate measurements and the median of these values was considered to represent the threshold. Threshold values <1 g that were not observed in sham-operated animals were considered to represent hypersensitivity.

Assessment of Aversive Avoidance Behavior

Place-avoidance test was performed, as described earlier,[2,3,23] to obtain a measure of affective pain induced by mechanical stimulation of the neuropathic hind paw. Before testing, the animals were habituated to the test conditions by spending 1 to 2 hours daily for 2 days in the test box. In the actual testing, the rat was placed within a Plexiglas chamber (60 × 30 × 30 cm) one half of which was painted black on the external surface placed upon an elevated metal grid. The rats were placed over the midline of the chamber and stimulation of the plantar surface of the hind paw initiated with a 180-g monofilament once every 15 seconds for 15 minutes. When residing within the dark side of the chamber, the injured or sham-operated hind paw (left-operated paw) was stimulated. Conversely, the nonoperated hind paw (right-intact paw) was stimulated when residing within the light side of the chamber. Throughout the 30-minute test period, rats were allowed unrestricted movement throughout the chamber. The percent time spent in the dark side of the chamber during the 30-minute observation period was determined in each condition for each animal. It is assumed that the more aversive the mechanical stimulation of the hind paw, the more the animal spends time in the light side of the chamber; ie, the place-avoidance test is considered to assess affective-emotional pain behavior.[23] It should be noted that if the operation does not influence the sensibility of the limb, then the place-avoidance test using the current study design (stimulation of the operated paw in the dark and unoperated in the light) assesses influence of exposure to light per se rather than emotional pain behavior.

Placement of Guide Cannulae for Intracerebral Drug Injections

The animals had a guide cannula for drug administration into the AMY or the nonamygdaloid control site. The guide cannula was either in the left (ipsilateral to the nerve injury or sham operation) or the right hemisphere (contralateral to the nerve injury or sham operation). For placement of the guide cannula (30 gauge), the skull was exposed and a hole drilled for its placement in anesthetized animals during the same operation in which the peripheral nerve was ligated or sham operated. The desired injection target in the left or right AMY was in the capsule lateral of central nucleus of the amygdala (CeA): 2.12 mm posterior from the bregma, 4.30 mm lateral from the midline, and 7.80 mm ventral from the dura mater.[33] The control injection site that does not express CRF-BP[35] was in the right internal capsule: 2.12 mm posterior from bregma, 3.6 mm lateral from the midline, and 5 mm ventral from the dura mater.[33] The tip of the guide cannula was positioned 2 mm above the desired injection site. The cannula was fixed into the skull using a dental screw and dental cement. Drug administration to the brain and experimental protocols started 1 week after fixation of the guide cannula to the skull. A dummy cannula was placed in the guide cannula, except when drug administrations were performed.

Drugs

The CRF-BP ligand inhibitor human/rat CRF_{6-33} (.01, .03, .1 or .3 μg in .5 μl) that has no action of its own on the CRF receptors and the nonselective CRF receptor antagonist α-helical CRF_{9-41} (1 μg in .5 μl) were purchased from Sigma (St. Louis, MO). For amygdaloid injections, CRF_{6-33} and CRF_{9-41} were dissolved in saline before each experiment. Physiological saline (OrionPharma, Espoo, Finland) was used for control injections. The drug doses were selected on previously published results[36] and preliminary experiments.

Drug Administration Procedure

Unilateral infusions of drugs, or an equivalent volume of saline, were made by using 30-gauge injection needles connected to a 10-μl Hamilton microsyringe by polyethylene (PE-20) tubing. The injection needles protruded 2.0 mm beyond the cannula tips and a .5-μl injection volume was injected. The animals were gently restrained during the infusion procedure. The duration of injection was 30 seconds. Injection needle was retained within the cannula for an additional 20 seconds after drug infusion to maximize diffusion and to prevent backflow of the drug into the cannula.

Course of Study

In all animals, nerve injury or sham operation was in the left hind limb and the intracerebral drug injection was performed in the left or right hemisphere. Six experimental groups of rats were tested: 1) SNI animals (n = 9) with amygdaloid injections contralateral to the nerve injury; 2) SNI animals (n = 5) with amygdaloid injections ipsilateral to the nerve injury; 3) sham-operated animals (n = 7) with amygdaloid injections contralateral to the sham operation; 4) sham-operated animals (n = 4) with amygdaloid injections ipsilateral to the nerve injury; 5) SNI animals (n = 4) with nonamygdaloid injections contralateral to the nerve injury; and 6) sham-operated animals (n = 4) with nonamygdaloid injections contralateral to the sham operation. Experiments were performed 1 to 3 weeks following nerve injury or sham operation. The drug treatment conditions were saline, CRF_{6-33} at a dose of .01 μg, .03 μg, .1 μg, or .3 μg, CRF_{9-41} at a dose of 1 μg, or CRF_{9-41} at a dose of 1 μg 1 minute before administration of CRF_{6-33} at a dose of .03 μg. Drugs were administered 15 minutes before the start of withdrawal-threshold assessment, which was immediately followed by the place-avoidance test. The duration of each session was 1 hour 15 minutes, except for control sessions, in which the time course of the CRF_{6-33}-induced effect was studied for 30 minutes. In each experimental group, each drug condition was assessed in a separate day. The interval in testing different drug conditions in 1 animal was at least 2 days. Each animal participated in 3 to 4 experimental conditions. The order of testing different drug conditions was varied within the groups to avoid serial effects.

Histology

At the end of the experiment, rats were sacrificed by an overdose of pentobarbital and the brain was removed and immersed in 4% formaldehyde. Coronal sections of the brain were cut to verify the site of injection according to the atlas of Paxinos and Watson.[33]

Statistical Analysis

Withdrawal-threshold data are presented as median and interquartile range, while place-aversion test data are presented as mean and S.E.M. Withdrawal-threshold data were analyzed using Kruskal-Wallis test followed by Dunn's test (comparison of 3 or more groups), or Mann-Whitney *U* test (comparison of 2 groups). The place-aversion data were analyzed using 1- or 2-way analysis of variance followed by a t-test with a Bonferroni correction for multiple comparisons (comparison of 3 or more groups), or t-test (comparison of 2 groups). $P < .05$ was considered to represent a significant difference.

Results

Verification of Neuropathic Hypersensitivity

The monofilament-induced limb-withdrawal threshold was significantly decreased in the nerve-injured limb (Fig 1). Nerve injury did not influence the withdrawal threshold of the contralateral limb as suggested by the finding that withdrawal thresholds contralateral to nerve injury and sham operation were identical (Fig 1).

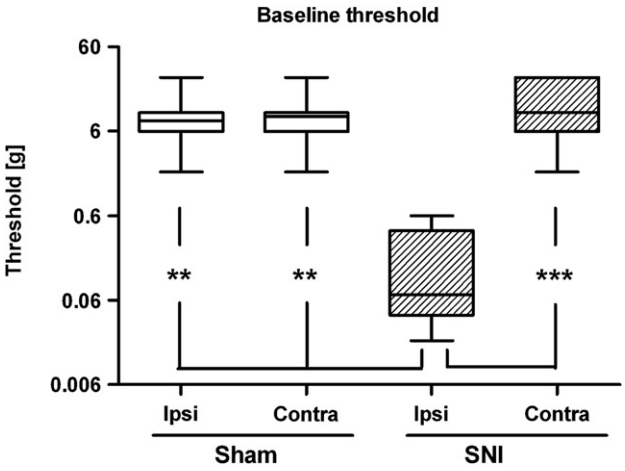


Figure 1. Hind limb-withdrawal thresholds to monofilament stimulation of the paw in sham-operated (Sham) and nerve-injured (SNI) animals assessed 1 week after operations. Ipsi = ipsilateral to operation, Contra = contralateral to operation. The boxes show median thresholds with interquartile ranges, and whiskers show the range. n_{Sham} = 8, n_{SNI} = 9. $^{**}P < .01$, $^{***}P < .005$ (Dunn's test).

Modulation of Sensory-Discriminative Pain Response by CRF_{6-33}

Monofilament-induced limb-withdrawal threshold was used to assess sensory-discriminative pain response. In sham-operated animals, CRF-BP inhibitor, CRF_{6-33}, at doses .01 to .3 μg in the right CeA, produced a decrease of the limb-withdrawal threshold both in the sham-operated left limb (KW = 10.8, $P < .03$; Fig 2A) and the unoperated right limb (KW = 20.59, $P < .0005$; Fig 2B); the reduction of the withdrawal threshold in both limbs was nonmonotonic, being strongest following administration of CRF_{6-33} at the dose of .03 μg. In the left CeA of sham control animals, in contrast, CRF_{6-33} at doses .01 to .3 μg failed to influence the withdrawal threshold of the sham-operated left limb (KW = .78; Fig 2C) or the unoperated right limb (KW = 2.1; Fig 2D). When injected in the control site (the right internal capsule), CRF_{6-33} at a dose of .03μg failed to produce a change in the threshold of the sham-operated left limb (Mann-Whitney U = 6.0; Fig 2E) or the unoperated right limb (Mann-Whitney U = 6.0; Fig 2F).

Following pretreatment of the right CeA of sham control animals with a CRF receptor antagonist (CRF_{9-41}, at a dose of 1 μg), the decrease of the withdrawal threshold of the right unoperated limb induced by administration of .03 μg of CRF_{6-33} was not any more significant (Fig 2G). CRF_{9-41} alone at the dose of 1 μg in the right CeA failed to influence the withdrawal threshold in sham-operated animals (Fig 2G).

In SNI animals, CRF_{6-33}, at doses .01 to .3 μg in the right CeA, produced a significant decrease of the withdrawal threshold in the injured left limb (KW = 10.99, $P < .03$; Fig 3A); in all SNI animals, CRF_{6-33} at doses .03 and .1 μg in the right CeA induced a decrease of the withdrawal threshold of the injured left limb to the lowest stimulus force level (.008 g) that was used. The influence by CRF_{6-33} at doses .01 to .3 μg in the right CeA of SNI animals was short of significance on the withdrawal threshold of the unoperated right limb (KW = 8.69, $P = .07$; Fig 3B); it should be noted, however, that SNI animals appeared to avoid putting weight on the injured left limb after treatment of the right CeA with CRF_{6-33}. When CRF_{6-33} was administered at doses .01 to .3 μg in the left CeA of SNI animals, it failed to influence the withdrawal threshold of the injured left limb (KW = 5.2; Fig 3C). The withdrawal threshold of the unoperated right limb, however, was significantly influenced by CRF_{6-33} in the left CeA of SNI animals (KW = 12.0, $P < .02$; Fig 3D); at the dose of .03 μg, CRF_{6-33} in the left CeA produced a significant reduction of threshold in the unoperated right limb of SNI animals. When injected in the control site (the right internal capsule), CRF_{6-33} at a dose of .03 μg failed to produce a change in the withdrawal threshold of the injured left limb (Mann-Whitney U = 4.5; Fig 3E) or the uninjured right limb (Mann-Whitney U = 5.5; Fig 3F).

Pretreatment of the right CeA of SNI animals with CRF_{9-41} at a dose of 1 μg failed to influence the withdrawal threshold of the injured left limb (KW = 4.69; Fig 3G). Pretreatment of the right CeA with CRF_{9-41} completely reversed the CRF_{6-33}-induced decrease of threshold of the injured limb in the SNI group (KW = 8.76, $P < .04$; Fig 3H). Administration of CRF_{9-41} in the right CeA of SNI animals at a dose of 1 μg alone had no effect on the withdrawal threshold of the uninjured right or injured left limb (Figs 3G and 3H).

Time course of the effect by .03 μg of CRF_{6-33} in the left CeA on the limb-withdrawal threshold of the unoperated right limb was studied in 4 SNI animals. In these animals, CRF_{6-33} produced a significant decrease of the threshold (KW = 11.5, $P < .05$) that was most prominent 10 and 15 minutes following its amygdaloid administration (Fig 4).

Modulation of Emotional/Affectivelike Pain Response by CRF_{6-33}

In the place-avoidance test, the SNI group spent less time in the dark than the sham group as indicated by comparison of saline-treated sham and SNI animals (main effect of SNI: $F_{1,22}$ = 134.96, $P < .0001$; Figs 5A and 5B). Amygdaloid administration of CRF_{6-33} at the high dose of .3 μg tended to produce sedativelike effect in some animals and, therefore, the place-avoidance results obtained with this dose were not taken into account in the analysis.

In the sham group, administration of CRF_{6-33} in the CeA at doses .01 to .1 μg failed to produce a change in time spent in the dark (main effect of drug dose: $F_{3,34}$ = 1.62; Fig 5A), independent of whether CRF_{6-33} was administered in the right or left CeA (main effect of injection side: $F_{1,34}$ = 2.22). In the SNI group, amygdaloid administration of CRF_{6-33} at doses .01 to .1 μg had a dose-related influence on time spent in the dark ($F_{3,34}$ = 13.32, $P < .0001$; Fig 5B), and the CRF_{6-33}-induced change in the place-avoidance behavior was significantly different following injection in the left

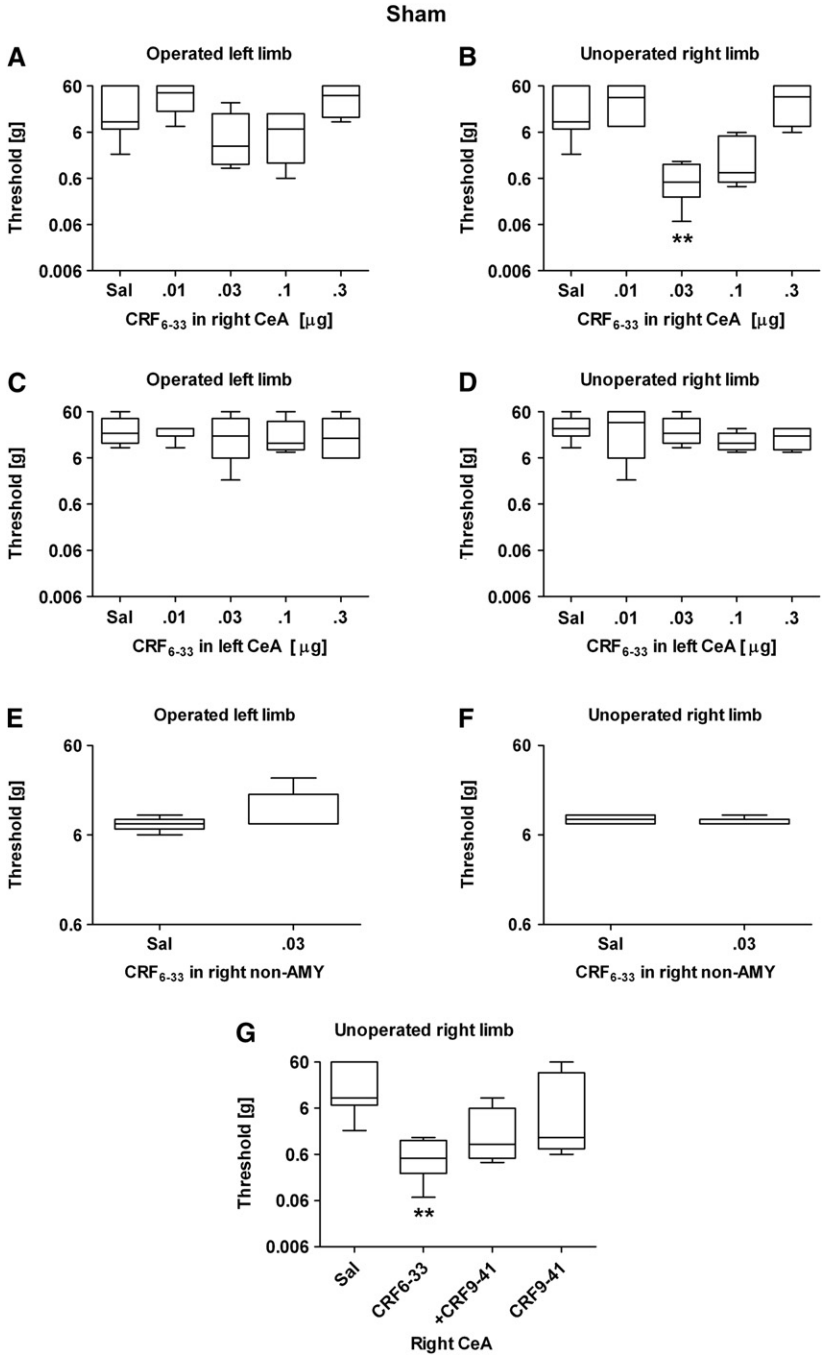


Figure 2. Withdrawal thresholds to monofilament stimulation of the sham-operated left (**A,C,E**) and the unoperated right (**B,D,F,G**) limb in the sham control group after administration of studied compounds into the right (**A,B,G**) or left (**C,D**) central nucleus of the amygdala (CeA), or to a control site in the right internal capsule (**E,F**). Sal = saline. In **G**, CRF_{6-33} = CRF_{6-33} at a dose of .03 μg, +CRF_{6-33} = .1 μg of CRF_{9-41} followed by .03 μg of CRF_{6-33}, CRF_{9-41} = .1 μg of CRF_{9-41} alone. The boxes show median thresholds with interquartile ranges, and whiskers show the range. In **A** and **B**, n = 4 to 10; in **C-F**, n = 4; and in **G**, n = 5 to 6. $^{**}P < .01$ (Dunn's test; reference: the corresponding saline condition).

versus right CeA ($F_{1,34}$ = 5.63, $P < .03$; Fig 5B); independent of the injection side, CRF_{6-33} at doses .01 to .03 μg produced a dose-related increase in the time spent in the dark, while a higher dose of .1 μg in the right but not left CeA had a weaker effect.

When injected in the control site (the right internal capsule), CRF_{6-33} at a dose of .03 μg failed to influence the place-avoidance behavior in the sham-operated (t_3 = .2; Fig 5C) or nerve-injured (t_3 = .3; Fig 5D) animals.

Pretreatment of the right CeA with a nonselective CRF receptor antagonist, CRF_{9-41} at a dose of 1 μg, completely reversed the CRF_{6-33}-induced increase in the time spent in the dark in the SNI group (Fig 5E). Administration of CRF_{9-41} alone at a dose of 1 μg in the right CeA had no significant effect on the time spent in the dark in the SNI group (Fig 5E).

Fig 5F shows the time course of the place-avoidance behavior following administration of saline or .03 μg of

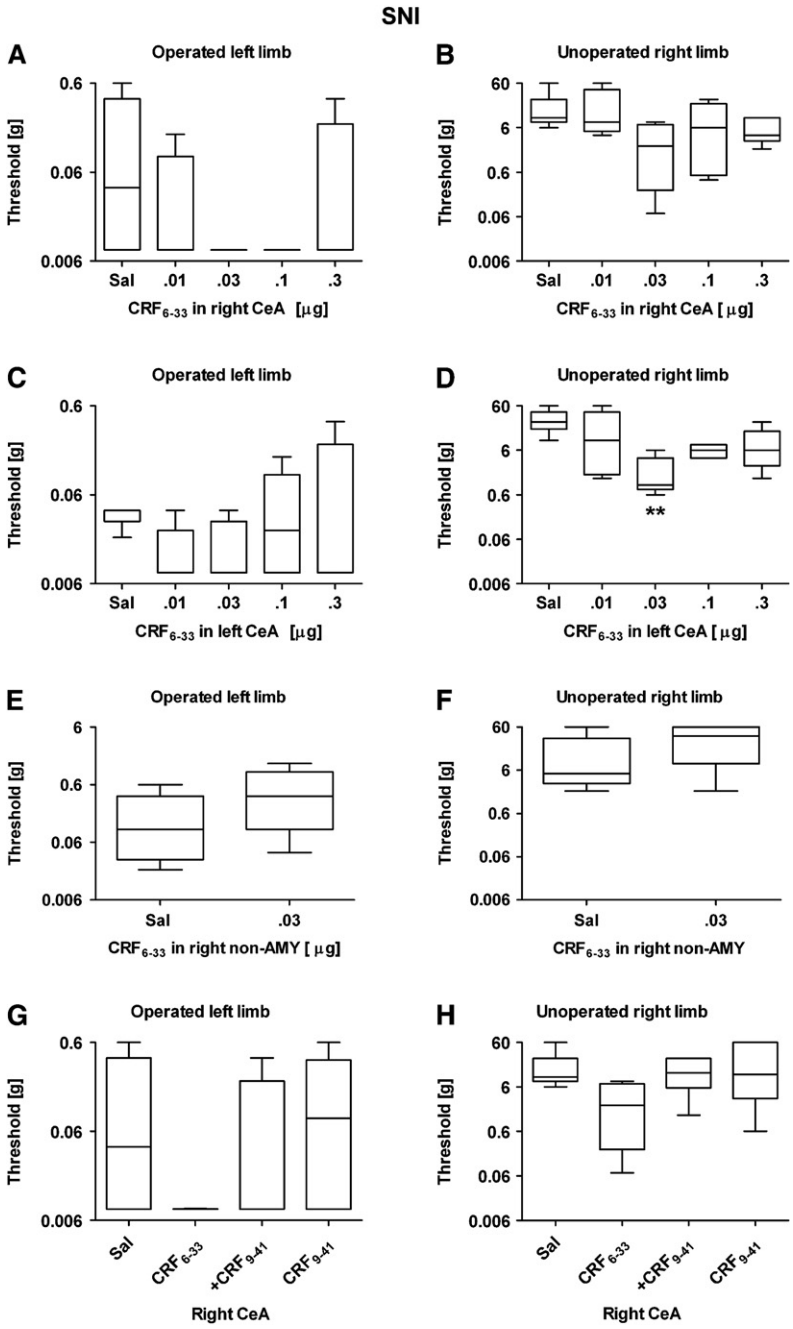


Figure 3. Withdrawal thresholds to monofilament stimulation of the nerve-injured left (**A,C,E,G**) and the uninjured right (**B,D,F,H**) limb in the neuropathic (SNI) group after administration of studied compounds in the right (**A,B,G,H**) or left (**C,D**) central nucleus of the amygdala (CeA), or in a control site in the right internal capsule (**E,F**). Sal = saline. In **G** and **H**, CRF_{6-33} = CRF_{6-33} at a dose of .03 μg, +CRF_{6-33} = .1 μg of CRF_{9-41} followed by .03 μg of CRF_{6-33}, CRF_{9-41} = .1 μg of CRF_{9-41} alone. The boxes show median thresholds with interquartile ranges, and whiskers show the range. In **A** and **B**, n = 5 to 7; in **C** and **D**, n = 4 to 5; in **E** and **F**, n = 4; and in **G** and **H**, n = 4 to 6. $^{**}P < .01$ (Dunn's test; reference: the corresponding saline condition).

CRF_{6-33} in the right CeA of SNI animals; the CRF_{6-33}-induced change in place-avoidance ($F_{1,54}$ = 23.5, $P < .0001$) lasted until the end of the test.

Histology

Histologically verified amygdaloid injections sites and control injection sites in the internal capsule are shown in Figs 6 and 7.

Discussion

Facilitation of Mechanical Sensitivity by Inhibition of CRF-BP

CRF_{6-33} in the CeA produced a decrease of the limb-withdrawal threshold to mechanical stimulation (ie, mechanical hypersensitivity effect) both in nerve-injured and sham-operated animals. CRF_{6-33} inhibits CRF-BP, and this is expected to increase the level of free

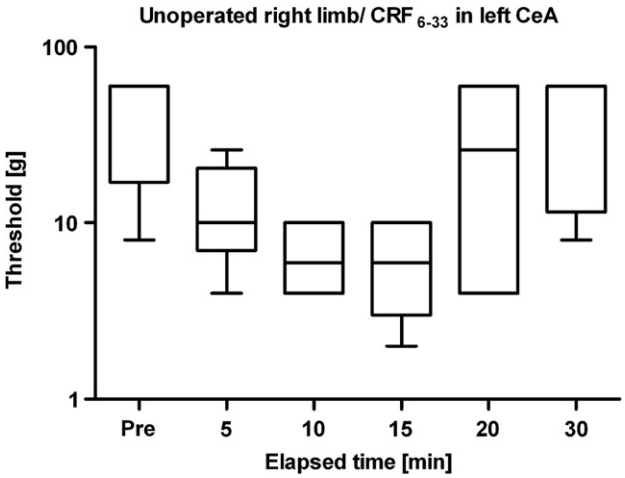


Figure 4. Time course of the effect by administration of CRF_{6-33} at a dose of .03 μg in the left CeA on the monofilament-induced withdrawal threshold of the unoperated right limb in neuropathic animals. The boxes show median thresholds with interquartile ranges, and whiskers show the range (n = 4). Pre = threshold before administration of CRF_{6-33}.

endogenous CRF. Therefore, it may be concluded that increased level of free CRF in the CeA produced the mechanical hypersensitivity effect that presumably reflects facilitation of the sensory aspect of pain. The mechanical hypersensitivity effect was attenuated by a nonselective CRF receptor antagonist, CRF_{9-41}, suggesting that amygdaloid CRF receptors were involved in mediating the mechanical hypersensitivity effect. CRF_{9-41} failed to produce a complete reversal of the CRF_{6-33}-induced hypersensitivity effect. This finding suggests that the dose of CRF_{9-41} may have been too low for complete reversal or that mechanisms independent of CRF receptors contributed to the CRF_{6-33}-induced hypersensitivity.

CRF_{6-33} in the CeA had the strongest pronociceptive effect on the monofilament-induced limb-withdrawal response at the dose of .03 μg, whereas with a further increase of the dose the pronociceptive effect was weaker. This finding suggests that an increase of endogenous CRF level in the CeA first produced a mechanical hypersensitivity effect that disappeared with a further increase of the endogenous CRF level. This is in line with earlier results showing that a low dose of exogenous CRF in the CeA facilitated pain-related responses,[17,18] whereas with an increase in the dose of exogenous CRF the facilitation disappeared and was replaced by suppression of pain-related responses;[9,17,18] the pain-facilitatory effect induced by exogenous CRF in the CeA was mediated by CRF1 receptors and the inhibitory effect by CRF2 receptors.[18] The lateral capsular division (CeLC) of the central nucleus of the CeA has been shown to be the main terminal area of a spino-parabrachio-amygdaloid nociceptive pathway.[6] Additionally, several thalamic areas project to the CeLC providing additional sources of nociceptive input.[7] These afferent connections together with the demonstration of nociceptive neurons in the CeLC[7,29-31] are in line with the concept that the CeLC is the nociceptive subdivision of the CeA. Efferent connections of the CeLC are primarily and extensively to the substantia innominata and less densely to other brain areas, such as the bed nucleus of the stria terminalis and the posterior hypothalamus.[7] Among areas that receive efferent connections from the substantia innominata are the thalamus, the hypothalamus, the substantia nigra, and the periaqueductal gray, which all could play a role in the regulation of aversive behavior or pain following administration of CRF_{6-33} in the CeA.[7] Interestingly, the medullary dorsal reticular nucleus that is implicated in descending facilitation of pain[26] may also play a role in the pronociceptive action induced by CRF_{6-33} in the CeA, since it receives afferent connections from both the CeA and the substantia innominata.[1]

In the present study, amygdaloid administration of a nonselective CRF receptor antagonist alone failed to influence withdrawal thresholds of nerve-injured or sham-operated animals. This finding suggests that amygdaloid CRF receptors don't have a tonic influence on cutaneous sensitivity in neuropathic or control animals. Previously, a decrease of pain-related responses has been reported in arthritic but not control conditions following amygdaloid administration of a selective CRF1 receptor antagonist,[17,18] whereas another study reported that amygdaloid administration of a nonselective CRF receptor antagonist decreased pain sensitivity in healthy animals.[9] Together, these findings suggest that the contribution of amygdaloid CRF to tonic regulation of pain varies with the experimental condition.

Suppression of Emotional Painlike Behavior by Inhibition of CRF-BP

CRF_{6-33} in the CeA induced an increase in time the animals spent in the dark compartment of the place-avoidance test. The increase in time that the nerve-injured animals spent in the dark, where the injured limb was stimulated, may be considered to reflect CRF_{6-33}-induced suppression of emotional painlike behavior rather than increased anxiety, because it was associated with a CRF_{6-33}-induced facilitation of the monofilament-evoked sensory pain response from the injured limb; ie, following amygdaloid CRF_{6-33} treatment, nerve-injured animals were expected to experience more pain in the dark. Consequently, nerve-injured animals were expected to avoid the dark compartment, unless CRF_{6-33} treatment attenuated unpleasantness evoked by the monofilament-induced sensory-pain response from the injured paw.

In the present study, amygdaloid administration of CRF_{6-33} in nerve-injured animals induced the strongest suppression of emotional painlike behavior at the dose of .03 μg, whereas a lower or a higher dose tended to induce a weaker effect. The CRF_{6-33}-induced suppression of emotional painlike behavior was completely reversed by a nonselective CRF receptor antagonist. These findings suggest that a small-to-moderate increase of free endogenous CRF in the CeA suppresses the emotional aspect of pain, due to action on CRF receptors. The suppression of emotional painlike behavior was accompanied by

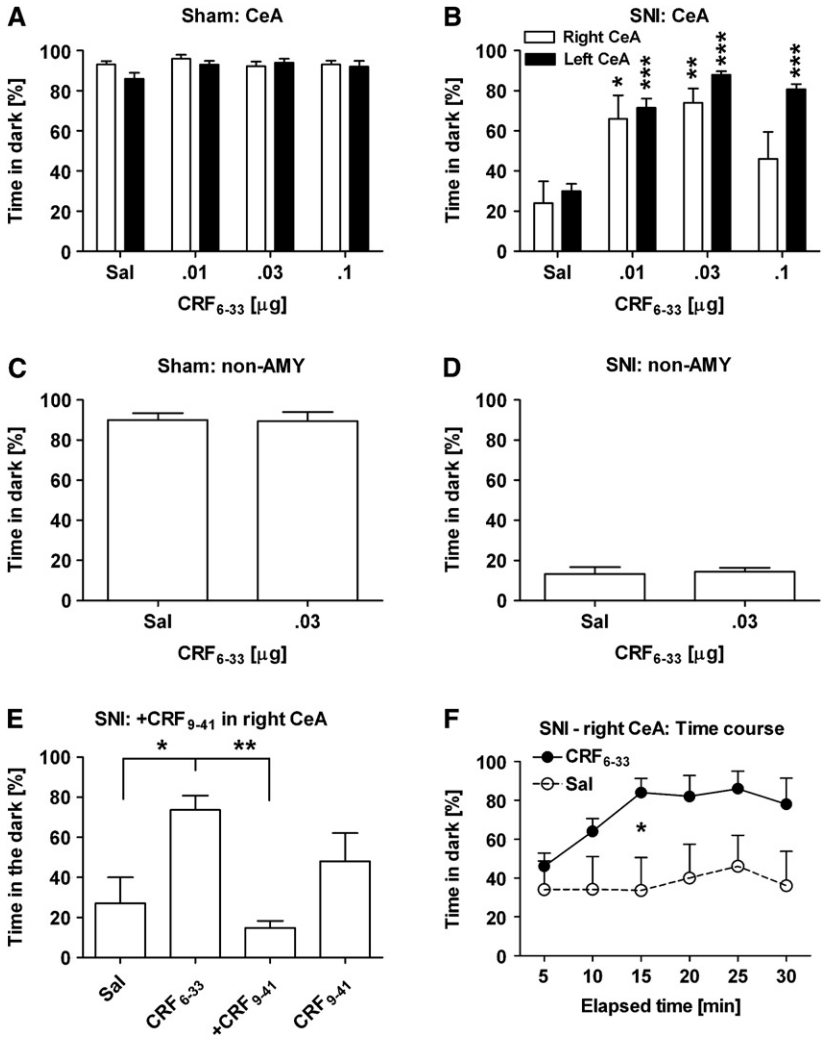


Figure 5. Emotional painlike behavior as revealed by an aversive place-conditioning test in sham-operated (Sham; **A,C**) and nerve-injured (SNI; **B,D,E,F**) animals following drug treatment of the CeA (**A,B,E,F**) or the control site in the right internal capsule (**C,D**). For the SNI groups, the more the animals spend time in the dark compartment, the less they are assumed to have emotional/affective pain associated with mechanical stimulation of the injured paw in the dark. Due to study design, the test assesses only unpleasantness associated with light exposure, not pain, in the sham group. Sal = saline. In **E** and **F**, CRF_{6-33} = CRF_{6-33} at a dose of .03 μg, +CRF_{6-33} = .1 μg of CRF_{9-41} followed by .03 μg of CRF_{6-33}, CRF_{9-41} = .1 μg of CRF_{9-41} alone. Open bars show results following drug injections in the right hemisphere and filled ones following drug injections in the left hemisphere. The graphs show mean and S.E.M. (In **A**, n = 4 to 10; in **B**, n = 4 to 6; in **C** and **D**, n = 4; and in **E** and **F**, n = 5 to 6). *P < .05, **P < .01, ***P < .001 (t-test with a Bonferroni correction for multiple comparisons; reference: the corresponding saline condition).

facilitation of the sensory aspect of pain, as revealed by a decrease in the limb-withdrawal threshold. While the facilitation of the sensory aspect of pain by an increase in free CRF in the CeA may be explained by action through descending pathways, the suppression of emotional painlike behavior is likely to be explained by intra-amygdaloid mechanisms or by action on supraspinal circuitries influencing other brain structures involved in emotional processing of pain.[29,31]

Concerning potential intra-amygdaloid downstream mechanisms, CRF has increased GABA release through action on amygdaloid CRF1 receptors under in vitro conditions.[4] Moreover, GABAergic compounds in the amygdala of neuropathic animals have produced selective modulation of the affective component of pain.[34] Furthermore, group III metabotropic glutamate receptors 7 and 8 in the amygdala have induced dissociative effects on sensory and affective components of pain.[32]

Hemispheric Lateralization of the Amygdala-Induced Pain Modulation

Recent studies indicate that the right CeA has a dominant role in processing of pain-related signals in inflamed animals.[8,19] In line with this, administration of CRF_{6-33} in the right but not left CeA had a pronociceptive effect in sham-operated animals of the present study. In nerve-injured animals, however, CRF_{6-33} increased sensory-like pain and decreased emotional-like pain, independent of whether CRF_{6-33} was administered in the left or the right CeA. This finding suggests that hemispheric lateralization of amygdaloid pain

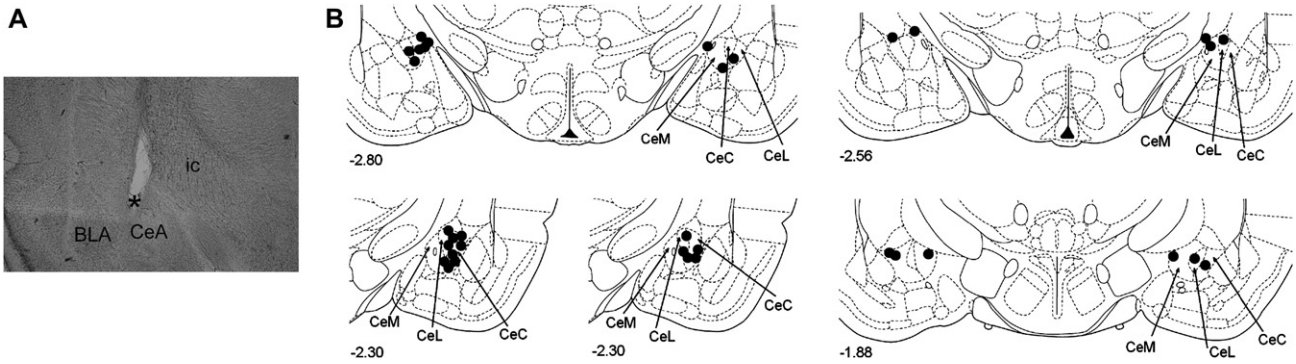


Figure 6. An example of a Nissl-stained coronal section of the injection site (asterisk) in the amygdala (**A**). Schematic illustrations showing injection sites in the amygdala (**B**). CeA, Central nucleus of the amygdala; BLA, basolateral nucleus of the amygdala; ic, internal capsule; CeC, Capsular division of the central nucleus of amygdala; CeL, Lateral division of the central nucleus of amygdala; CeM, Medial division of the central nucleus of amygdala. Distance of the coronal section from the bregma is shown in the lower left corner of each graph in mm. Right side in the graphs represents right hemisphere.

processing is lost following peripheral nerve injury. Previous studies have demonstrated that peripheral nerve injury induces molecular,[38] morphological,[14] and functional[16] changes in the CeA. It remains to be studied which pathophysiological changes underlie the loss of hemispheric lateralization in amygdaloid pain processing in peripheral neuropathy.

Caveats

One limitation of this study is that only 1 behavioral test was used for assessing sensory pain and 1 test for assessing emotional pain. Moreover, due to indirect assessment of pain and emotions from limb-withdrawal responses and place-avoidance behavior, one should be cautious when making interpretations from experimental animal studies to pain, emotions, or emotional pain. It might be argued that since amygdaloid CRF1 receptors are involved in generation of anxiety,[20] neuropathic animals treated with CRF_{6-33} spent more time in the dark compartment of the place-avoidance test due to increased anxiety rather than decreased unpleasantness caused by monofilament stimulation of the injured paw in the dark. This argument, however, is not supported by the finding that the mechanical hypersensitivity effect induced by amygdaloid treatment with CRF_{6-33} failed to increase time spent in the dark in sham-operated animals, although the sham-operated animals had an equal baseline threshold in the limb ipsi- as well as contra-lateral to sham operations. Moreover, it has been shown earlier that CRF_{6-33}, unlike a full agonist of the CRF receptor, does not have anxiogenic or aversive properties.[15] Still, further studies are needed to exclude the possibility that a nonspecific change in anxiety-induced behavioral hyperreactivity contributed to cutaneous hypersensitivity in the CRF_{6-33}-treated neuropathic animals; the extremely low threshold ($\leq$ .008 g) in the nerve-injured limb of CRF_{6-33}-treated animals might reflect, at least partly, general hyperreactivity.

Weight bearing of the limb may provide a significant confounding factor in the assessment of the monofilament-induced withdrawal threshold.[22] In the present study, the increased hypersensitivity and decreased weight bearing of the nerve-injured left limb following administration of CRF_{6-33} in the right CeA may have masked hypersensitivity effect in the uninjured right limb that had to bear more weight.

Previous studies suggest that CRF and CRF receptors may have a pain-modulatory role not only in the AMY but also in various locations outside of AMY.[24] The currently used injection volume of .5 μl was likely to spread to an area that was about 1 mm from the tip of the injection needle.[28] While the center of the injection was in the CeA, the spread of the injected CRF_{6-33} covered an area that included all amygdaloid subnuclei, the substantia innominata, and part of the striatum. Due to this spread, one needs to be cautious with interpretations concerning the contribution of the CeA versus other adjacent structures to the CRF_{6-33}-induced actions. There are, however, several lines of evidence suggesting that the CRF_{6-33}-induced modulation of pain behavior in the present study was due to action on the CeA rather than the adjacent structures. Neuroanatomical studies indicate that CRF-BP, CRF, and CRF receptors are localized

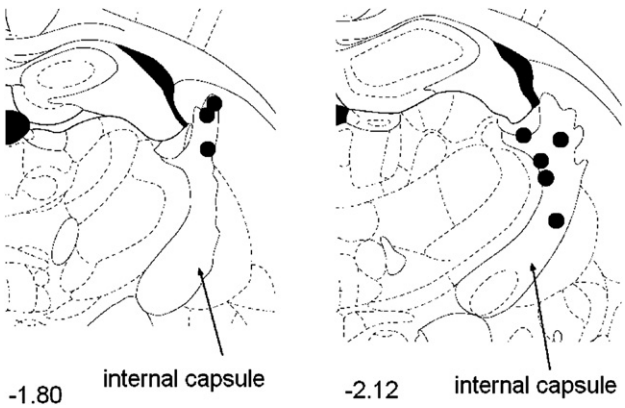


Figure 7. Schematic illustration showing injection sites in the internal capsule that was the control injection site. Distance of the coronal section from the bregma is shown in the lower left corner of each graph in mm. Right side in the graph represents right hemisphere.

in the CeA, particularly in its subdivision CeLC.[5,37] Importantly, functional studies have demonstrated that endogenous activation of CRF1 receptors and the consequent synaptic facilitation in the CeLC contribute to facilitation of pain-related neuronal and behavioral responses in inflammatory conditions.[11,17,18] Moreover, peripheral nerve injury induced synaptic facilitation of pain-related inputs to the CeLC,[16] and pain behavior was modulated by administration of $GABA_A$- or glutamatergic compounds in the CeA of neuropathic animals.[3,34] Together, these findings suggest that modulation of pain behavior by amygdaloid administration of CRF_{6-33} in the present study was due to action on CRF receptors in the CeA rather than adjacent structures. This proposal is further supported by the failure to induce behavioral changes by administration of CRF_{6-33} in a control site, the right internal capsule, although the internal capsule may not have been as adequate a control site for the CeA as substantia innominata or the basolateral nucleus of the AMY.

Conclusions

The present results suggest that endogenous CRF in the CeA of neuropathic animals may differentially influence sensory and emotional aspects of pain due to action that involves amygdaloid CRF receptors. Endogenous CRF in the CeA produced an enhancement of mechanical hypersensitivity effect at the spinal cord level and this was accompanied by decreased emotional painlike behavior. In control conditions, endogenous CRF in the right CeA had a dominant role in pain modulation, whereas in nerve-injured animals endogenous CRF contributed to pain modulation in the left as well as right CeA.

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Short communication

Influence of amygdaloid glutamatergic receptors on sensory and emotional pain-related behavior in the neuropathic rat

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ABSTRACT

The role of amygdaloid glutamatergic receptors (GluRs) in maintenance of the sensory versus emotional component of neuropathic pain was studied by assessing monofilament-induced limb withdrawal response (sensory pain) and aversive place-conditioning behavior (emotional pain) following amygdaloid administration of various glutamatergic compounds in nerve-injured animals. The results indicate that endogenous activation of amygdaloid group I metabotropic GluRs, $mGluR_1$ and $mGluR_5$, and the NMDA-R contributes to maintenance of sensory and emotional components of neuropathic pain. The predominant effect by amygdaloid group I mGluRs was facilitation of emotional-like pain behavior.

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The amygdala has an important role in emotions [14] and contributes to processing and modulation of pain [9]. Recent studies indicate that peripheral nerve injury that is associated with chronic pain induces neural plasticity in the amygdala as shown by increased postsynaptic currents evoked by ascending inputs [5] and generation of new amygdala neurons [4]. Additionally, glutamatergic stimulation of the amygdala in nerve-injured animals increases the discharge rate of pronociceptive medullary neurons [1], decreases the discharge rate of antinociceptive pontine neurons [15], and promotes emotional-like pain behavior [1]. These findings support the hypothesis that neural plasticity in the amygdala that involves a change in the function of amygdaloid glutamate receptors plays a role in maintenance of chronic neuropathic pain. However, the contribution of various subtypes of amygdaloid glutamate receptors to the sensory versus emotional (affective-motivational) component of neuropathic pain is still poorly known. In the present study, we investigated the roles of amygdaloid group I metabotropic glutamate receptors (mGluRs) and NMDA-Rs in endogenous regulation of mechanically induced limb withdrawal response (an index for the sensory component of pain) and aversive place-conditioning behavior (an index for the emotional component of pain) in animals with a peripheral neuropathy.

The experiments were performed in adult, male Hanover-Wistar rats weighing 180–190 g at the beginning of the experiment (Harlan, Horst, The Netherlands). The experimental protocol was accepted by the Institutional Ethics Committee and the experiments were performed according to the guidelines of European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

The unilateral axotomy and ligation of the tibial and common peroneal nerves were performed under pentobarbitone anesthesia (50 mg/kg i.p.) as described in detail earlier [2,3]. After the surgery, the animals were allowed to recover before the actual testing that was performed either 1 or 8 weeks after the operation. Development of hypersensitivity was verified behaviorally in animals habituated to the experimental conditions 1–2 h daily for 2–3 days. Only animals that developed tactile allodynia-like symptoms (the limb withdrawal threshold to monofilament stimulation $\leq$1 g) in the sural nerve area of the injured limb were considered further in this study. For control purpose, a group of animals were sham-operated; in the sham group, the operation was identical, except that the nerves were not ligated. Sham operation as well as nerve ligation was performed in the left side.

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The animals were installed with stainless steel guide cannulae (26 gauge; PlasticsOne, Roanoke, VA) for drug administration into the amygdala ipsi- and contralateral to the spared nerve injury under pentobarbitone anesthesia (50 mg/kg i.p.) at least one week before behavioral testing. For placement of the guide cannulae, the skull was exposed and holes drilled for their placement. The desired injection sites were in the central nucleus of the amygdala (CeA): 7.12 mm anterior from the ear bar, 4.00 mm lateral (left and right) from the midline, and 8.00 mm ventral from the dura mater [11]. The tips of the guide cannulae were positioned 2 mm above the desired injection site. The guide cannulae were fixed into the skull using dental screws and dental cement. Dummy cannulae were placed in the guide cannulae, when the animal was not tested.

Drugs or saline control were microinjected into the amygdala through a 33-gauge stainless steel injection cannula (PlasticsOne) inserted through and protruding 2 mm beyond the tip of the guide cannula. The microinjection was made using a 10 μl Hamilton syringe (Hamilton Company, Bonaduz, Switzerland) that was connected to the injection cannula by polyethylene (PE-10) tubing. The volume of injection was 0.5 μl. At this volume, the spread of the injected drugs within the brain was at least 1 mm [8]. The efficacy of injection was monitored by watching the movement of a small air bubble through the tubing. The injection lasted 30 s and the injection cannula was left in place for an additional 30 s to minimize flow of the drug solution back up the injector track.

Rate of the limb withdrawal response to repetitive monofilament stimulation of the sural nerve area of the injured limb at the force of 1.4 g (North Coast Medical, Inc., Morgan Hill, CA) was used as an index for the sensory component of pain. The monofilament stimulation was performed five times at about 2 s intervals. Response rate of 100% indicates that the animal withdrew the limb at every stimulus presentation, whereas response rate of 0% indicates that animal did not withdraw its limb at any of the stimulus presentations. Place-avoidance test adapted from that described earlier [6] was used to obtain a measure of emotional pain induced by mechanical stimulation of the injured hind paw. Before testing, the animals were habituated to the test conditions by spending 1–2 h daily for 2 days in the test box. In the actual testing, the rat was placed within a Plexiglas chamber (60 cm × 30 cm × 30 cm; one half of which was painted black on the external surface) placed upon an elevated metal grid. The rats were placed over the midline of the chamber and stimulation of the plantar surface of the hind paw was performed with a 60 g monofilament (North Coast Medical, Inc.) once every 15 s for 15 min. When residing within the dark side of the chamber the injured or sham-operated hind paw was stimulated. Conversely, the non-operated hind paw was stimulated when residing within the light side of the chamber. Throughout the 15 min test period rats were allowed unrestricted movement throughout the chamber. The percent time spent in the light side of the chamber during the 15 min observation period was determined in each condition for each animal. It is assumed that the more aversive the mechanical stimulation of the hind paw, the more the animal spends time in the light side of the chamber; i.e., the place-avoidance test is considered to assess emotional pain behavior [6].

(S)-3,5-dihydroxyphenylglycine (DHPG; an $mGluR_{1/5}$ agonist), (RS)-2-chloro-5-hydroxy (CHPG; an $mGluR_5$ agonist), 6-methyl-2-(phenylethynyl)pyridine (MPEP; an $mGluR_5$ antagonist), (+)-MK-801 hydrogen maleate (MK-801; an NMDA-R antagonist) were purchased from Sigma (St. Louis, MO) and 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt; an $mGluR_1$ antagonist) was purchased from Tocris (Bristol, UK). Physiological saline was used for control injections. Drugs were dissolved in saline, except for CPCCOEt that was dissolved in DMSO. In the choice of drug doses and time points for testing, previously published results [1,13] and preliminary experiments were taken into account.

In the bilateral treatment groups, the drug conditions were saline, DMSO, DHPG at the dose of 10 nmol/amygdala (20 nmol/animal), MPEP at the dose of 50 nmol/amygdala (100 nmol/animal), MK-801 at the dose of 3 nmol/amygdala (6 nmol/animal), and CPCCOEt at the dose of 20 nmol or 40 nmol/amygdala (40 nmol or 80 nmol/animal), respectively. In the unilateral treatment groups, drug conditions were saline, 20 nmol of CPCCOEt, or 40 nmol of CPCCOEt either ipsi- or contralateral to nerve injury. In all experimental conditions, drugs were administered into the amygdala 5 min before the start of the place-avoidance test. Duration of the place-avoidance test was 15 min; i.e., place-avoidance test was performed 5–20 min after amygdaloid administration of the studied compound. Limb withdrawal response to repetitive stimulation of the operated paw was assessed immediately after the end of the place-avoidance test; i.e., limb withdrawal test was performed 20 min after amygdaloid injection of the studied compounds. In each experimental group, each drug condition was assessed in a separate day, 1–2 weeks following nerve injury. Each animal participated in 3–4 drug testing sessions. The interval between different drug conditions in one animal was at least 2 days. The order of testing different drug conditions was varied within the groups to avoid serial effects.

Data are presented as mean ± S.E.M. One- or two-way ANOVA followed by Tukey's test, *t*-test with a Bonferroni correction for multiple comparisons, or unpaired *t*-test (differences between two experimental groups) were used in statistical analysis of the data. $P < 0.05$ was considered to represent a significant difference.

Nerve injury induced marked changes in sensory and emotional-like pain behaviors as indicated by the limb withdrawal response and aversive place-conditioning tests, respectively. In sham-operated control animals ($n = 6$), repetitive monofilament stimulation of the sural nerve area at the force of 1.4 g produced no responses (not shown), whereas in nerve-injured animals, the limb withdrawal rate following bilateral injection of saline was 92 ± 5% ($n = 5$; Fig. 1A). In the aversive place-conditioning test, sham-operated animals spent only 5 ± 1% ($n = 6$) of the test time in the light compartment (not shown), whereas saline-treated nerve-injured animals spent 40 ± 8% ($n = 5$) of the test time in the light compartment (Fig. 1B). A separate control experiment in nerve-injured animals ($n = 5$) indicated that the limb withdrawal response to repetitive presentation of a monofilament at a force of 1.4 g was of the same magnitude independent whether the limb withdrawal test was performed before or after the aversive place-avoidance test ($t_8 = 0.8$, *t*-test; not shown). Due to lack of on-going sensory or emotional-like pain behavior in sham-operated animals, pain-modulatory influence by endogenous activation of amygdaloid glutamatergic receptors was studied with specific receptor antagonists only in nerve-injured animals.

Bilateral administration of DHPG, an $mGluR_{1/5}$ agonist into the CeA (20 nmol/animal), failed to influence limb withdrawal response (Fig. 1A); due to a high baseline value, however, it may not be possible to exclude a DHPG-induced increase in the limb withdrawal response. Bilateral administration of DHPG (20 nmol/animal) increased emotional-like pain behavior (Fig. 1B). Bilateral administration of CPCCOEt, an $mGluR_1$ antagonist, produced a dose-related (40–80 nmol/animal) decrease in the limb withdrawal response ($F_{2,10} = 31.2$, $P < 0.0001$; Fig. 1C) and emotional-like pain behavior ($F_{2,10} = 7.4$, $P < 0.02$; Fig. 1D); at a lower dose (40 nmol/animal), CPCCOEt produced a significant reduction only in the emotional-like pain behavior. Vehicle for CPCCOEt was DMSO. When compared with saline, DMSO alone ($n = 4$) did not influence the limb withdrawal response ($t_7 = 0.45$; not shown) or emotional-like pain behavior ($t_7 = 0.27$; not shown). Bilateral administration of MPEP, an $mGluR_5$ antagonist, failed to reduce limb withdrawal responses

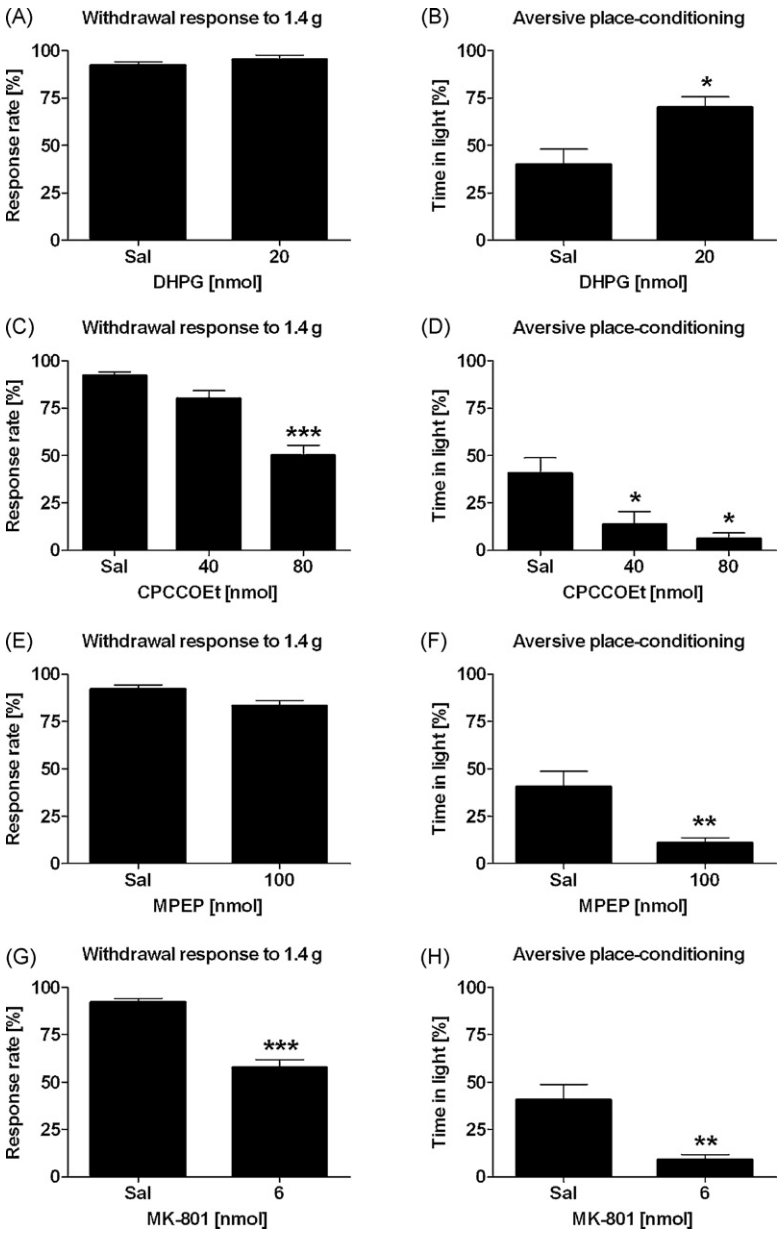


Fig. 1. An index for the sensory component of pain, hind limb withdrawal response elicited by repetitive mechanical stimulation of the nerve-injured paw (left column) and an index for the emotional component of pain, aversive place-conditioning behavior (right column) following bilateral amygdaloid injection of saline (Sal) or various glutamatergic compounds. The doses/animal of the injected glutamatergic compounds are shown in the *X*-axis. A decrease in the limb withdrawal response rate (left columns) and time spent in the light compartment (right columns) are considered to indicate a decrease in sensory and emotional-like pain, respectively. DHPG is an $mGluR_{1/5}$ agonist, CPCCOEt an $mGluR_1$ antagonist, MPEP an $mGluR_5$ antagonist, and MK-801 an NMDA-R antagonist. Error bars represent S.E.M. ($n = 4–8$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ (in C and D, Tukey's test, in other graphs, *t*-test; reference: the corresponding Sal-group).

at a dose (100 nmol/animal; Fig. 1E) that was enough to produce a significant attenuation of the emotional-like pain behavior (Fig. 1F). Bilateral administration of MK-801, an NMDA-R antagonist (6 nmol/animal), produced a significant attenuation of both the limb withdrawal response (Fig. 1G) and the emotional-like pain behavior (Fig. 1H).

CPCCOEt (20–40 nmol/side) or saline was administered unilaterally to study pain-modulatory roles of the CeA ipsi- versus contralateral to nerve injury. Limb withdrawal response was reduced in a dose-related fashion by unilateral injections of CPCCOEt ($F_{2,37} = 32,7$, $P < 0.0001$; Fig. 2A). The suppression of the limb withdrawal response was stronger following amygdaloid administration of CPCCOEt contra- than ipsilateral to nerve injury ($F_{1,37} = 7.55$, $P < 0.01$), independent of the dose ($F_{2,37} = 2.65$). Unilateral administration of CPCCOEt suppressed also emotional-like pain behavior in a dose-related fashion ($F_{2,37} = 8.5$, $P < 0.001$; Fig. 2B). Suppression of emotional-like pain behavior was of the same magnitude following unilateral injection of CPCCOEt ipsi- as contralateral to nerve injury ($F_{1,37} = 0.23$). At the currently used doses, the studied compounds failed to produce any obvious side-effects. Histological analysis indicated that amygdaloid injection sites were in or adjacent to the CeA (Fig. 3).

The present results indicate that amygdaloid group I and NMDA receptors contribute to maintenance of sensory and emotional-like pain in peripheral neuropathy. The finding that group I mGluR antagonists attenuated emotional-like pain behavior at a lower dose than limb withdrawal responses suggests that amygdaloid group I mGluRs may have a more important role in promotion of emotional-like pain than sensory aspects of pain. This finding also suggests that the modulation of emotional-like pain behav-

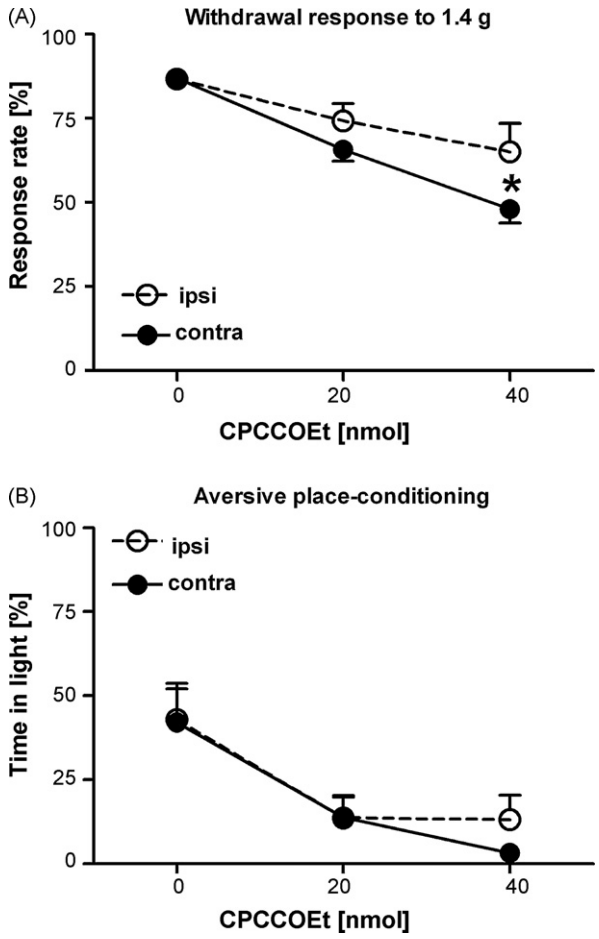


Fig. 2. Hind limb withdrawal response elicited by mechanical stimulation of the nerve-injured paw (A) and aversive place-conditioning behavior (B) following unilateral amygdaloid injection of saline or CPCCOEt (an $mGluR_1$ antagonist) at the dose of 20 or 40 nmol/animal. Ipsi = amygdaloid injection ipsilateral to nerve injury, contra = amygdaloid injection contralateral to nerve injury. Error bars represent S.E.M. ($n = 4–9$). $^*P < 0.05$ (t-test with a Bonferroni correction; comparison between corresponding values in the ipsi- versus contra-group).

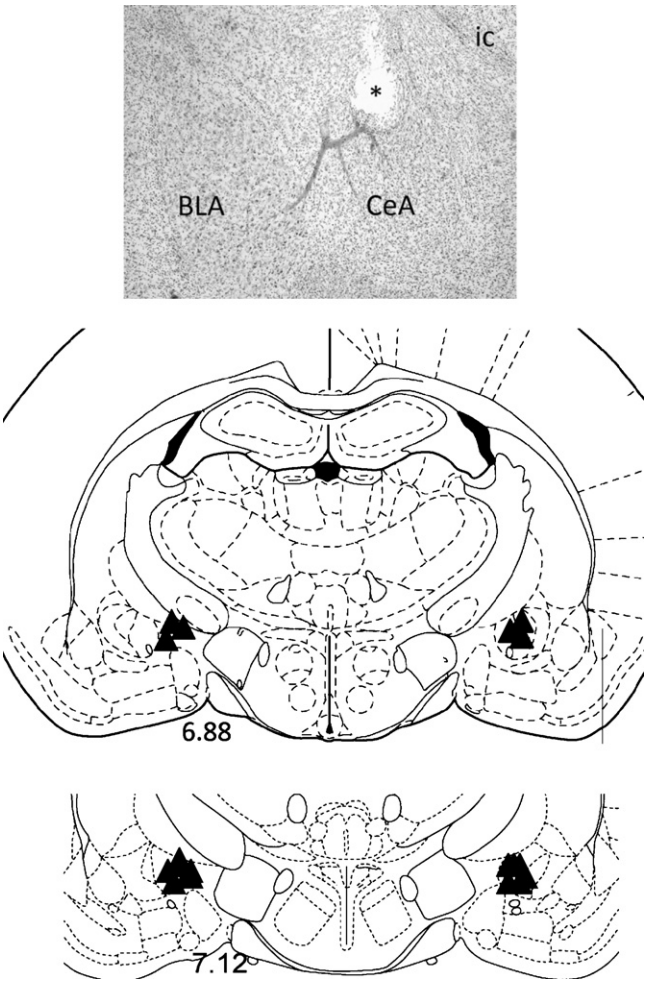


Fig. 3. Injection sites in the amygdala. Upper graph shows an example of a lesion induced by the injection needle in a Nissl-stained coronal section. Triangles in lower schematic diagrams represent injections sites (each triangle represents 1–3 sites). BLA, basolateral amygdaloid nucleus; CeA, central amygdaloid nucleus; ic, internal capsule; and *, tip of the cannula.

ior by amygdaloid administration of compounds acting on group I mGluRs is predominantly a direct effect on mechanisms underlying emotional pain rather than indirect effect due to enhancement of pain-related sensory signals.

A previous electrophysiological study demonstrated plasticity of synaptic inputs from the parabrachial nucleus to the CeA, and this synaptic plasticity was independent of the NMDA receptor in peripheral neuropathy [5], unlike in inflammatory conditions [7]. In the present study, an NMDA-R antagonist in the CeA facilitated both sensory and emotional-like pain behavior in nerve-injured animals. A possible explanation for these findings is that the amygdaloid NMDA-R plays a role in maintenance of neuropathic symptoms by facilitating inputs from the basolateral amygdala or synaptic signaling between interneurons within the CeA rather than facilitating the ascending spino-parabrachial-amygdala input. It should be noted that nerve injury may induce pathophysiological changes also in the function of various other amygdaloid neurotransmitter receptors, such as $GABA_A$-R [12] that may contribute to neuropathic symptoms.

Suppression of spinal withdrawal response was slightly stronger following administration of the $mGluR_1$ antagonist into the CeA contra- than ipsilateral to nerve injury, while the suppression of emotional-like pain was of the same magnitude following ipsi- as contralateral administration. This difference, together with the differential dose-dependence in the modulation of sensory and emotional-like pain by group I mGluR antagonists, supports the hypothesis that amygdaloid mechanisms promoting sensory and emotional-like pain in neuropathy are, at least partly, different.

While earlier studies have shown that under physiological conditions the amygdala has a role in induction of analgesia [for review see Ref. [9]], the present results add to the accumulating evidence indicating that under inflammatory [7,10] and neuropathic [1,5,15] conditions amygdaloid glutamatergic receptors may promote pain. Endogenous activation of the amygdaloid group I mGluRs may be of particular importance for the promotion of emotional-like neuropathic pain as revealed by the predominant suppression of the aversive place-conditioning behavior following amygdaloid administration of group I mGluR antagonists in nerve-injured animals.

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Descending effect on spinal nociception by amygdaloid glutamate varies with the submodality of noxious test stimulation

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HIGHLIGHTS

- Glutamate in the central amygdala induced spinal antinociception in healthy rats.
- Mechanical antinociceptive effect was predominantly contralateral.
- Heat antinociception was of shorter duration and bilateral.
- Amygdaloid NMDA receptor mediated mechanical but not heat antinociception.
- Spinal antinociceptive effect did not vary with the brain hemisphere.

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ABSTRACT

Amygdala has an important role in the processing of primary emotions, such as fear. Additionally, amygdala is involved in processing and modulation of pain. While the amygdala, particularly its central nucleus (CeA), has been shown to contribute to pain control, the descending pain regulation by the CeA is still only partly characterized. Here heat and mechanical nociception was tested in both hind limbs of healthy rats with a chronic guide cannula for microinjection of glutamate into the CeA of the left or right hemisphere. The aim was to assess whether the descending pain regulatory effect by glutamate in the amygdala varies with the submodality or the body side of nociceptive testing, brain hemisphere or the amygdaloid glutamate receptor. Motor performance was assessed with the Rotarod test. Amygdaloid glutamate, independent of the treated hemisphere, produced a dose-related heat and mechanical antinociception that varied with the submodality of testing. Heat antinociception was short lasting (minutes), bilateral and not reversed by blocking the amygdaloid NMDA receptor with MK-801. In contrast, mechanical antinociception lasted longer (>20 min), was predominantly contralateral and reversed by blocking the amygdaloid NMDA receptor. At an antinociceptive dose, amygdaloid glutamate failed to influence motor performance. The results indicate that independent of the brain hemisphere, the spatial extent and duration of the descending antinociceptive effect induced by amygdaloid glutamate varies with the amygdaloid glutamate receptor and the submodality of pain.

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1. Introduction

The amygdala exerts an important role in processing of primary emotions, such as fear. Amygdala, particularly its central nucleus (CeA), is also known to be involved in processing of emotional aspects of pain and in regulation of spinal nociception through its efferent connections to brainstem relay nuclei that project caudally [11,16]. Glutamatergic amygdaloid receptors are known to be involved in processing of pain-related signals [5,10]. The descending influence by glutamatergic receptors of the CeA has varied from facilitation to inhibition of spinal nociception depending on the type of the amygdaloid glutamate receptor, the behavioral assay and the pathophysiological condition of the animal [1,2,7,8,13,14,17]. The contribution of the CeA to nociceptive processing has varied with the brain hemisphere, the right CeA playing a major role in many [3,4,6,7] although not all experimental conditions [17]. While previous studies indicate that the glutamatergic system in the CeA plays a role in descending modulation of pain, many characteristics of the descending pain modulatory effect

Abbreviations: CeA, central nucleus of the amygdala; NMDA, N-methyl-D-aspartate.

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induced by glutamate in the amygdala of healthy control animals are still only partly known.

The aim of this study was to determine whether glutamate in the amygdala of healthy control animals modulates spinal nociception bi- or unilaterally, whether the descending effect varies with the submodality of noxious test stimulation or brain hemisphere, and whether amygdaloid NMDA and non-NMDA receptors have variable roles in the glutamate-induced descending effects.

2. Materials and methods

2.1. Animals

The experiments were performed with adult male Hannover-Wistar rats (Harland, Horst, The Netherlands) weighing 200–350 g. The experimental protocols were approved by the Experimental Animal Ethics Committee of the Provincial Government of Southern Finland (Hämeenlinna, Finland), and the experiments were performed according to the guidelines of European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to limit distress and to use only the number of animals necessary to produce reliable scientific data. Rats were housed in a 12-hour light/dark cycle with food and water access *ad libitum*.

2.2. Cannula insertion and drug injection procedure

The animals had a guide cannula for drug administration either into the right or left amygdala as described in detail earlier [3]. For placement of the guide cannula (26 gauge; PlasticsOne, Roanoke, VA, USA), the skull was exposed and a hole drilled for its placement under pentobarbitone anesthesia (50 ml/kg i.p.). The desired injection target in the left or right amygdala was in the capsule lateral of the central nucleus of amygdala (CeA): 2.1 mm posterior from the bregma, 4.3 mm lateral from the midline, and 7.8 mm ventral from the dura mater [15]. The control injection site was in the right internal capsule: 2.1 mm posterior from bregma, 3.6 mm lateral from the midline, and 5.0 mm ventral from the dura mater. The tip of the guide cannula was positioned 2 mm above the desired injection site. The cannula was fixed into the skull using a dental screw and dental cement. Drug administration to the brain and experimental protocols started 1 week after fixation of the guide cannula to the skull. A dummy cannula was placed in the guide cannula, except when drug administrations were performed.

2.3. Drugs and their administration procedure

Glutamate (L-glutamic acid monosodium salt) and the NMDA receptor antagonist (+)-MK-801 maleate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Glutamate was administered at the dose of 32 μg or 100 μg, while MK-801 was administered at the dose of 2 μg. Physiological saline (OrionPharma, Espoo, Finland) was used for control injections.

Unilateral infusions of drugs, or an equivalent volume of saline, were made by using 33 gauge injection needles (PlasticsOne) connected to a 10 μl Hamilton microsyringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) by polyethylene (PE-10) tubing. The injection needles protruded 2.0 mm beyond the cannula tips and a 0.5 μl volume was injected. The animals were gently restrained during the infusion procedure. The duration of injection was 30 s. Injection needle was retained within the cannula for an additional 20 s after drug infusion to maximize diffusion and to prevent backflow of the drug into the cannula. At the currently used injection volume, the spread of injection may be close to 1 mm [9], due to which the drug application method allows concluding whether the drug-induced effect originates in the amygdala rather than pinpointing the effect to one of its subnuclei. However, since the CeA is involved in nociception and provides efferent connections of the amygdala to the brainstem [11], it may be argued that the present results on descending regulation of nociception reflect drug-induced actions on the CeA rather than on its other subnuclei.

2.4. Assessment of pain behavior

Before assessment of pain behavior, the rats were habituated to the testing conditions at least in three one hour sessions during three consecutive days. Mechanical pain behavior was assessed using a calibrated series of monofilaments that in the current experiment produced forces ranging from 1 to 300 g (North Coast Medical, Inc., Morgan Hill, CA, USA). During testing, rats were on a grid, free to move inside a transparent box. The monofilaments were applied below the grid to the foot pad with increasing force until the rat withdrew its hind limb. The lowest force producing a withdrawal response to all of its five consecutive presentations was considered the threshold. In each condition, the monofilament-induced withdrawal threshold was assessed separately in both hind limbs.

Heat nociception was assessed by determining limb withdrawal latency induced by heat applied to the plantar skin using radiant heat equipment (Plantar test model 7370, Ugo Basile, Varese, Italy). The cut-off point was set at 15 s. Rats were free to move inside a transparent box of thermal plantar test device.

2.5. Rotarod test

To exclude a motor effect of the glutamate injection into the left or right CeA, motor activity of the rats was assessed in the Rotarod test. After starting the Rotarod test device (Ugo Basile), it increased its speed each second by 2 revolutions per minute (rpm). The maximum revolution speed at which the rats were able to stay on the drum was determined one minute after saline and glutamate administrations.

2.6. Course of study

Experiments were performed at least a week following unilateral installation of the brain cannula into the left or the right CeA. Mechanical pain threshold, noxious heat-evoked withdrawal latency and motor performance were evaluated on separate days. In half of the animals, the experiment started with assessment of heat nociception and in half with assessment of mechanical nociception. The Rotarod test was always performed as the last test. Mechanical and heat nociception was tested in the following drug-treatment conditions: saline, glutamate at the dose of 32 μg or 100 μg, MK-801 at the dose of 2 μg followed 5 min later by glutamate at the dose of 100 μg, or MK-801 at the dose of 2 μg alone. Pain behavior was assessed 1, 10, 20, 40 and 60 min after drug administration as well as before it. The order of testing the drug conditions was randomized using Quickcalcs software of Graph Pad (http://www.graphpad.com/quickcalcs/randMenu/). The interval between testing the different experimental conditions in the same animal was two days.

2.7. Histology

At the end of the experiment, rats were sacrificed by an overdose of pentobarbital and the brain was removed and immersed in 4% formaldehyde. Coronal sections of the brain were cut to verify the site of injection according to the atlas of Paxinos and Watson [15] (Fig. 1).

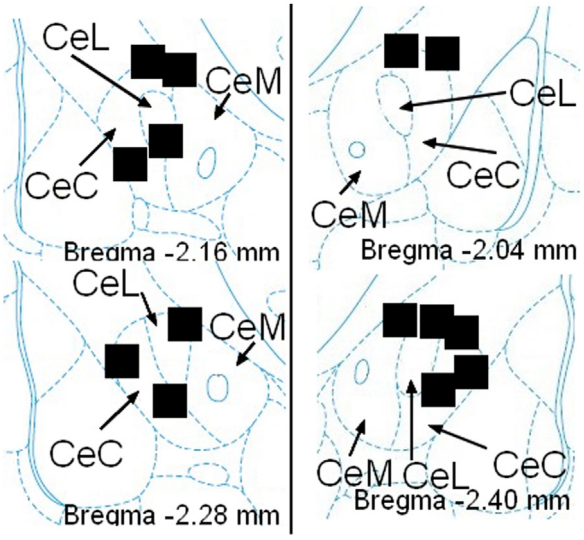


Fig. 1. Schematic representation of the site of cannula placement inside the left or right central nucleus of amygdala (CeA). Each symbol represents 1–3 overlapping injection sites in the amygdala. CeC: capsular nucleus of the CeA; CeM: medial division of the CeA; CeL: lateral division of the CeA.

2.8. Statistical analyses

Parametric data (heat-evoked withdrawal latencies) were analyzed using repeated one-way ANOVA or mixed two-way ANOVA followed by *t*-test with a Bonferroni correction for multiple comparisons, or (withdrawal latencies in less than three groups and motor performance) by paired *t*-test. Non-parametric data (monofilament-induced withdrawal threshold) were analyzed using Friedman's test followed by Dunn's test, or Wilcoxon signed rank test (less than three groups). $P < .05$ was considered to represent a significant difference.

3. Results

3.1. Dose-dependence of the modulation of nociception by glutamate in the CeA

Injection of glutamate into the right CeA induced a dose-related (32–100 μg) thermal antinociceptive effect as revealed by the increase of the heat-evoked withdrawal latency (main effect of glutamate: $F_{2,10} = 4.13$, $P = .049$; Fig. 2A). The glutamate-induced thermal antinociceptive effect was not significantly different between the ipsilateral (right) and contralateral (left) limb (main effect of the side of the cutaneous testing site: $F_{1,5} = 3.07$, $P = .140$), nor did the magnitude of the glutamate-induced thermal antinociception vary significantly with the side of thermal testing (interaction between glutamate treatment and side of testing: $F_{2,10} = 3.20$, $P = .084$).

Injection of glutamate into the right CeA produced a dose-related (32–100 μg) increase of the monofilament-induced withdrawal threshold of the contralateral (left) limb (Fr = 11.14, $P = .0001$) while glutamate in the right CeA failed to influence the threshold of the ipsilateral (right) limb (Fr = 0.14, $P = .956$; Fig. 2B).

3.2. Effects of the treated hemisphere or the side of the cutaneous test site

To assess whether the amygdaloid glutamate-induced effect varies with the hemisphere or the side of testing, 100 μg of glutamate or saline was administered into the right *versus* left CeA and pain behavior was elicited by heat or mechanical stimulation of the ipsilateral or contralateral hind paw. The magnitude of heat antinociception was not significantly different when glutamate was administered into the left or the right CeA (main effect of the treated hemisphere: $F_{1,11} = .04$; Fig. 2C). The contralateral heat antinociception was slightly greater (contra- *versus* ipsilateral test side: $F_{1,11} = 4.84$, $P = .050$), independent of the treated hemisphere (interaction between the treated hemisphere and the test side: $F_{1,11} = .23$; Fig. 2C).

Mechanical antinociceptive effect varied with the experiment condition (KW = 19.9, $P = .0002$; Fig. 2D). *Post hoc* testing indicated that the mechanical antinociceptive effect was significantly stronger in the contralateral than ipsilateral limb. In the ipsilateral limb, the mechanical pain threshold increase following amygdaloid glutamate injection was negligible. This is indicated by the finding that following unilateral glutamate administrations, the median mechanical pain threshold of the ipsilateral limb was not above the 90% percentile of the threshold in saline-treated animals. The mechanical antinociceptive effect was not significantly different between glutamate administrations into the left *versus* right CeA (Fig. 2D).

3.3. Time course of antinociception

Time course of amygdaloid glutamate-induced antinociception was assessed by administering 100 μg of glutamate or saline into the left CeA and by determining pain sensitivity to thermal and mechanical stimulation in the contralateral limb. Heat-induced withdrawal latency following amygdaloid treatment varied with time ($F_{5,15} = 4.9$, $P = .007$) and the amygdaloid treatment ($F_{5,15} = 4.3$, $P = .013$). *Post hoc* testing indicated that glutamate induced a significant thermal antinociceptive effect one min after the injection and the thermal pain sensitivity was back to normal by 10 min after the injection (Fig. 3A). The baseline latency was not significantly changed between the experimental sessions. This is shown by the finding that difference in the mean heat-evoked baseline latency between the first and last testing day was not significantly different in a group of animals with guide cannula in the left CeA ($t5 = .59$, $P = .578$). Mechanical antinociceptive effect also had a short onset but it lasted at least for 20 min after the injection (Fr = 16.3, $P = .006$; Fig. 3B). Complete recovery of the mechanical pain threshold was observed 60 min after amygdaloid glutamate administration.

3.4. Role of the amygdaloid NMDA receptor in the glutamate-induced antinociception

To assess whether the amygdaloid glutamate-induced thermal or mechanical antinociceptive effect is mediated by the NMDA receptor, the CeA was pretreated with 2 μg of MK-801, an NMDA receptor antagonist, before administering 100 μg of glutamate into the CeA. Amygdaloid drug treatments had a significant effect on heat nociception ($F_{3,12} = 16.1$, $P < .0001$). *Post hoc* tests indicated that while glutamate alone significantly attenuated heat-evoked pain behavior, pretreatment of the CeA with MK-801 failed to reduce the magnitude of the glutamate-induced antinociceptive effect (Fig. 3C). MK-801 alone at the currently used dose had no effect on heat nociception. Mechanical pain threshold was also significantly influenced by amygdaloid drug treatments (Fr = 28.8, $P < .0001$). *Post hoc* testing indicated that pretreatment of the right CeA with MK-801 (2 μg) reversed the elevation of the mechanical pain threshold induced by 100 μg of glutamate in the CeA (Fig. 3D).

3.5. Motor performance following glutamate in the CeA

To assess potential motor effects induced by amygdaloid glutamate, Rotarod test was performed. In the saline control condition, the animals were able to stay at the Rotarod drum up to the

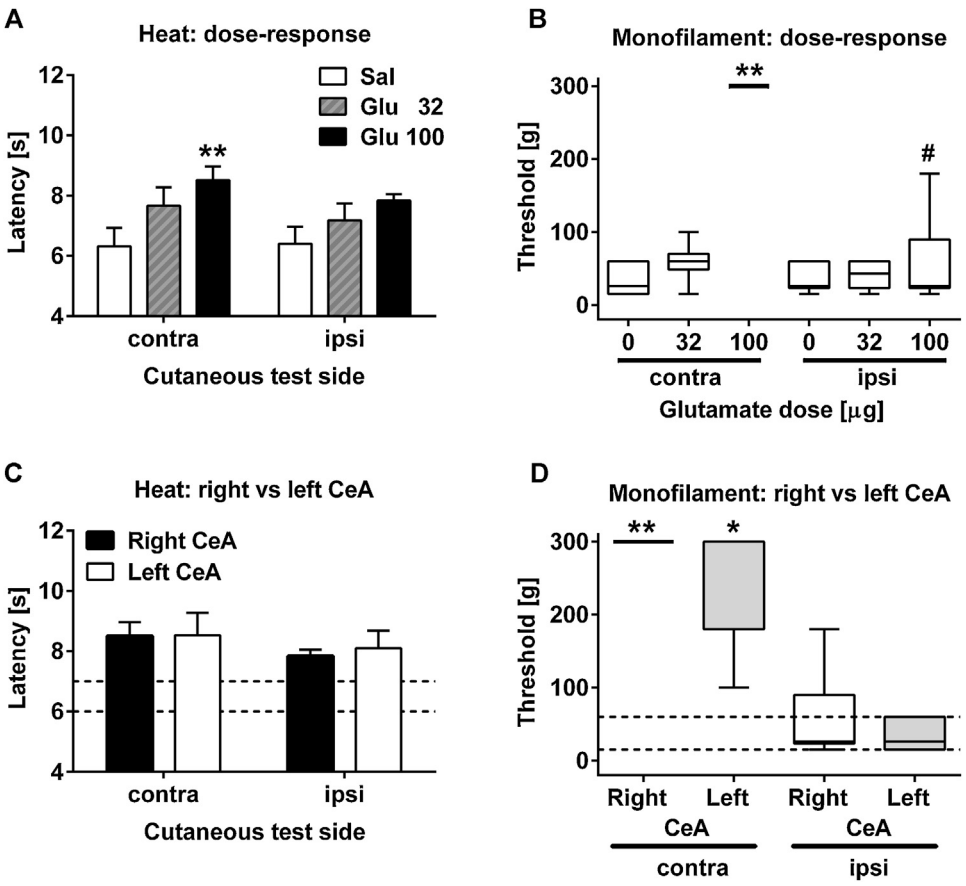


Fig. 2. Influence of the amygdaloid glutamate dose (A, B) and the treated brain hemisphere (C, D) on spinal nociceptive withdrawal response evoked by heat (A, C) or mechanical stimulation (B, D). In A and C, the bars represent the mean withdrawal latency and the error bars S.E.M. In B and D, the boxes show median thresholds with interquartile ranges, and whiskers show the range. The broken horizontal lines represent the 95% confidence limits of the withdrawal latency (C) or the range from the 10% to the 90% percentile of the withdrawal threshold (D) in saline-treated animals. In each group, $n = 6–7$. Sal or 0 μg = saline, and Glu 32–100 represent glutamate doses in μg. In C and D, glutamate was administered into the central nucleus of the amygdala (CeA) only at the dose of 100 μg. contra = contralateral to the treated CeA, ipsi = ipsilateral to the treated CeA. $^{*}P < .05$, $^{**}P < .01$ (reference in A and B was the corresponding Sal group whereas in D the reference was the corresponding ipsi group; in A, *t*-test with a Bonferroni correction and in B and D, Dunn's test). $^{\#}P < .05$ (reference: the corresponding contra group, Dunn's test).

revolving speed of 35.7 + 3.2 rpm (+S.E.M., $n = 10$). In the glutamate conditions, the animals were able to stay at the Rotarod drum up to the revolving speed of 32.3 + 2.4 rpm ($n = 10$). Glutamate (100 μg) in the CeA failed to have a significant effect on the performance in the Rotarod test ($F_{1,7} = 0.9$), independent whether glutamate was administered to the left or right CeA ($F_{1,7} = 0.5$).

3.6. Control injection site

When compared with vehicle, glutamate (100 μg) in the control injection site (the right internal capsule; $n = 6$) failed to produce a significant change in the mechanically evoked withdrawal threshold of the contralateral hind limb ($W = 5.0$, $P = .688$, not shown). While glutamate in the control injection site did not influence pain behavior, in one animal it induced motor side effects.

4. Discussion

The present results indicate that glutamate administration into the amygdala attenuated spinally-organized pain behavior in healthy control rats. This is in line with earlier findings indicating that activation of the CeA with glutamate or electric stimulation suppressed spinal withdrawal reflexes in healthy controls [8,12]. Earlier studies assessing the effects of subtype specific glutamatergic compounds in the CeA indicate that the metabotropic glutamate receptor $mGluR_8$ suppresses spinal withdrawal reflexes in inflamed animals [13,14] while the $mGluR_{1/5}$ [7] and $mGluR_7$ [13] facilitate spinal nociception. The MK-801-reversible attenuation of the spinal withdrawal reflex evoked by mechanical but not heat stimulation in the present study suggests that the NMDA receptor in the CeA contributes predominantly to the mechanical antinociceptive effect. This descending antinociceptive action mediated by the amygdaloid NMDA receptor is not driven in a tonic fashion in healthy controls, as shown by lack of effect by MK-801 alone. In contrast to the current findings on spinal antinociception, earlier studies have demonstrated that the amygdaloid NMDA and non-NMDA receptors are involved in tonic promotion of pain affect-like behavior in healthy controls [17] and in chronic neuropathy [1,2].

While there is accumulating evidence indicating that the processing of pain-related signals is lateralized to the right CeA [3,4,6,7], the lateralization may not apply to all aspects of the amygdaloid pain-processing as indicated by the present finding that the descending modulation of spinal nociception by glutamate in the right amygdala was not significantly different from that in the left amygdala. In line with this, it was recently reported that NMDA or non-NMDA receptor antagonists in the right CeA had equal effects on pain affect as in the left CeA [17]. Interestingly, the spinal antinociceptive effect by unilateral glutamate administration into the CeA was bilateral for heat-evoked responses and predominantly contralateral for mechanically evoked responses. Moreover, the opposite effect (mechanical hypersensitivity) by an $mGluR_{1/5}$ agonist in the CeA was also contralateral [7]. Together,

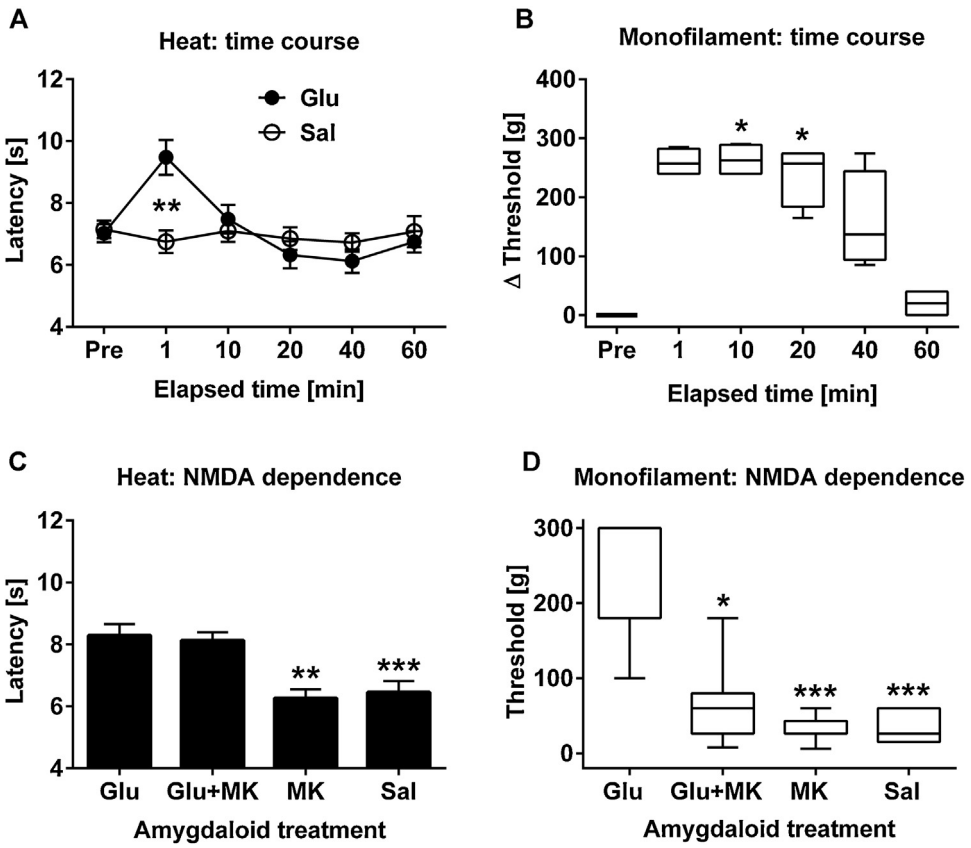


Fig. 3. Time course of the amygdaloid glutamate-induced descending antinociceptive effect (A, B) and its attempted reversal by blocking the amygdaloid NMDA receptors (C, D). Graphs A and C show the mean heat-evoked withdrawal latency (+S.E.M.) while graphs B and D show the changes in the mechanically evoked withdrawal threshold. Graph B shows the glutamate-induced change of the withdrawal threshold and *Y*-axis values >0 g represent glutamate-induced increases when compared with the corresponding saline-values. In B and D, the boxes show the median and its interquartile range, while the whiskers show the range from the minimum to the maximum value. In A and B, $n = 6–7$ in each group, and in C and D, $n = 13$ in each group. Glu = 100 μg of glutamate, Sal = saline control, MK = 2 μg of MK-801, Glu + MK = 100 μg of glutamate + 2 μg of MK-801, Pre = before drug administration. In A and B, drugs were administered into the left central nucleus of the amygdala (CeA). In C and D, drug administrations into the left ($n = 7$) and right ($n = 6$) CeA are pooled. In all graphs, noxious test stimulation was applied to the hind limb contralateral to the drug-treated CeA. $^{*}P < .05$, $^{**}P < .01$ (reference: the corresponding Sal-value in A, the Pre-value in B, and the Glu value in C and D; *t*-test with a Bonferroni correction in A and C, and Dunn's test in B and D).

these findings suggest that the descending glutamatergic effect of the CeA on mechanically evoked spinal nociceptive responses is predominantly contralateral, independent of the direction of the modulatory effect. The variation of the CeA-induced descending modulatory effect with the submodality of noxious test stimulation adds to the earlier findings indicating that the descending effect may vary with the submodality of pain also when it originates in the midbrain periaqueductal gray or the rostroventromedial medulla, relay nuclei mediating the descending effect from the CeA to the spinal dorsal horn [16].

5. Conclusions

Independent of the brain hemisphere, unilateral glutamate administration into the CeA of healthy control animals produces an NMDA receptor-mediated spinal antinociceptive effect against mechanical stimulation contralaterally and a short-lasting non-NMDA receptor-mediated spinal antinociceptive effect against heat stimulation bilaterally.

Conflict of interest

The authors are not aware of any conflict of interest related to this study.

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Research report

Bidirectional amygdaloid control of neuropathic hypersensitivity mediated by descending serotonergic pathways acting on spinal 5-HT_3 and 5-HT_{1A} receptors

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HIGHLIGHTS

- Amygdaloid glutamate modulates neuropathic hypersensitivity bidirectionally.
- A low glutamate dose increases neuropathic hypersensitivity.
- The increase of hypersensitivity is mediated by the spinal 5-HT_3 receptor.
- A high glutamate dose suppresses neuropathic hypersensitivity.
- The decrease of hypersensitivity is mediated by the spinal 5-HT_{1A} receptor.

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ABSTRACT

Amygdala is involved in processing of primary emotions and particularly its central nucleus (CeA) also in pain control. Here we studied mechanisms mediating the descending control of mechanical hypersensitivity by the CeA in rats with a peripheral neuropathy in the left hind limb. For drug administrations, the animals had a guide cannula in the right CeA and an intrathecal catheter or another guide cannula in the medullary raphe. Hypersensitivity was tested with monofilaments. Glutamate administration in the CeA produced a bidirectional effect on hypersensitivity that varied from an increase at a low-dose (9 μg) to a reduction at high doses (30–100 μg). The increase but not the reduction of hypersensitivity was prevented by blocking the amygdaloid NMDA receptor with a dose of MK-801 that alone had no effects. The glutamate-induced increase in hypersensitivity was reversed by blocking the spinal 5-HT_3 receptor with ondansetron, whereas the reduction in hypersensitivity was reversed by blocking the spinal 5-HT_{1A} receptor with WAY-100635. Both the increase and decrease of hypersensitivity induced by amygdaloid glutamate treatment were reversed by medullary administration of a 5-HT_{1A} agonist, 8-OH-DPAT, that presumably produced autoinhibition of serotonergic cell bodies in the medullary raphe. The results indicate that depending on the dose, glutamate in the CeA has a descending facilitatory or inhibitory effect on neuropathic pain hypersensitivity. Serotoninergic raphe neurons are involved in mediating both of these effects. Spinally, the 5-HT_3 receptor contributes to the increase and the 5-HT_{1A} receptor to the decrease of neuropathic hypersensitivity induced by amygdaloid glutamate.

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1. Introduction

Amygdala is considered a key structure in the processing of primary emotional responses such as fear [22]. Additionally, a subdivision of the central nucleus of the amygdala (CeA) is involved in processing and modulation of nociceptive signals through its reciprocal connections with various cortical and subcortical areas [27]. Peripheral nerve injuries that produce chronic pain and cutaneous hypersensitivity have been shown to induce structural [12] and functional [11,15] changes of the amygdala. It may be expected

Abbreviations: CeA, central nucleus of amygdala; i.t., intrathecal; NMDA, N-methyl-D-aspartate; 8-OH-DPAT, 8-hydroxy-2-dipropylaminotetralin; RVM, rostral ventromedial medulla.

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that the peripheral nerve injury-induced changes in the amygdala influence processing and regulation of pain by the amygdala. In line with this, there is already evidence suggesting that the amygdala contributes to descending regulation of neuropathic symptoms as revealed e.g. by exacerbation of hypersensitivity following activation of the amygdaloid type I metabotropic glutamate receptor [1,2] and corticotropin-releasing factor receptor [5], or decreased hypersensitivity following activation of the amygdaloid $GABA_A$ receptor [33].

The amygdala modulates neuronal activity in the spinal dorsal horn particularly through the CeA–periaqueductal gray (PAG)–rostroventromedial medulla (RVM)–spinal cord pathway but it may also modulate spinal signals through direct or indirect action on other descending pain control systems, such as the noradrenergic coeruleospinal pathway (see for reviews [25,27,35]). Previous electrophysiological studies have suggested that among mechanisms underlying the CeA-induced descending exacerbation of neuropathic hypersensitivity may be increased activity of pronociceptive On-like RVM cells in the CeA–PAG–RVM–spinal cord pathway [1] and the CeA-induced suppressed neuronal activity in the coeruleospinal pathway [47]. However, neurochemical mechanisms mediating the CeA-induced descending modulation of neuropathic hypersensitivity are still poorly known. For example, it is still not clear whether a serotonergic subgroup of spinally-projecting RVM neurons provides a relay for the descending pain control by the CeA–PAG–RVM–spinal cord pathway.

Here we studied neurotransmitter mechanisms relaying the CeA-induced descending modulation of neuropathic hypersensitivity. Therefore, we first characterized the effect of glutamate administration into the CeA on neuropathic pain hypersensitivity as revealed by mechanically evoked withdrawal reflexes in an experimental animal model of peripheral neuropathy. To assess potential role of amygdaloid NMDA receptors in descending controls, we attempted to prevent the glutamate-induced effects by amygdaloid administration of an NMDA receptor antagonist at a dose that alone had no significant effect. To determine spinal neurotransmitter mechanisms underlying the CeA-induced descending modulation of pain hypersensitivity, we attempted to reverse the modulatory effect with spinally administered neurotransmitter receptor antagonists. To assess further whether serotonergic medullary neurons are involved in relaying the descending modulation from the CeA to the spinal cord, we also attempted to reverse the CeA-induced descending modulation by intramedullary microinjections of a 5-HT_{1A} agonist that was expected to reduce firing of spinally projecting serotonergic neurons, due to activation of autoinhibitory 5-HT_{1A} receptors on their cell bodies [40].

2. Materials and methods

2.1. Animals

The experiments were performed with 36 adult male Hannover-Wistar rats (Harlan, Horst, The Netherlands) weighing 200–260 g. The experimental protocols were approved by the Experimental Animal Ethics Committee of the Provincial Government of Southern Finland (Hämeenlinna, Finland), and the experiments were performed according to the guidelines of European Communities Council Directive of 22 September 2010 (2010/63/EU). All efforts were made to limit distress and to use only the number of animals necessary to produce reliable scientific data. Rats were housed in a 12-h light/dark cycle with food and water access ad libitum. Experiments were performed during light cycle.

2.2. Surgical procedures for producing neuropathy

For inducing neuropathy, the spared nerve injury (SNI) model, as described by Decosterd and Woolf [8], was adopted. Prior to surgery, the rat was anaesthetized with sodium pentobarbital (OrionPharma, Espoo, Finland), administered intraperitoneally at the dose of 60 mg/kg. Further doses of pentobarbitone were given at the dose of 15–20 mg/kg as needed to keep the depth of anesthesia deep enough so that the animal did not react to noxious stimulation. An incision was subsequently made into the skin on the lateral surface of the left thigh, followed by a section through the biceps femoris muscle to expose the sciatic nerve and its terminal branches: the sural, common peroneal and tibial nerves. The common peroneal and tibial nerves were then tightly ligated with 4–0 silk, sectioned distal to the ligation and 3–4 mm of the distal nerve stump removed. The sural nerve was left intact and care was exercised not to stretch it. To prevent postoperative pain, animals were treated with 0.01 mg/kg of buprenorphine twice daily for 1–3 days and they were allowed to recover for at least a week before the experiments. Only animals with tactile allodynia-like hypersensitivity (hind limb withdrawal threshold to monofilament stimulation in the operated side $\leq$4 g, which is below the lower 95% confidence limit of the threshold in unoperated control animals) were selected for this study. SNI model produced mechanical hypersensitivity in all animals of the present study.

2.3. Preparation and procedure for cerebral drug injections

The animals had a guide cannula for drug administration into the central nucleus of the right amygdala as described in detail earlier [5]. The right amygdala was chosen as a target in this study, since previous experimental animals studies indicate that the right amygdala has a more important role in pain processing than the left amygdala [7,16,18], although not in all conditions [42]. Additionally, earlier evidence suggested that the descending effect by the amygdala is stronger on the contra-than ipsilateral side [5,18], which finding further supported the choice of the right amygdala for unilateral injections in the experiments in which peripheral nerve injury was in the left hind limb. For placement of the guide cannula (26 gauge; PlasticsOne, Roanoke, VA, USA), the skull was exposed and a hole drilled for its placement under pentobarbitone anesthesia (60 ml/kg i.p.). The desired injection target in the right amygdala was in the capsule lateral of the central nucleus of amygdala (CeA): 2.1 mm posterior from the bregma, 4.3 mm lateral from the midline, and 7.8 mm ventral from the dura mater [32]. One group of animals had an additional guide cannula for drug injections into the rostral ventromedial medulla (RVM), where the desired injection site was: 11.0 mm posterior from the bregma, in the midline, and 10.5 mm ventral from the dura mater [32]. To exclude the possibility that glutamate induced its effects due to wide spread from the injection site, a separate group of animals had a guide cannula in a control injection site, the right internal capsule: 2.1 mm posterior from the bregma, 3.6 mm lateral from the midline, and 5.0 mm ventral from the dura mater [32]. In all injection locations, the tip of the guide cannula was positioned 2 mm above the desired injection site. The cannula was fixed into the skull using a dental screw and dental cement. Drug administration to the brain and experimental protocols started 1 week after fixation of the guide cannula to the skull. A dummy cannula was placed in the guide cannula, except when drug administrations were performed.

Intracerebral infusions of drugs, or an equivalent volume of saline, were made by using 33 gauge injection needles (PlasticsOne) connected to a 10 μl Hamilton microsyringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) by polyethylene (PE-10) tubing. The injection needles protruded 2.0 mm beyond the cannula tips and a 0.5 μl volume was injected into the CeA and a volume of 1.0 μl

into the rostral RVM. The animals were gently restrained during the infusion procedure. The duration of injection was 30 s. Injection needle was retained within the cannula for an additional 20 s after drug infusion to maximize diffusion and to prevent backflow of the drug into the cannula.

2.4. Preparation and procedure for intrathecal drug injections

In addition to a chronic guide cannula for drug injections into the right CeA, a group of animals had an intrathecal (i.t.) catheter for drug delivery to the lumbar spinal cord level. The catheter (Intramedic PE-10, Becton Dickinson and Company, Sparks, MD, USA) was installed into the lumbar level of the spinal cord under pentobarbital anesthesia (60 mg/kg i.p.) as described in detail elsewhere [44]. I.t. catheter was installed in the same procedure as nerve injury at least two weeks before actual drug testing. Following recovery from anesthesia, the correct placing of the catheter was verified by administering lidocaine (4%, 7–10 μl followed by a 10 μl of saline for flushing) with a 50 μl Hamilton syringe (Hamilton Bonaduz AG). Only those rats that had no motor impairment before lidocaine injection but had a bilateral paralysis of hind limbs following i.t. administration of lidocaine were studied further. When the studied drug was administered i.t., the volume of drug injections was 5 μl that was followed by a flush with 15 μl of saline.

2.5. Drugs

Glutamate (L-glutamic acid monosodium salt), the 5-HT_{1A} receptor antagonist WAY-100635, the 5-HT_3 receptor antagonist ondansetron, the dopamine D2 receptor antagonist raclopride, the opioid receptor antagonist naloxone HCl, the 5-HT_{1A} receptor agonist 8-OH-DPAT, and the NMDA receptor antagonist (+)-MK-801 maleate were purchased from Sigma–Aldrich (St. Louis, MO, USA). The α_2-adrenoceptor antagonist atipamezole was obtained from OrionPharma (Turku, Finland). Physiological saline was used for control injections. The doses of drugs were chosen based on previous studies and preliminary experiments. Based on earlier findings, the doses of i.t. administered receptor antagonists (WAY-100635 at 3 μg [49]; atipamezole at 5 μg [37]; raclopride at 1 μg [48]; naloxone at 1 μg [36]) had little if any effect on pain behavior in control animals while suppressing neuropathic hypersensitivity or the receptor agonist-induced effects. Our preliminary experiments (unpublished results) indicated that i.t. administration of ondansetron at the dose of 5 μg or RVM administration of 8-OH-DPAT at the dose of .125 μg failed to influence baseline pain behavior in controls.

2.6. Assessment of pain behavior

Observations by clinical neurologists indicate that nerve injury often produces tactile allodynia or hypersensitivity to mechanical stimulation of the skin (e.g., with brush) and only occasionally heat hyperalgesia [41]. Therefore, the focus of this study was in the assessment of tactile allodynia-like hypersensitivity by determining a limb withdrawal response evoked by monofilament stimulation of the injured dermatome.

Before assessment of pain behavior, the rats were habituated to the testing conditions at least in three 1 h sessions during three consecutive days. Mechanical pain behavior (tactile allodynia-like behavior) was assessed using a calibrated series of monofilaments that in the current experiment produced forces ranging from 1 to 15 g (North Coast Medical, Inc., Morgan Hill, CA, USA). During testing, rats were on a grid, free to move inside a transparent box. The monofilaments were applied below the grid to the foot pad with increasing force until the rat withdrew its hind limb. At each time point, the paw ipsilateral to the spinal nerve ligation was stimulated five times at each stimulus force with an ascending series of calibrated monofilaments. At each stimulus force, the withdrawal response frequency was determined. When the animal responded by withdrawing the limb to each of the five repetitive monofilament stimulations, the response rate to the studied stimulus force was consider to be 100%. When the animal did not withdraw to any of the five repetitive stimulations, the response rate to the studied stimulus force was considered to be 0%. An increase in the withdrawal response rate was considered to represent mechanical hypersensitivity effect.

2.7. Course of study

In one group of animals, spared nerve injury and two brain cannulae (one for drug injections into the CeA and one for injections into the RVM) were installed at the same time. In another group of animals, spared nerve injury, one brain cannula (for drug injections into the CeA) and i.t. catheter were installed at the same time. The effect of glutamate in a control injection site was assessed in a group of animals with the guide cannula in the right internal capsule. Experiments started 7–10 days following the operations.

In each drug treatment condition, mechanical hypersensitivity was assessed in the injured hind limb before the treatment and 5, 15, 30 and 60 min after the treatment. The order of testing different drug treatment conditions was varied to avoid serial effects. The effect of vehicle and three doses of glutamate was first assessed in a counterbalanced order (i.e., ascending series of doses was tested in half of the animals, and a descending series in the other half). After that, the effects of glutamate after i.t. pretreatments with receptor antagonists or the effects of i.t. treatments with receptor antagonists alone were tested; the testing of antagonists alone or with glutamate were varied between the animals. Then, the group of animals with two brain cannulae were tested so that they were injected in counterbalanced order with 6-OH-DPAT into RVM alone, 6-OH-DPAT into RVM followed by a low dose of glutamate into CeA, or 6-OH-DPAT into RVM followed by a high dose of glutamate into CeA. The effect MK-801 in CeA was tested in one group of animals (that was used also in assessing the effect of an intrathecally administered receptor antagonist) using the dose of 1 μg. The effect of two lower doses of amygdaloid MK-801 was assessed in another group followed by assessment of the co-administration of the lowest dose of MK-801 with a low or high dose of amygdaloid glutamate (in counterbalanced order). Finally, the effect of glutamate administration at various doses in the control injections site (the internal capsule) was assessed in counterbalanced order. Each animal was tested in 4–6 drug treatment conditions. The interval between different test conditions with each rat was 2–3 days. Comparison of pre-treatment responses was used to exclude the possibility that the drug treatments produced long-term effects. For example, when comparing pre-treatment responses of a group of six animals that were tested in six different conditions, the pre-treatment responses were not significantly different between the different treatment days (main effect of time: $F_{5,5} = .636$, $P = .583$).

All the drug doses that were used in the present study are described in Section 3. When attempting to reverse the amygdaloid glutamate-induced effects with i.t., intra-amygdaloid or intramedullary administration of drugs, the i.t., intra-amygdaloid or intramedullary treatment was performed five min before the amygdaloid glutamate or vehicle treatment. While the effect of glutamate alone was assessed using various stimulus forces, to reduce complexity of data analyses, the effects of drug combinations were assessed on the response elicited by the monofilament producing an innocuous force of 4 g, the response to which was considered to reflect tactile allodynia-like behavior.

2.8. Histology

At the end of the experiment, rats were sacrificed by an overdose of pentobarbital and the brain was removed and immersed in 4% formaldehyde. Coronal sections of the brain were cut to verify the site of injection according to the atlas of Paxinos and Watson [32] (Figs. 1 and 2).

2.9. Statistical analyses

Data were analyzed using one-way ANOVA or mixed two-way ANOVA followed by Tukey's test. $P < .05$ was considered to represent a significant difference.

3. Results

3.1. Dose-related bidirectional effect on hypersensitivity by amygdaloid glutamate

Glutamate treatment of the right CeA significantly influenced mechanical hypersensitivity in the injured (left) hind limb (main effect of drug: $F_{2,75} = 26.90$, $P < .0001$; Fig. 3A), and the drug-induced effect varied with time (interaction between drug and time: $F_{8,75} = 3.12$, $P = .0043$). Post hoc testing indicated that a low dose of glutamate (9 μg) increased hypersensitivity with a peak effect at 5 min after its administration, whereas a high dose (100 μg) attenuated hypersensitivity, with a peak effect at 15 min after its administration (Fig. 3A).

To assess whether the modulation of mechanical hypersensitivity by amygdaloid glutamate varies with the test stimulus intensity, the drug effect was assessed at various stimulus forces (1–10 g) producing a submaximal withdrawal response. (A stimulus force of 15 g evoked the maximum response and therefore, it was not included in the analyses, although the response to it is shown in the graphs.) Amygdaloid administration of a low dose (9 μg) of glutamate significantly enhanced mechanical hypersensitivity (main effect of drug: $F_{1,60} = 53.06$, $P < .0001$; Fig. 3B), independent of the stimulus force (interaction between drug and stimulus force: $F_{5,60} = 1.42$). Similarly, amygdaloid administration of a high dose (100 μg) of glutamate significantly attenuated mechanical hypersensitivity ($F_{1,60} = 43.4$, $P < .0001$; Fig. 3C), independent of the stimulus force ($F_{5,60} = 1.56$).

Dose-dependence of the amygdaloid glutamate-induced modulation of mechanical hypersensitivity was assessed at the test stimulus force of 4 g using three different doses of glutamate varying from 9 μg to 100 μg, and saline for control injections. Glutamate had a dose-related effect on mechanical hypersensitivity ($F_{3,26} = 16.18$, $P < .0001$; Fig. 3D). Post hoc testing indicated that at the dose of 9 μg amygdaloid glutamate administration significantly increased mechanical hypersensitivity, whereas at the doses of 30 μg or 100 μg it significantly suppressed mechanical hypersensitivity (Fig. 3D).

3.2. Spinal neurotransmitter receptor mediating the antihypersensitivity effect of amygdaloid glutamate

To study spinal mechanisms of the amygdala-induced antihypersensitivity effect, antagonists of various neurotransmitter receptors that are known to suppress nociception were administered i.t. while 100 μg of glutamate was administered in the CeA. I.t. administration of WAY-100635, a 5-HT_{1A} receptor antagonist, at a dose of 3 μg completely reversed the antihypersensitivity effect induced by amygdaloid administration of glutamate (Fig. 4A). I.t. administration of 5 μg of atipamezole, an α_2-adrenoceptor antagonist, failed to attenuate the mechanical antihypersensitivity effect induced by amygdaloid administration of glutamate (Fig. 4B). Nor did i.t. treatment with 1 μg of raclopride, a dopamine D2 receptor antagonist (Fig. 4C), or 1 μg of naloxone, an opioid receptor antagonist (Fig. 4D), produce a significant attenuation of the mechanical antihypersensitivity effect induced by amygdaloid administration of 100 μg of glutamate. At the currently used doses, the receptor antagonists WAY-100635, atipamezole, raclopride or naloxone

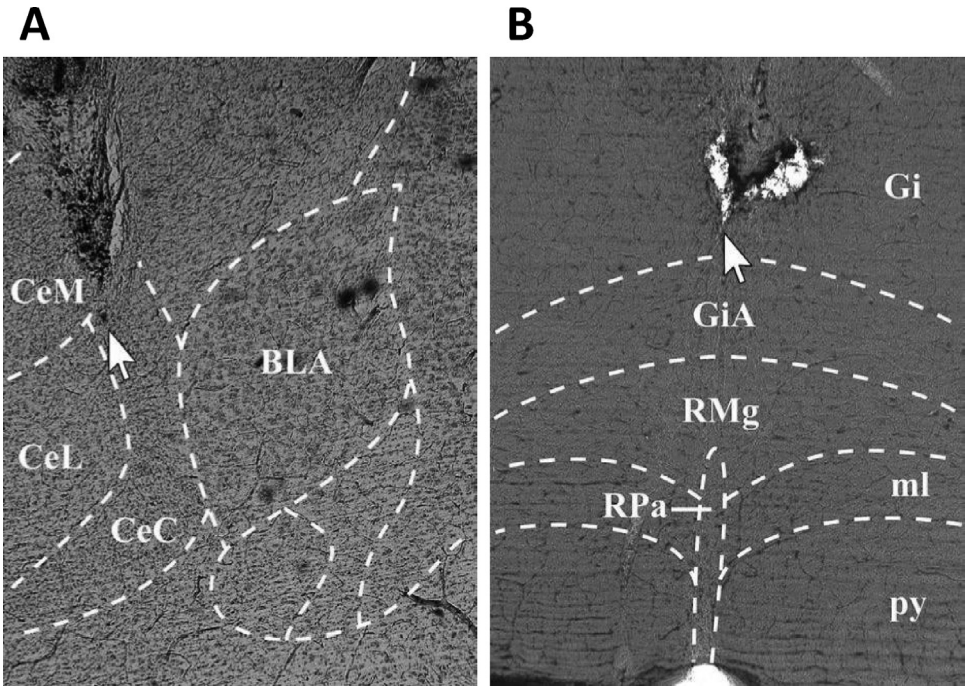


Fig. 1. Photographic examples of injection sites in the right amygdala (A) and the rostral ventromedial medulla (B). The arrows indicate the injection sites. CeM = medial division of the central amygdaloid nucleus, CeL = lateral division of the central amygdaloid nucleus, CeC = capsular division of the central amygdaloid nucleus, BLA = anterior basolateral amygdaloid nucleus, Gi = gigantocellular nucleus, GiA = gigantocellular nucleus pars alpha, RMg = raphe magnus nucleus, RPa = raphe pallidus nucelus, ml = medial lemniscus, py = pyramidal tract.

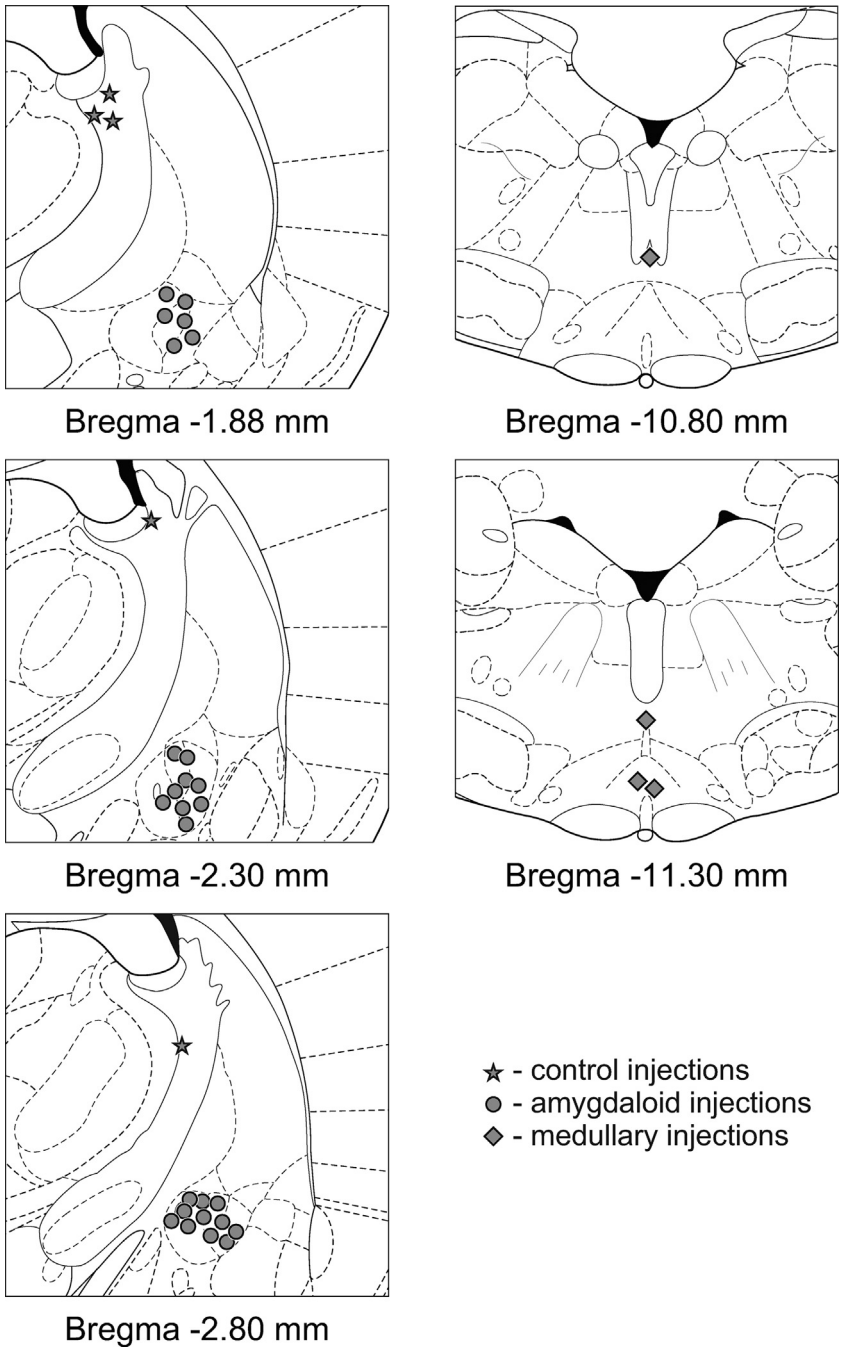


Fig. 2. Injection sites in the right amygdala (solid circles), the rostroventromedial medulla (solid squares), and the right internal capsule (solid stars). Each symbol represents 1–2 injection sites.

had no significant effects when they were administered alone (Fig. 4A–D).

3.3. Spinal neurotransmitter receptor mediating the hypersensitivity effect of amygdaloid glutamate

To study spinal mechanisms of the amygdala-induced hypersensitivity effect, an antagonist of the 5-HT_3 receptor that is known to contribute to the maintenance of pain hypersensitivity was administered i.t. while 9 μg of glutamate was administered in the CeA. I.t. treatment with ondansetron, a 5-HT_3 receptor antagonist, at a dose of 5 μg completely reversed the increase of hypersensitivity induced by amygdaloid administration of 9 μg of glutamate (Fig. 5A). Ondansetron alone at the dose of 5 μg had no significant effect on mechanical hypersensitivity (Fig. 5A).

3.4. Role of amygdaloid NMDA receptors in the glutamate-induced descending effects

Experiments described in Section 3.1 indicated that amygdaloid administration of glutamate produces a phasic increase or decrease of mechanical hypersensitivity, depending on the dose. To study whether the amygdaloid NMDA receptor contributes to

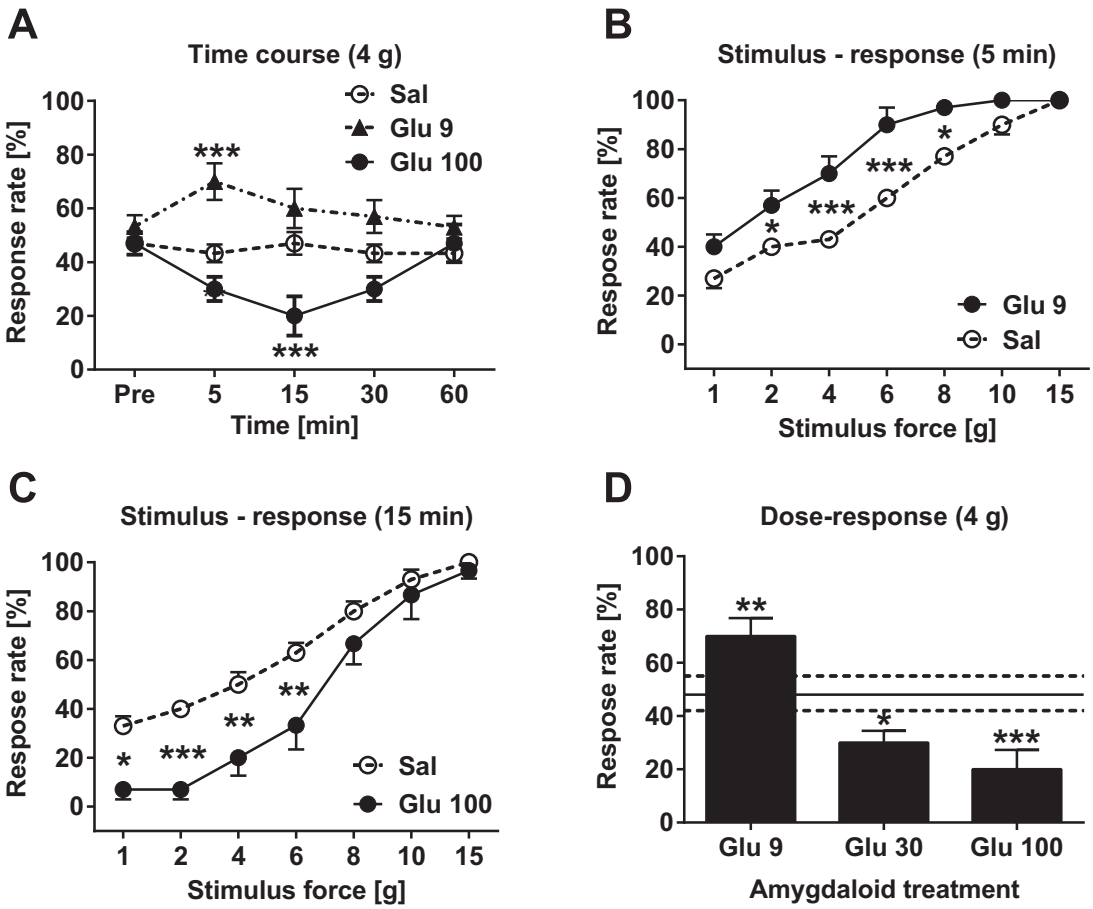


Fig. 3. Influence of amygdaloid glutamate administration on mechanical hypersensitivity in the nerve-injured hind limb. (A) Time course of effect induced by a low and a high dose of glutamate. (B) Change in stimulus–response function following administration of a low dose of glutamate (C) Change in stimulus–response function following administration of a high dose of glutamate. (D) Dose–response effect of amygdaloid glutamate. *Y*-axis represents withdrawal response rate to repetitive mechanical stimulation of the hind paw in the nerve-injured limb. Sal, saline, Glu 9-100, glutamate doses in μg. In A and D, stimulus force was 4 g. In (B) testing was performed 5 min after Glu/Sal administration and in C 15 min after Glu/Sal administration representing time points of the maximum effects for these doses. Error bars represent S.E.M. ($n = 6$ in each graph). In D, the horizontal lines represent the mean response and its 95% confidence limits in saline-treated animals. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .005$ (Tukey's test; reference: the corresponding value in the saline-treated group).

the increase or reduction of neuropathic hypersensitivity induced by amygdaloid glutamate administration or to tonic endogenous drive of neuropathic hypersensitive, MK-801, an NMDA receptor antagonist, was microinjected into the CeA. MK-801 alone in the CeA produced a dose-related (.1–1.0 μg) decrease of mechanical hypersensitivity ($F_{3,26} = 8.80$, $P = .0003$; Fig. 5B). Post hoc testing indicated that while MK-801 alone at the doses of .3 μg and 1.0 μg produced a significant decrease of hypersensitivity, MK-801 alone at the dose of .1 μg had no significant effect on mechanical hypersensitivity. The decreases of mechanical hypersensitivity induced by .3–1 μg of MK-801 were of the same magnitude as that induced by amygdaloid administration of 100 μg of glutamate (Fig. 5B).

The attempts to prevent the increase and decrease of mechanical hypersensitivity induced by administration of a low or high dose of glutamate, respectively, were performed using amygdaloid pretreatment with MK-801 at the dose of .1 μg that alone had no significant effect. Pretreatment of the CeA with MK-801 (.1 μg) completely reversed the increase of mechanical hypersensitivity induced by amygdaloid administration of a low (9 μg) dose of glutamate (Fig. 5C). However, pretreatment of the CeA with MK-801 (.1 μg) failed to attenuate the reduction of hypersensitivity induced by amygdaloid administration of a high (100 μg) dose of glutamate (Fig. 5D).

3.5. Serotonergic medullary raphe neurons in the relay of descending hyper- and antihypersensitivity effect

To determine whether serotonergic raphe neurons in the RVM are mediating the descending hypersensitivity- or/and the antihypersensitivity effect induced by amygdaloid glutamate, 8-OH-DPAT, a 5-HT_{1A} receptor agonist, was microinjected into the RVM. Administration of 8-OH-DPAT was expected to reduce firing in serotonergic neurons, through action on 5-HT_{1A} autoreceptors on their cell bodies [40]. RVM administration of 8-OH-DPAT at a dose of .125 μg reversed both the hypersensitivity effect induced by amygdaloid administration of 9 μg of glutamate (Fig. 6A) and the antihypersensitivity effect induced by amygdaloid administration of 100 μg of glutamate (Fig. 6B). RVM administration of 8-OH-DPAT alone at the dose of .125 g failed to have a significant effect on hypersensitivity (Fig. 6A and B).

3.6. Glutamate injection into a brainstem control injection site

In a separate group of SNI animals ($n = 5$), glutamate was microinjected into the right internal capsule. In the control injection site, glutamate at either the low (9 μg; Fig. 7A) or high (100 μg; Fig. 7B) dose had no significant influence on mechanical hypersensitivity when compared with a pooled vehicle group, but a

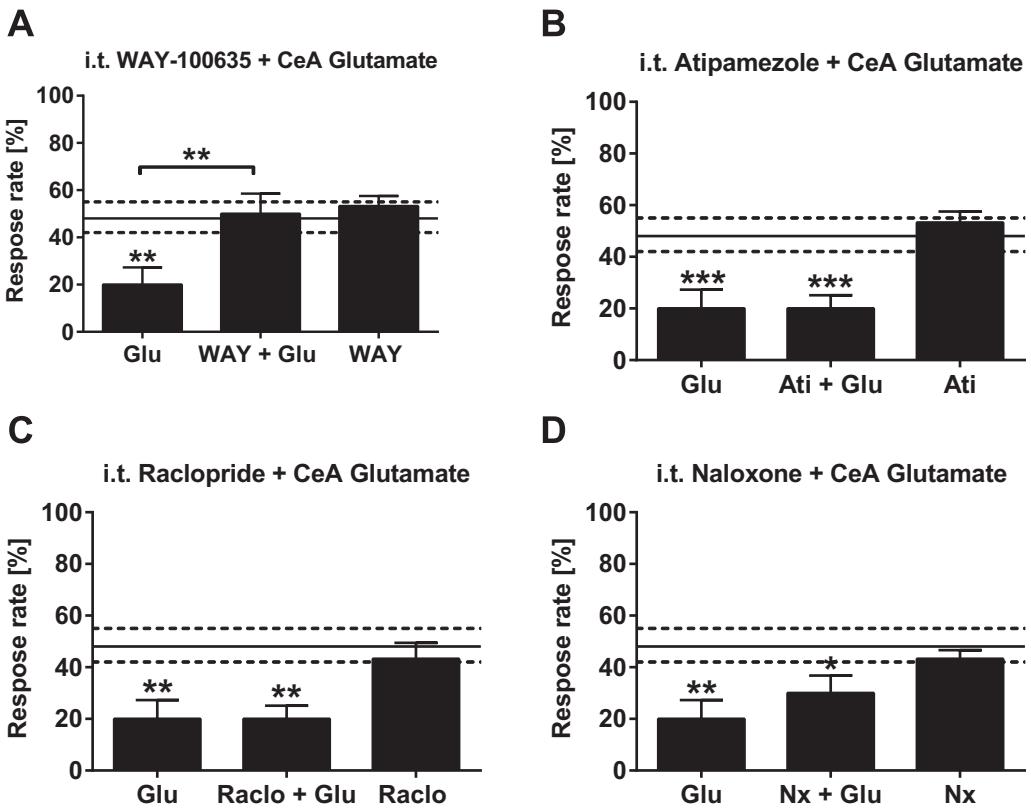


Fig. 4. Attempts to reverse the amygdaloid glutamate-induced antihypersensitivity effect by intrathecal pretreatments with selective receptor antagonists. (A) Blocking the spinal 5-HT_{1A} receptors with 3 μg of WAY-100635 (WAY). (B) Blocking the spinal α_2-adrenoceptors with 5 μg of atipamezole (Ati). (C) Blocking the spinal dopamine D2 receptors with 1 μg of raclopride (Raclo). (D) Blocking the spinal opioid receptors with 1 μg of naloxone (Nx). Main effects of drug treatment were significant in all conditions (graph A: $F_{3,26} = 6.97$, $P = .0014$; graph B: $F_{3,26} = 13.91$, $P < .0001$; graph C: $F_{3,26} = 9.63$, $P = .0002$; graph D: $F_{3,26} = 7.28$, $P = .0011$). *Y*-axis represents withdrawal response rate to repetitive mechanical stimulation of the hind paw in the nerve-injured limb. Glu, amygdaloid glutamate administration at the dose of 100 μg that was administered either alone (Glu) or following intrathecal pretreatment with a receptor antagonist (e.g., WAY + Glu). CeA, central nucleus of the amygdala. Error bars represent S.E.M. ($n = 6$ in each graph). In each graph, the horizontal lines represent the mean response and its 95% confidence limits in saline-treated animals (a pooled control group, $n = 12$). $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .005$ (Tukey's test; reference: unless specified, the corresponding value in the saline-treated group).

significantly weaker pro- or antinociceptive effect than the corresponding glutamate dose in the CeA.

4. Discussion

4.1. Descending regulation of neuropathic hypersensitivity by amygdaloid glutamate: role of the serotonergic system

The present results indicate that glutamate administration into the CeA produces bidirectional modulation of neuropathic hypersensitivity. At a low dose, amygdaloid glutamate administration increased hypersensitivity whereas at higher doses it reduced hypersensitivity. The CeA, a main output nucleus of the amygdala [22,27], projects to the midbrain periaqueductal gray that has descending projections to the spinal dorsal horn through a relay in the RVM [35]. A subpopulation of the RVM neurons is serotonergic and provides the serotonergic innervation of the spinal cord; serotonergic neurons with spinal projections have been demonstrated in various subareas of the RVM including the nucleus paragigantocellularis, nucleus gigantocellularis as well as raphe magnus [20]. In the present study, RVM administration of a 5-HT_{1A} receptor agonist that was expected to hyperpolarize serotonergic cell bodies in the medullary injection area ([40]; however [17]) reversed both the increase and decrease of neuropathic hypersensitivity induced by amygdaloid glutamate treatment. This finding suggests that serotonergic neurons in the RVM were relaying both the descending facilitatory and inhibitory effects induced by amygdaloid glutamate treatment.

At the spinal cord level, blocking the 5-HT_3 receptor reversed the increase in hypersensitivity induced by amygdaloid administration of a low dose of glutamate. This finding suggests that the spinal 5-HT_3 receptor mediated the CeA-induced increase of neuropathic hypersensitivity. This is in line with some earlier results in experimental animals indicating that the spinal 5-HT_3 receptor has a pronociceptive role in neuropathy [9,28,46] as well as in some other chronic pain conditions [13,45]. However, there is also evidence suggesting that the spinal 5-HT_3 receptor may decrease rather than increase pain behavior (see for review [25]), which discrepancy may be due to complexity and plasticity of the circuitry involving the spinal 5-HT_3 receptor [14] as well as other differences in experimental conditions.

Blocking the spinal 5-HT_{1A} receptor reversed the antihypersensitivity effect induced by amygdaloid administration of a high dose of glutamate suggesting that the spinal 5-HT_{1A} receptor was involved in mediating the CeA-induced decrease in hypersensitivity. This is in line with earlier studies showing that the spinal 5-HT_{1A} receptor suppresses pain-related responses in various experimental conditions [10,50], including peripheral neuropathy [49].

It still remains to be studied which mechanism explains that amygdaloid treatment with a low and high dose of glutamate produced activation of medullospinal serotonergic pathways acting predominantly on the spinal 5-HT_3 versus 5-HT_{1A} receptor, respectively. Among potential explanations is that low and high glutamate doses in the CeA led to activation of two different populations of amygdaloid glutamate receptors and neurons, which may have led to activation of two different populations of

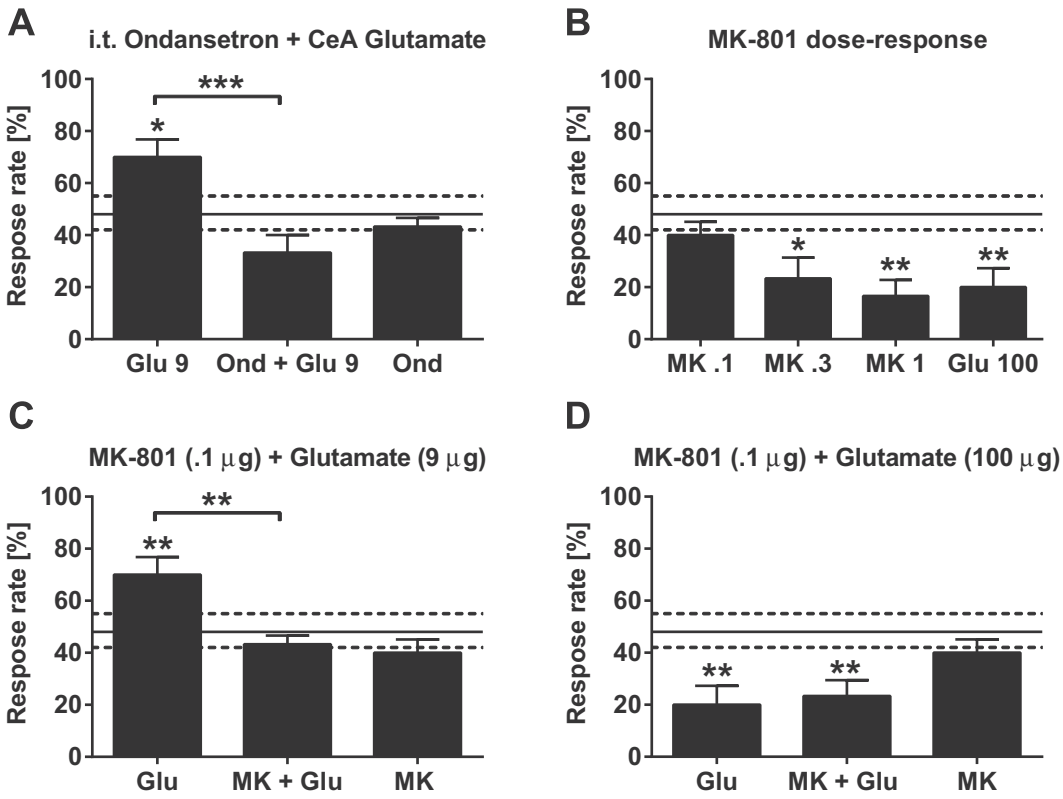


Fig. 5. (A) Attempt to reverse the amygdaloid glutamate-induced exacerbation of hypersensitivity by intrathecal pretreatment with 5 μg of ondansetron (Ond), a 5-HT_3 receptor antagonist. (B) Dose-related attenuation of hypersensitivity induced by amygdaloid administration of MK-801 (MK), an NMDA receptor antagonist, and a comparison with the antihypersensitivity effect induced by amygdaloid administration of a high dose of glutamate alone. (C) Attempt to reverse the low (9 μg) glutamate dose-induced increase of hypersensitivity by amygdaloid pretreatment with .1 μg of MK-801. (D) Attempt to reverse the high (100 μg) glutamate dose-induced decrease of hypersensitivity by amygdaloid pretreatment with .1 μg of MK-801. Main effects of drug treatments were significant in all conditions (A: $F_{3,26} = 8.83$, $P = .0003$; B: $F_{4,31} = 7.35$, $P = .0003$; C: $F_{3,26} = 7.73$, $P = .0008$; D: $F_{3,26} = 8.15$, $P = .0005$). *Y*-axis represents withdrawal response rate to repetitive mechanical stimulation of the hind paw in the nerve-injured limb. Glu 9 and 100, amygdaloid glutamate administration at the dose of 9 or 100 μg. In C and D, Glu represents amygdaloid glutamate administration at the dose of 100 μg. CeA, central nucleus of the amygdala. Error bars represent S.E.M. ($n = 6$ in each graph). In each graph, the horizontal lines represent the mean response and its 95% confidence limits in saline-treated animals (a pooled control group: $n = 12$). $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .005$ (Tukey's test; reference: unless specified, the corresponding value in the saline-treated group).

serotonergic neurons in the medullary raphe. In line with this, it has been shown that the pain regulatory effect may vary with amygdaloid glutamate receptor subtype from pro- to antinociception [30,31]. Alternatively, with an increase of the activity in raphe-spinal neurons from low to high level, the net spinal effect changed from the 5-HT_3 receptor-mediated pronociception to the 5-HT_{1A} receptor-mediated antinociception. Interestingly, an earlier study demonstrated that the descending influence of glutamate administration in the RVM was changed from pronociceptive to antinociceptive with an increase of the glutamate dose [51], which finding resembles the present finding on the dose-related effect of glutamate in the CeA. It should also be noted that mechanism underlying the antinociceptive action induced by midbrain injections of morphine varied with the morphine dose [3].

Regarding non-serotonergic pain control systems, we tested whether the noradrenergic and dopaminergic ones or the spinal opioidergic system contributed to the decrease of pain hypersensitivity induced by amygdaloid glutamate treatment. Blocking the spinal α_2-adrenoceptors, dopamine D2 receptors, or opioid receptors failed to reduce the decrease of neuropathic hypersensitivity induced by amygdaloid glutamate treatment. This finding indicates that while the descending pathways acting on spinal noradrenergic, dopaminergic and opioidergic receptors are known to be involved in the control of pain [25,34,35], none of them has a major role in the descending antihypersensitivity effect induced by amygdaloid glutamate.

4.2. Phasic versus tonic regulation of hypersensitivity by the amygdala and the role of amygdaloid NMDA receptors

Pretreatment of the amygdala with a low dose of an NMDA receptor antagonist alone had no significant effect on hypersensitivity, but it reversed the increase of hypersensitivity induced by a low dose of glutamate, without influencing the decrease of hypersensitivity induced by a high dose of glutamate. This finding suggests that the amygdaloid NMDA receptor might contribute to the glutamate-induced increase but not decrease of neuropathic hypersensitivity.

The increase and decrease of neuropathic hypersensitivity by amygdaloid glutamate treatment represent phasic modulation of the sensory signal. In the present as in our earlier study [2] blocking the NMDA receptor in the CeA of neuropathic animals, unlike in healthy controls [6], reduced limb withdrawal responses. This finding suggests that endogenous glutamate release, acting on the amygdaloid NMDA receptor, has a tonic pronociceptive action in peripheral neuropathy. However, it should be emphasized that there is abundant evidence indicating that metabotropic glutamate receptors in the amygdala that were not studied in the present experiment exert an important role in regulation of pain behavior (e.g., [1,18,23,30,31]). On the other hand, in line with present findings, there is also evidence indicating that amygdaloid NMDA receptors contribute to regulation of pain behavior (e.g., [2,43]).

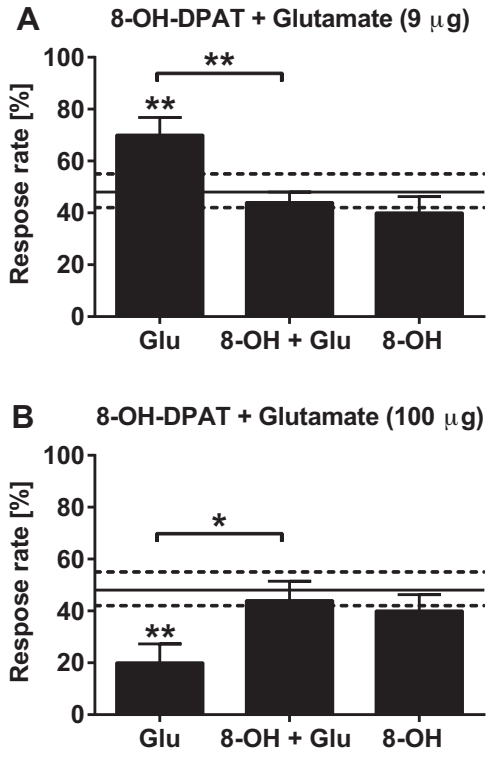


Fig. 6. Attempts to influence the amygdaloid glutamate-induced modulation of hypersensitivity by medullary administration of .125 μg of 8-OH-DPAT (8-OH), a 5-HT_{1A} receptor agonist that presumably autoinhibits serotonergic raphe neurons. (A) Attempt to reduce the increase of hypersensitivity induced by 9 μg of glutamate. (B) Attempt to reduce the decrease of hypersensitivity induced by 100 μg of glutamate. In both conditions, main effect of drug was significant (A: $F_{3,24} = 6.73$, $P = .0019$; B: $F_{3,24} = 5.66$, $P = .0045$). *Y*-axis represents withdrawal response rate to repetitive mechanical stimulation of the hind paw in the nerve-injured limb. Glu 9 and 100, amygdaloid glutamate administration at the dose of 9 or 100 μg. Error bars represent S.E.M. ($n = 5$–6 in each graph). In each graph, the horizontal lines represent the mean response and its 95% confidence limits in saline-treated animals (a pooled control group, $n = 12$). $^{*}P < .05$, $^{**}P < .01$ (Tukey's test; reference: unless specified, the corresponding value in the saline-treated group).

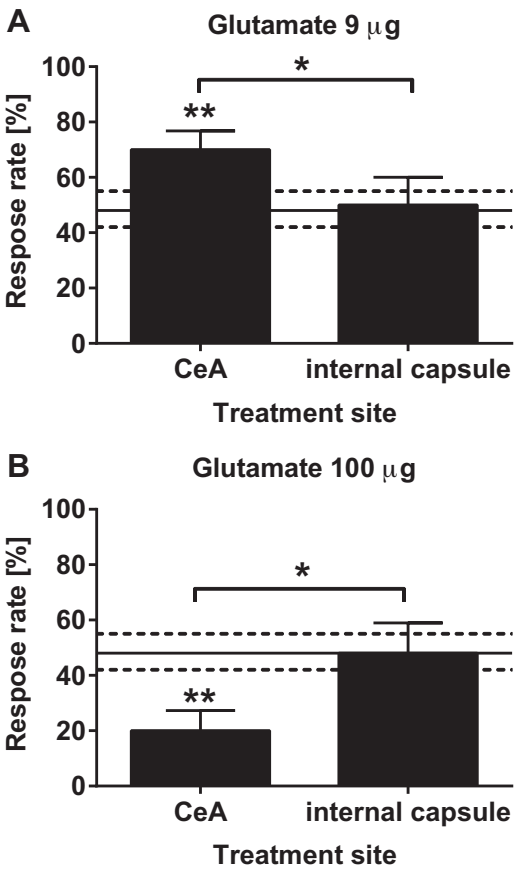


Fig. 7. Glutamate at a low (A) or high (B) dose in a control injection site (internal capsule) failed to produce a change in mechanical hypersensitivity. In both experimental conditions, the main effect of drug treatment was significant (A: $F_{2,20} = 6.74$, $P = .0058$; B: $F_{2,20} = 10.89$, $P = .0006$). *Y*-axis represents withdrawal response rate to repetitive mechanical stimulation of the hind paw in the nerve-injured limb. CeA, central nucleus of the amygdala. Error bars represent S.E.M. ($n = 6$ in the CeA groups and $n = 5$ in the internal capsule groups). In each graph, the horizontal lines represent the mean response and its 95% confidence limits in saline-treated animals (a pooled control group, $n = 12$). $^{*}P < .05$, $^{**}P < .01$, $^{***}P < 005$ (Tukey's test; reference: unless specified, the corresponding value in the saline-treated control group).

Interestingly, spinal administration of ondansetron, a 5-HT_3 receptor antagonist, at the dose that reversed the increase of hypersensitivity induced by amygdaloid administration of a low dose of glutamate failed to reduce baseline hypersensitivity in neuropathic animals. This result, which is in line with the failure to influence baseline mechanical or thermal hypersensitivity by i.t. administration of ondansetron in the spinal nerve ligation-induced model of peripheral neuropathy [38], raises the possibility that a mechanism other than the descending serotonergic system acting on the 5-HT_3 may contribute to the mediation of the tonic pronociceptive action induced by endogenous release of glutamate in the amygdala. However, there are also reports indicating that blocking the spinal 5-HT_3 receptor with ondansetron has decreased baseline pain behavior [9] or baseline responses of nociceptive spinal dorsal neurons [46] in neuropathic animals indicating that at least in some experimental conditions, the spinal 5-HT_3 receptor has a tonic pronociceptive effect in neuropathy.

4.3. Limitations

In the present study, the focus was on mechanical hypersensitivity assessed by spinally organized withdrawal reflex responses evoked by monofilament stimulation of the nerve-injured limb. This index of pain hypersensitivity represents sensory-discriminative rather than affective-motivational (or emotional) component of pain. Therefore, we cannot exclude the possibility that the effect of amygdaloid glutamate treatment on affective-like pain, assessed e.g. by aversive place-conditioning [21] or vocalization responses (e.g., [23,30,42]) might have been at least partly different as the present results on sensory aspects of neuropathic pain. While amygdala plays an important role in regulation of supraspinally-organized affective-like pain behavior, present and previous results indicate that amygdala may also contribute to descending control of pain-related sensory signals both in physiological [6,19,29] and pathophysiological conditions [2,18].

Glutamate was administered into the CeA at the volume of 0.5 μl, which injection volume is expected to spread about 1 mm [26], thereby covering also other subnuclei of the amygdala. Therefore, we cannot exclude the contribution of other amygdaloid subnuclei to the present findings. However, since the CeA is the main output nucleus of the amygdala providing efferent projections to the brainstem areas involved in descending pain control [22,27], it may be argued that the CeA was involved in the descending regulation of hypersensitivity induced by amygdaloid glutamate treatments in the present study. In line with this, a high or a low dose of glutamate in the present control injection site, the right internal capsule, did not influence neuropathic hypersensitivity as glutamate in the CeA did, nor did amygdaloid glutamate administration at the currently used high dose influence motor behavior of control animals in our previous study [6].

It might be argued that the reversal of the amygdaloid glutamate-induced descending effect by RVM administration of a 5-HT_{1A} receptor agonist were due to secondary effects on 5-HT_{1A} heteroreceptors expressed by non-serotonergic neurons rather than autoinhibition of 5-HT neurons in the RVM. However, together the findings that RVM administration of 5-HT_{1A} receptor agonist as well as i.t. administration of selective 5-HT receptor antagonists reversed the amygdaloid glutamate-induced effects support the proposal that serotonergic rather than non-serotonergic RVM neurons were relaying the amygdaloid glutamate-induced effects to the spinal dorsal horn.

In this study, the focus was on subtypes 1A and 3 of the spinal 5-HT receptor. There are also other subtypes of the 5-HT receptor that have been shown to play a role in regulation of pain-related signals in the spinal dorsal horn [25]. It remains to be studied whether subtypes other than 1A and 3 of the spinal 5-HT receptor might contribute to the amygdala-induced descending modulation of pain hypersensitivity.

4.4. Conclusions and potential implications

The present results indicate that the CeA can modulate neuropathic symptoms in a bidirectional fashion varying from exacerbation to suppression of mechanical hypersensitivity. The CeA-induced increase of hypersensitivity is mediated by medullospinal serotonergic pathways acting on the spinal 5-HT_3 receptor whereas the CeA-induced antihypersensitivity effect is mediated by medullospinal serotonergic pathways acting on the spinal 5-HT_{1A} receptor.

Chronic neuropathic pain is associated with affective and cognitive disorders and vice versa, affect and cognition may influence neuropathic pain [24]. Since the amygdala has a key role in processing of affects and it also processes and regulates pain, it is among potential neural links between comorbidity of neuropathic pain and affective disorders [4]. It may be hypothesized that the CeA-induced regulation of neuropathic hypersensitivity by the descending serotonergic system provides a plausible relay mechanism for bidirectional affective modulation of pain sensitivity [39].

Conflicts of interest

The authors declare that they are not aware of any conflicts of interest concerning this study.

Acknowledgements

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Involvement of the periaqueductal gray in the descending antinociceptive effect induced by the central nucleus of amygdala

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Abstract

Among many functions of the central nucleus of the amygdala (CeA) is processing and control of nociception. Here we studied whether descending control of mechanical nociception by glutamate in the CeA of healthy control animals is induced by amygdaloid NMDA receptors and relayed through the midbrain periaqueductal gray (PAG). Mechanical nociception in the hind paws was assessed by calibrated monofilaments in rats with a chronic guide cannula for glutamate administration in the right CeA and another one for inducing local anesthesia in the PAG. In a separate electrophysiological study, ON-like PAG neurons giving an excitatory response to noxious pinch of the tail were recorded in anesthetized rats following administration of glutamate into the CeA. A high dose of glutamate (100 µg) in the CeA induced mechanical antinociception in the contra- but not ipsilateral hind limb. Antinociception was reversed by pretreatment of the CeA with an NMDA receptor antagonist or by local anesthesia of the PAG. Discharge rate of ON-like PAG neurons was increased by a high dose of glutamate (100 µg) in the CeA and this increase was reversed by pretreatment of the CeA with an NMDA receptor antagonist. The results indicate that a high dose of glutamate acting on NMDA receptors in the CeA of healthy animals induces contralaterally mechanical antinociception through a circuitry relaying in the PAG where activation of ON-like neurons is associated with the descending antinociceptive effect.

Introduction

Central nucleus of the amygdala (CeA), the main output nucleus of the amygdala, is involved in processing of primary emotions, such as fear[1], but among its many functions is processing and regulation of pain- and nociception-related responses[2]. Concerning descending regulation of nociception, CeA has efferent projections to brainstem structures involved in descending control of nociception. Among them are the midbrain periaqueductal gray (PAG)[3,4] and the pontine locus coeruleus (LC)[5]. It has been reported that attenuation of spinal heat nociception induced by electric stimulation of the CeA was abolished following local anesthesia of the PAG[6] indicating that the CeA projection to the PAG rather than e.g. the LC relayed the descending antinociceptive effect. Descending control mechanisms of spinal nociception may vary with submodality of test stimulation as indicated by the finding that transection of the spinal cord had opposite effects on mechanical and heat nociception[7]. Therefore, the earlier finding that blocking the PAG abolished the CeA-induced heat antinociception[6] does not exclude the possibility that the CeA-induced descending control of mechanical nociception is relayed by brainstem structures other than the PAG.

Recently we showed that a high dose of glutamate in the CeA suppresses mechanical nociception in the contralateral limb and that this descending antinociceptive effect was reversed by pretreatment of the CeA with an NMDA receptor antagonist[8]. In the present study, we determined whether this amygdaloid NMDA receptor-induced mechanical antinociceptive effect is relayed by the PAG. Therefore, we assessed whether local anesthesia of the PAG abolishes the mechanical antinociceptive effect induced by NMDA receptor-mediated action of glutamate the CeA. Moreover, the response of nociceptive PAG neurons to amygdaloid glutamate was determined to have a neuronal correlate of a possible relay mechanism.

Methods

Animals

Adult male Hannover-Wistar rats (Harlan, Horst, the Netherlands), weighing 200 to 350 g, were used to perform the experiments. The Experimental Animal Ethics Committee of the Provincial Government of Southern Finland (Hämeenlinna, Finland) approved the study protocol, and the experiments were performed according to the guidelines of European Communities Council Directive of 22 September 2010 (2010/63/EU). All efforts were made to limit distress and to use only the number of animals necessary to produce reliable scientific data. Rats were housed in a 12-hour light/dark cycle with food and water access *ad libitum*.

Guide cannula insertion and drug injection procedure

The animals had a guide cannula for drug administration into the right CeA as described in detail earlier[9]. The right CeA was chosen as a target in this study, since previous experimental animals studies indicate that the right CeA has a more important role in pain processing than the left CeA[10-12], although not in all conditions[13]. For placement of the guide cannulae (26 gauge; PlasticsOne, Roanoke, VA, USA), the animal was placed into a standard stereotaxic frame, skull was exposed and two holes drilled for their placement under pentobarbitone anesthesia (60 ml/kg i.p.). One of the guide cannulae was placed in the right CeA: 6.8 mm anterior from the interaural line, 4.2 mm lateral from the midline, and 7.9 mm ventral from the dura mater[14]. The other guide cannula was placed into the PAG: 2.0 mm anterior from the interaural line, 0.8 mm lateral from the midline, and 5-6 mm ventral from the dura mater. The guide cannulae were kept in place with dental cement and two dental screws inserted in the skull bone. For treatment of postoperative pain, the animals were given buprenorphine at a dose of 0.01 mg/kg intramuscularly starting during the operation and continuing twice daily for three days postoperatively. The rats had one week time to recover from the operation before starting the behavioral experiment.

Intracerebral injections of drugs or saline were performed at the volume of 0.5µl by using 30-gauge injection (PlasticsOne) needle connected to a 10-µl Hamilton microsyringe (Hamilton Company, Bonaduz, Switzerland) by polyethylene (PE-20) tubing. The injection needle protruded 2.0 mm beyond the cannula tips. The animals were gently restrained during the injection procedure. The injection lasted 20 s after which the injection needle was kept in place for another 20 s to maximize diffusion and to prevent back flow of the drug into the cannula.

Assessment of limb-withdrawal threshold to mechanical stimulation

Rats were habituated three days to the experimental conditions. For assessment of mechanical nociception, the limb withdrawal threshold was determined by mechanical stimulation of the right and left hind paw with calibrated monofilaments producing forces that varied from 0.008 g to 300 g. The experimental test consists in five consecutive stimulations of the foot pads per mono filaments, the lowest filament force producing 100% withdrawal response from the paw was considered as the withdrawal threshold.

Course of the behavioral study

Experiments were performed 1 to 2 weeks after cannula insertion. Mechanical nociception was tested in the following drug conditions: Saline or lidocaine (4%) in the PAG followed 5 minutes later by one of the following treatments of the CeA: saline alone, glutamate alone at the dose of 100µg, MK-801 alone at the dose of 2µg, or MK-801 at the dose of 2µg followed 5 minutes later by glutamate at the dose of 100µg. The order of testing these different drug treatment conditions was randomized using QuickCalcs software (www.graphpad.com).

Withdrawal threshold determination started 1 min following completion of the drug treatment procedure. The minimum interval in testing different drug conditions in the same animal was 1 day.

Electrophysiological Study

Electrophysiological recording was done under sodium pentobarbitone anesthesia at a dose of 60 mg/kg i.p. for the induction of anesthesia, then followed by administering 15-20 mg/kg/h or more, when required to maintain rats under stable anesthesia. The anesthetized animals were placed in a standard stereotaxic frame. The skull was exposed and two holes were drilled in the skull: one for the recording electrode to be inserted into the PAG (2 mm anterior from the interaural line, 0.8 mm lateral from the midline, 4.6-6.2 mm ventral from the dura mater) and the other hole for the guide cannula insertion into the CeA (6.8 mm anterior from the interaural line, 4.2 mm lateral from the midline, 7.9 mm ventral from the dura mater) according to the coordinates of the rat stereotaxic atlas of Paxinos and Watson[14].

The level of the anesthesia was frequently assessed by pinching the paws and ears, and supplemental doses of the anesthetic were given as needed. While the level of anesthesia may vary in spite of attempts to maintain anesthesia at a constant level, it is important to note that anesthesia was maintained in an identical manner in all experimental conditions. This was expected to minimize the contribution of the potential differences in anesthesia level to the differences in results between the experimental conditions within this study. The rats were spontaneously breathing. A warming blanket was used to maintain body temperature within physiological range.

A lacquer-coated tungsten electrode (tip impedance 3-10 MΩ at 1 kHz) was used to record extracellularly single neuron activity which was amplified and filtered using standard methods. A computer connected to a CED Micro 1401 interface and using Spike 2 software (Cambridge Electronic Design, Cambridge, U.K.) was used to perform the data sampling. Neurons were classified according their response to a noxious pinch of the tail with a hemostatic clamp that produced pain when applied to the experimenter's hand. Only neurons with an excitatory response to noxious pinch were

studied further. An earlier study described a neuron type called ON-cell in the PAG that gave an excitatory response to noxious heat prior to the heat-evoked spinal withdrawal reflex[15]. The PAG neurons recorded in the present study resembled those classified as PAG ON-cells the earlier study[15]. However, since in the present study withdrawal reflex was not assessed in parallel with the neuronal response and since the submodality of noxious test stimulation was different (pinch, instead of heat), the neurons recorded in the present study were called ON-like neurons.

Course of the electrophysiological study

After finding a PAG neuron that gave an excitatory response to noxious pinch of the tail, its spontaneous activity was recorded for at least one min before drug treatments. One of the drug treatment conditions was administration of saline followed 5 min later by glutamate at the dose of 100 µg. The other drug treatment condition was administration of MK-801 at the dose of 2 µg followed 5 min later by administration of glutamate at the dose of 100 µg. Neuronal discharge was recorded up to 15 min after drug administrations. The interval between testing the different drug treatment conditions was at least 60 min. The order of testing different drug treatment conditions was varied randomly.

Drugs

Glutamate and MK-801 (an NMDA receptor antagonist) were purchased from Sigma-Aldrich (St.Louis, MO, USA) and lidocaine (4%) from Astra-Zeneca (Södertälje, Sweden). Physiological saline (OrionPharma, Espoo, Finland) was used for control injections.

Histology

After completion of the experiments, rats were sacrificed by giving them a lethal dose of sodium pentobarbitone and the brain was immediately removed and placed in 10% formaldehyde. Coronal sections of the brain were made to verify recording and injection sites in the brain. A schematic representation of the sites of cannula placements in the right CeA as well as the cannula and recording electrode placements in the PAG are shown in Fig. 1.

Statistical Analyses

Behavioral data were analyzed using non-parametric Friedman's test followed by Dunn's test or when comparing two conditions, Wilcoxon's test. Neuronal data were analyzed using repeated-measures one-way ANOVA followed by Tukey's test. Statistical analyses were performed with the GraphPad Prism 5 software (GraphPad Inc., La Jolla, CA, USA). $P<0.05$ was considered to represent a significant difference.

Results

Behavioral study

Spinal antinociception induced by glutamate in the right CeA is prevented by blocking the PAG.

Drug treatments of the right CeA and PAG had a significant effect on withdrawal threshold in the contralateral hind limb ($Fr=14.86$, $P>0.0001$; Fig. 2A). Following pretreatment of the right PAG with saline, a high dose of glutamate (100µg) in the right CeA induced an increase of the mechanical withdrawal threshold in the contralateral hind limb (Fig. 2A), but failed to influence the threshold of the ipsilateral hind limb (Fig. 2B).

Pretreatment of the PAG with a local anesthetic (lidocaine 4%) prevented the increase of the mechanically evoked withdrawal threshold in the contralateral limb induced by a high dose of glutamate (100µg) in the right CeA (Fig. 2C). After local anesthesia of the PAG, the contralateral withdrawal threshold was not different between animals receiving saline or 100 µg of glutamate in the right CeA (Fig. 2C). None of the three drug administration conditions influenced the threshold of the ipsilateral hind limb ($Fr=3.391$, $P=0.1495$; Fig. 2D).

Following pretreatment of the right CeA with 2 µg of MK-801 (an NMDA receptor antagonist), 100 µg of glutamate in the CeA failed to induce an increase of the hind limb withdrawal threshold in the contralateral (Fig. 2E) or the ipsilateral (Fig. 2F) hind limb (Fig. 2E). Following treatment of the right CeA with 2 µg of MK-801 alone, the contralateral (Fig. 2E) and ipsilateral (Fig. 2F) hind limb withdrawal thresholds were within the interquartile range of the corresponding median threshold in saline-treated animals (Fig. 2E).

Electrophysiological study

Glutamate acting on NMDA receptors in the right CeA increases discharge of ON-like neurons in the PAG

Figure 3A shows an example of the response of an ON-like neuron of the PAG to drug treatments of the right CeA: Saline failed to influence the discharge rate of the PAG neuron, whereas 100 µg of glutamate induced a short-lasting increase of the ongoing discharge rate that was prevented by pretreatment of the CA with MK-801 at a dose of 2 µg that alone failed to influence the discharge rate of the PAG neuron. Over all studied neurons, drug treatments of the right CeA significantly influenced the discharge rate of ON-like neurons ($F_{1,5}$=8.95, P=0.0277; Fig.3B). *Post hoc* tests indicated that when compared with the effect of saline, the increase of the glutamate-induced increase of the discharge rate was significant, except in the condition in which CeA was pretreated with 2 µg of MK-801 before administration of glutamate (Fig. 3B).

Discussion

It has been shown earlier that heat antinociception induced by electrical stimulation of the CeA is relayed downwards through the PAG[6]. The present results extend this earlier finding by showing that the mechanical antinociceptive effect induced by a high dose of glutamate in the CeA is also relayed downwards through the PAG, as shown by reversal of the antinociceptive effect following local anesthesia of the PAG. Antinociceptive effect induced by glutamate in the CeA in behavioural experiments was accompanied by an increase in the discharge rate of ON-like PAG neurons giving an excitatory response to noxious pinch of the tail. Both the CeA-induced behavioural antinociceptive effect and the accompanying increase of the discharge rate of ON-like PAG cells were reversed by blocking amygdaloid NMDA receptors. Together, these findings suggest that amygdaloid NMDA receptors and ON-like PAG neurons are involved in the descending mechanical antinociceptive effect induced by a high dose of glutamate in the CeA. Interestingly, while activation of ON cells in the rostroventromedial medulla (RVM), an important relay between the PAG and the spinal dorsal horn, has been associated with pro- rather than antinociceptive actions[16], the present results suggest that activation of ON-like cells in the PAG is associated with mechanical antinociception. It remains to be studied whether descending projections of ON-like PAG cells drive antinociceptive OFF-cells and/or suppress pronociceptive ON-cells in the RVM.

It should be noted that the present study was performed in healthy control animals. In neuropathic animals, unlike in healthy controls, blocking the amygdaloid NMDA receptors with a dose of MK-801 that alone had no effect on spinal nociception failed to reverse the descending antinociceptive effect

induced by a high dose of glutamate in the CeA[17]. However, MK-801 at a dose producing no change in pain behaviour of neuropathic animals when administered alone reversed the pronociceptive effect induced by a low dose of glutamate in the CeA[17]. This finding suggests that the role of amygdaloid NMDA receptors in descending control of nociception in healthy controls differs from that in neuropathy. On the other hand, the dose of MK-801 that alone was ineffective in neuropathic animals was an order of magnitude lower than the corresponding dose in healthy controls (0.1 μg[17] versus 2.0 μg). Therefore, we cannot exclude the possibility that blocking the amygdaloid NMDA receptors with a high dose of MK-801 had reduced the descending antinociceptive effect induced by a high dose of glutamate in neuropathic animals of our previous study as well as in control animals of the present study.

The role of amygdaloid NMDA receptors in pain processing may vary depending on whether it is involved in mediating ascending nociceptive signals or descending controls and on the pathophysiological condition. This is indicated by the present finding that amygdaloid NMDA receptors induced a descending antinociceptive effect in healthy controls of the present study while earlier studies indicate that the descending pronociceptive effect induced by the CeA of neuropathic animals was at least partly mediated by amygdaloid NMDA receptors[17,18] and that peripheral neuropathy-induced synaptic plasticity leading to amplification of ascending nociceptive signals to the CeA was partly dependent on amygdaloid NMDA receptors in arthritic animals[19] but independent of amygdaloid NMDA receptors in neuropathic animals[20]. Moreover, it should be noted that the present study focused on nociceptive thresholds which represents sensory-discriminative aspects of pain. Amygdala, its NMDA receptors and projections to the PAG have been shown to contribute also to the control of pain affect[21].

Conclusions

The circuitry mediating the mechanical antinociceptive effect induced by a high dose of glutamate in the CeA of healthy rats involves amygdaloid NMDA receptors and activation of ON-like cells in the midbrain PAG.

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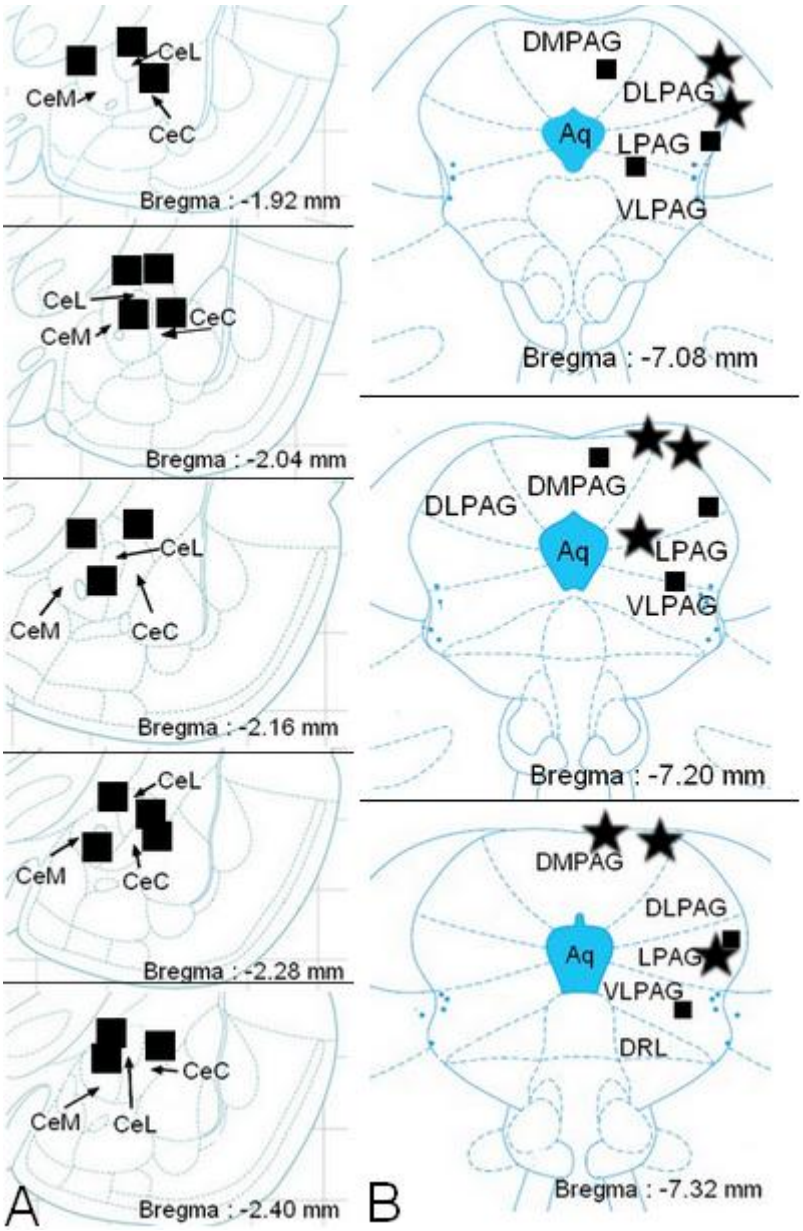


Fig. 1A: Schematic representation of the sites of cannula placements (black squares) in the right central nucleus of amygdala. CeC = Capsular nucleus of the central amygdala; CeM = Medial division of the central nucleus of amygdala; CeL = Lateral division of the central nucleus of amygdala.

Fig. 1B: Verified recording electrode sites (black squares) and injection cannula sites (black stars) in the periaqueductal gray. Each black square represents one electrode which has recorded between 1 to 3 neurones. Aq = aqueduct; DMPAG = Dorsomedial periaqueductal gray; DLPAG = Dorsolateral periaqueductal gray; LPAG= Lateral periaqueductal gray; VLPAG = Ventrolateral periaqueductal gray; DRL = Lateral part of the dorsal raphe nucleus.

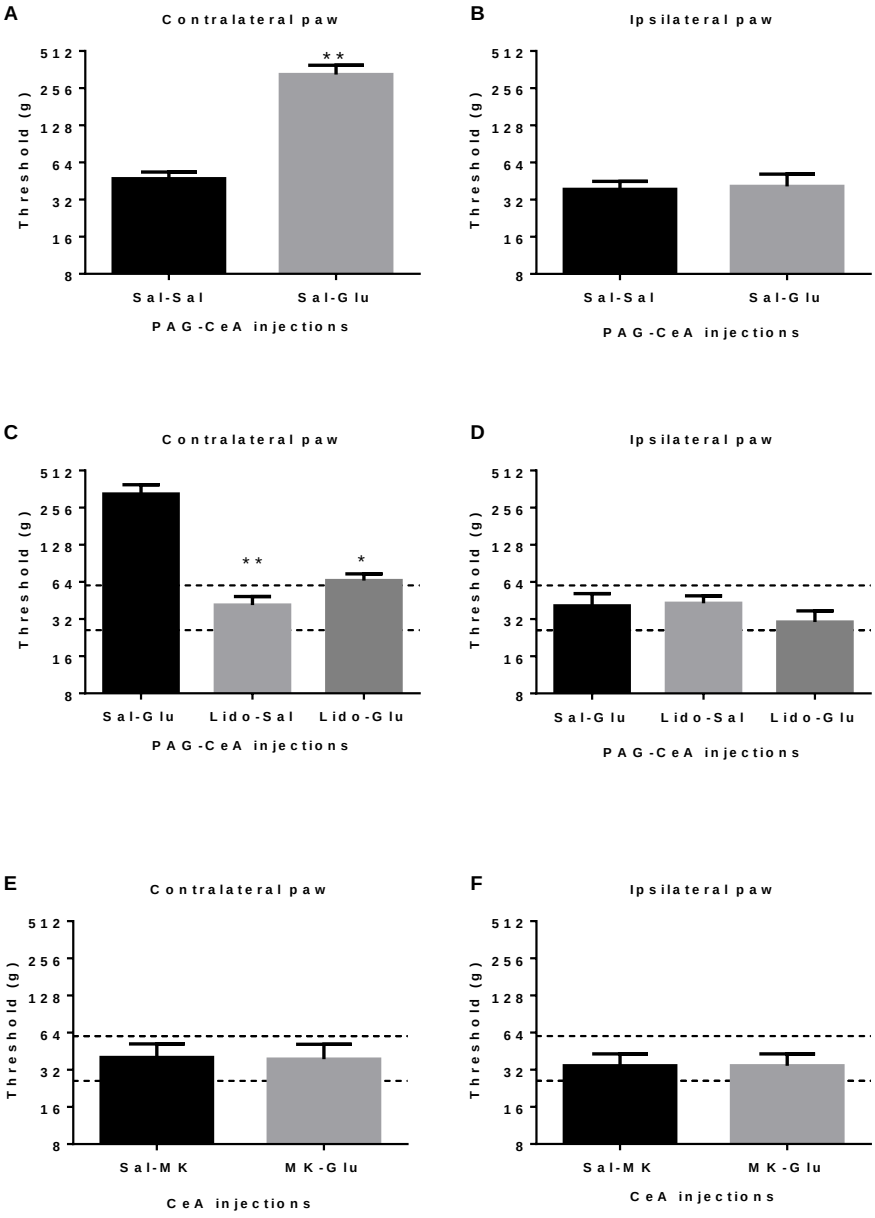


Fig. 2: Mechanical withdrawal thresholds in the hind limb contralateral (A, C, E) and ipsilateral (B, D, E) to the right central nucleus of the amygdala (CeA) and periaqueductal gray (PAG) into which drugs were microinjected. Treatment conditions were saline (Sal) in the PAG followed by saline or glutamate (Glu, 100 µg) in the CeA (A and B), saline or lidocaine (Lido, 4%) in the PAG followed by saline or glutamate in the CeA (C and D), and saline or MK-801 (MK, 2 µg) in the CeA followed saline or glutamate in the CeA (E and F). Broken horizontal lines represent the interquartile range of the median threshold in the corresponding saline-treated control group: Error bars represent S.E.M. (n=8 in graphs A-D, and n=4 in graphs E and F) *P<0.05, **P<0.01 (re: the Sal-Glu group; Willcoxon's test in A, and Dunn's test in C).

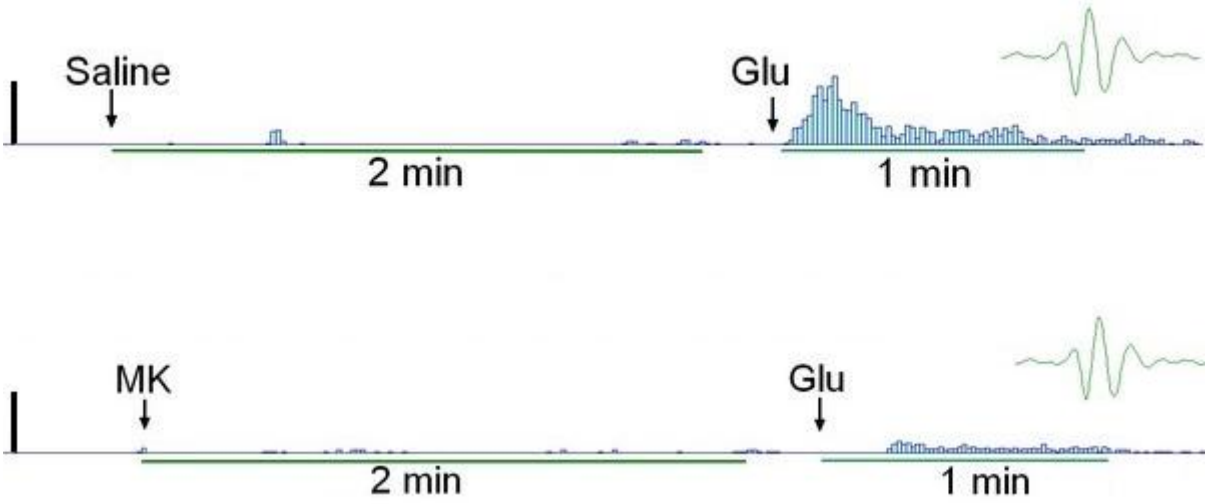


Fig. 3A: Examples of the response of a single ON-like neuron in the periaqueductal gray (PAG) following microinjections of drugs into the right central nucleus of the amygdala (CeA). Upper row shows that saline failed to influence the discharge rate whereas 100 µg of glutamate induced a short-lasting increase in the discharge rate. Lower row shows that MK-801 at the dose of 2 µg failed to influence the discharge rate, while it prevented the glutamate-induced increase of the discharge rate. The insets show the shape of the action potential in the studied PAG neuron and the vertical calibration bars represent 50 Hz.

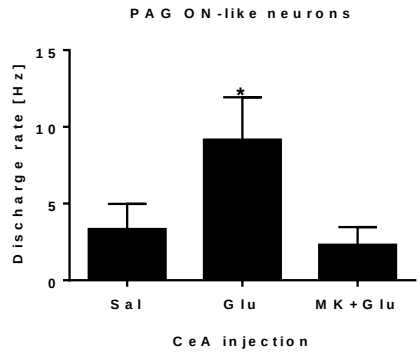


Fig. 3B: Mean responses of the ON-like PAG neurons to microinjections of drugs into the right CeA. Sal, saline; Glu, glutamate (100 µg); MK+Glu, MK-801 (2 µg) followed 2 min later by 100 µg of glutamate. Error bars represent S.E.M. (n=6). *P<0.05 (reference: the saline group; Tukey's test).