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.

*Shared first authorship

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).

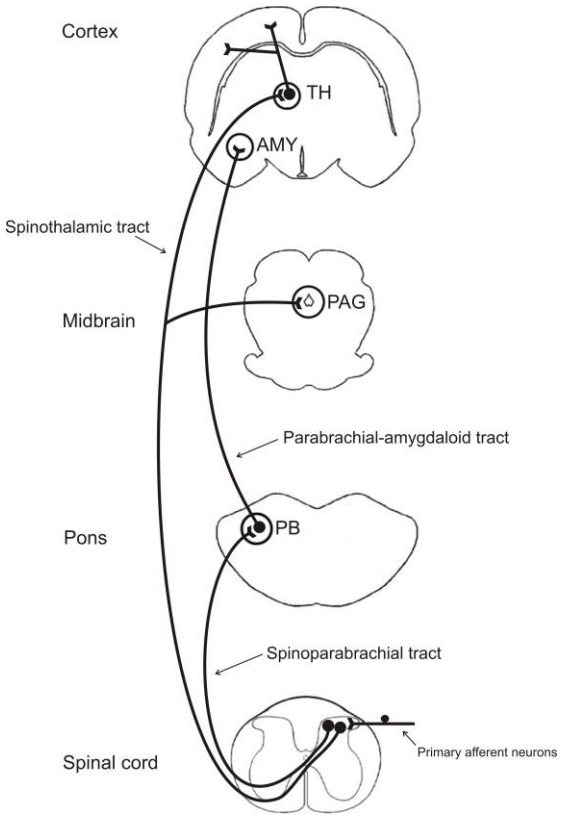

Fig. 1: Schematic representation of the spinothalamic and the spino-parabrachial-amygdaloid ascending pathways. TH = thalamus, AMY = amygdala, PAG = periaqueductal grey, PB = parabrachial nucleus.

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.

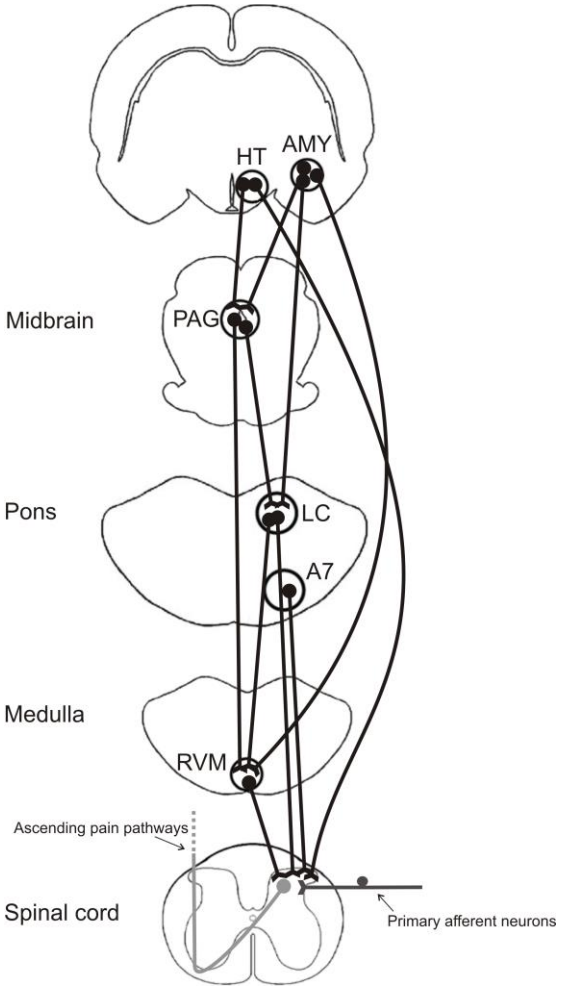

Fig. 2: Schematic representation of the main descending pain pathways. HT = hypothalamus, AMY = amygdala, PAG = periaqueductal grey, LC = Locus Coeruleus, RVM = rostral ventral medulla.

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].

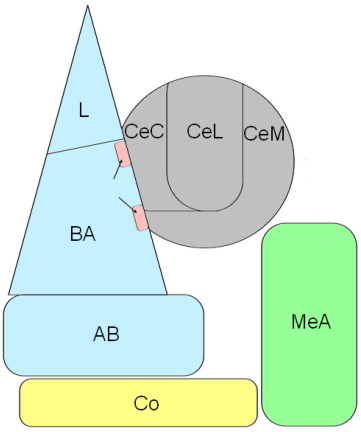

Fig. 3: Schematic representation of the main amygdala nuclei: BLA group (blue), central nucleus (grey), cortical nucleus (Co, yellow), medial nucleus (MeA, green) and the intercalated nuclei (ITC, pink).

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

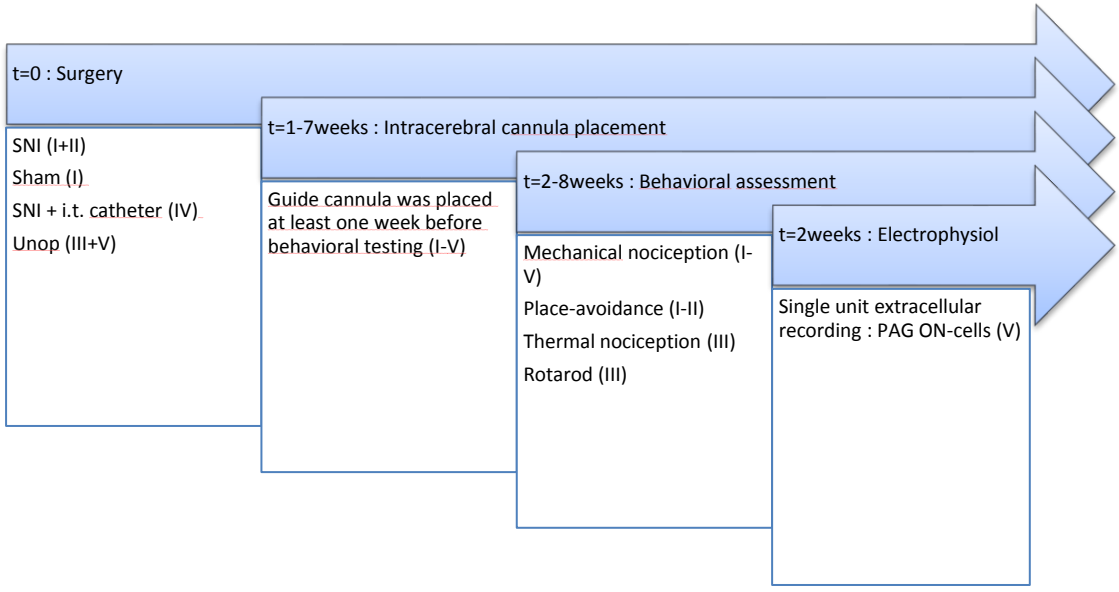

Fig. 4: Summary of the time course of the studies.

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).

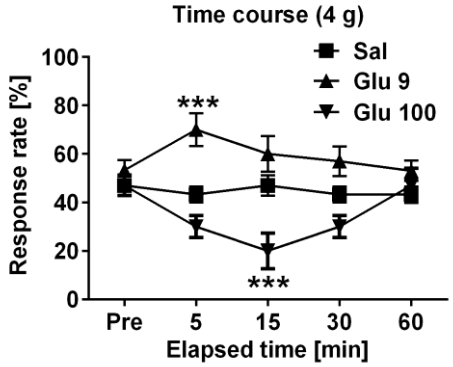

Fig. 5: Time course of the effect of glutamate 9µg and 100µg injections inside the right CeA on the nerve-injured limb withdrawal threshold induced by mechanical stimulus (monofilament of 4 g). Error bars represent S.E.M. ***p<0.005.

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).

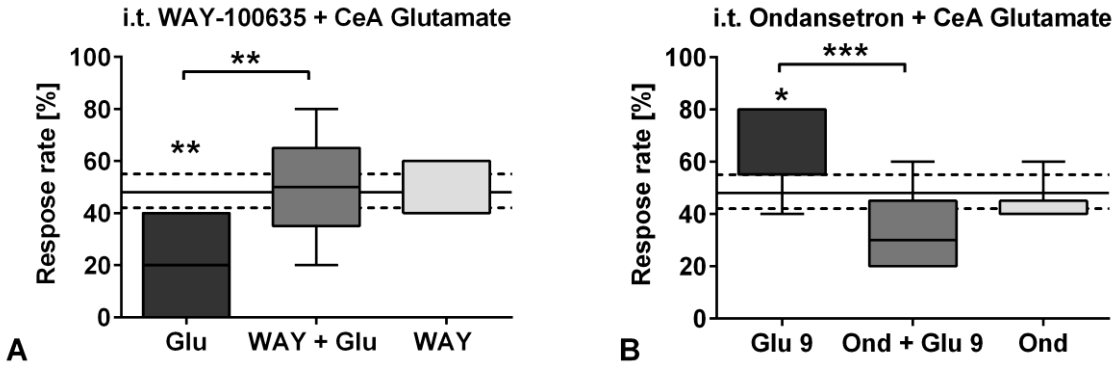

Fig. 6: A. Effect of blocking the spinal 5-HT_{1a} receptors with WAY-100635 (WAY) at 3µg on the reduction of the mechanical hypersensitivity mediated by CeA injection of glutamate (GLU) at 100µg. B. Effect of blocking the spinal 5-HT_3 receptors with ondansetron (Ond) at 5µg on the facilitation of mechanical 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).

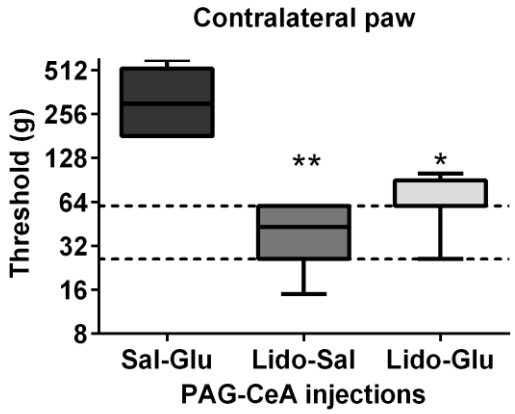

Fig. 7: Effect of pretreatment of the PAG with lidocaine (4%) on the mechanical antinociception mediated by glutamate (100µg) inside the CeA. Error bars represent S.E.M. Horizontal dash lines represent the interquartile range of the median threshold in the corresponding saline-treated animals. Sal =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

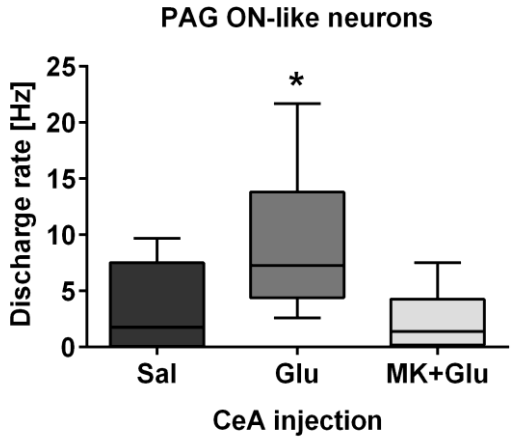

Fig. 8: Effect of CeA injection of glutamate (100µg) alone or pretreated with NMDA-receptors antagonist MK-801 (2µg) on the discharge rate of the PAG ON-Cells. Sal = saline, Glu = glutamate, MK = MK-801. *p<0.05.

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].

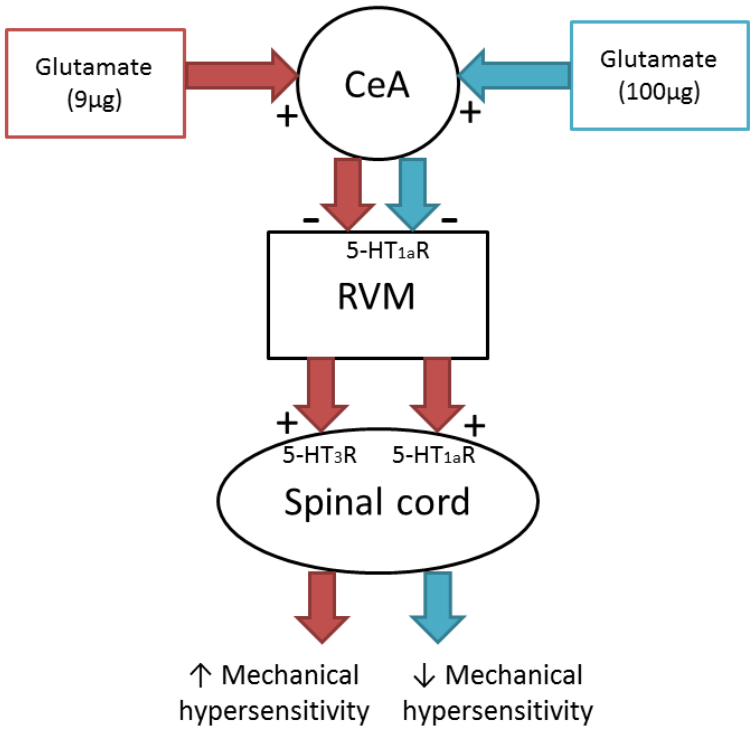

Fig. 2: Involvement of the serotoninergic descending pathways in the bidirectional modulation of the hypersensitivity induced by glutamate injection in the CeA in rats with peripheral neuropathy. + = facilitation, - = inhibition, ↑ = increase, ↓ = decrease.

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].

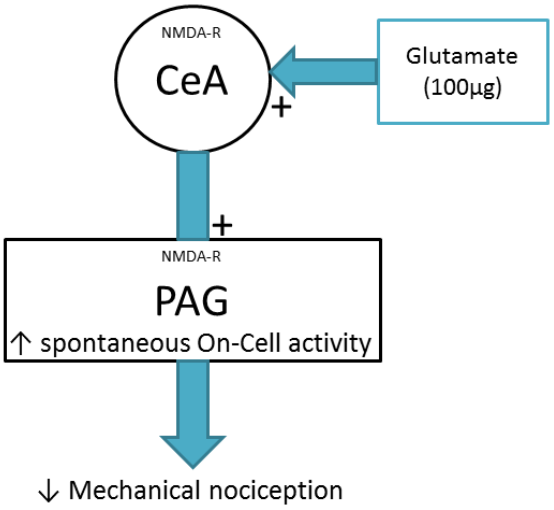

Fig. 3: Involvement of the PAG in descending mechanical antinociception induced by glutamate (100µg) in the CeA of healthy animals. + = facilitation, ↑ = increase, ↓ = decrease.

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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Original publications