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Professor Doutor Armando Almeida
e co-orientação de
Professor Doutor Antti Pertovaara

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"The greater danger for most of us
lies not in setting our aim too high and falling short;
but in setting our aim too low, and achieving our mark."
Michelangelo

"Live as if you were to die tomorrow. Learn as if you were to live forever."
Mahatma Gandhi

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"Friendship is a single soul dwelling in many *bodies."* Aristóteles

Abstract

Pain is a multidimensional experience with sensory-discriminative and motivational-affective dimensions. Neuropathic pain is caused by a primary lesion or dysfunction of the nervous tissue that leads to an anomalous nociceptive processing in pain centers. It results from a process of peripheral and central sensitization and is characterized by prolonged hyperalgesia, allodynia and spontaneous pain. The maintenance of these nerve injury-induced symptoms depends on abnormal discharge from peripheral nerves and pronociceptive changes in spinal and supraspinal mechanisms mediating and modulating pain-related signals. The aims of this work were: first, to elucidate what is the relative contribution of rostroventromedial medulla (RVM) ON- and OFF-cells to hypersensitivity observed in neuropathic pain; second, to evaluate if emotional disturbances and structural alterations in the amygdala (AMY) observed in human patients, were produced when inducing neuropathic pain to rats; third, to determine if structural alterations of the amygdala, associated to the peripheral nerve injury, influences RVM regulation of nociception; and finally, to analyze the alterations in response properties of amygdala nociceptive neurons to peripherally-evoked stimulation and what is the cortical influence in the neuropathic process.

In this work, we provide evidence that reinforce data indicating that RVM ON-cells are the main responsible for neuropathic hypersensitivity. Although both ON- and OFF- RVM cells were found to respond significantly more in neuropathic animals at a basal state, only ON-cell response was significantly different from Sham animals following noxious and non-noxious stimulation. The peripheral stimuli applied were: tail pinch and cold, since clinical studies indicate that hypersensitivity to these somatic stimuli are frequent after traumatic nerve injuries; and colo-rectal distention (CRD), because not much is known about the influence of somatic neuropathy on visceral processing. As RVM ON-cells are considered to be pronociceptive, they should have a major contribution in the hypersensitivity to peripheral stimulation observed in neuropathic animals.

We have demonstrated, for the first time, that animals with a peripheral neuropathy show depressive-like behaviour associated to AMY neuroplasticity. Importantly, AMY structural changes are mediated, at least in part, by an increase in the number of neurons, as a result of cell proliferation. Besides depressive-like behaviour, we also observed an increase in affective pain-related behaviour in neuropathic animals, assessed through the aversive place-conditioning test, which reinforces the association between prolonged neuropathic pain and altered emotional

behaviour. Moreover, emotional pain-related behaviour increased after AMY administration of a metabotropic glutamate receptor 1 and 5 ($mGluR_{1/5}$) agonist and decrease after the local injection of a metabotropic glutamate receptor 1 ($mGluR_1$) antagonist. The AMY administration of an $mGluR_{1/5}$ agonist has also induced an increase in the discharge rate of ON-cells in the RVM of nerve-injured animals, which was mainly due to the activation of $mGluR_1$. In what concerns the activity of AMY neurons, plastic alterations were present after neuropathy, with an increased spontaneous activity and a general decrease after peripheral stimulation.

When evaluating the cortical influence upon nociceptive behaviour of the neuropathic rat, the injection of glutamate (Glu) and of an NMDA-receptor (NMDA-r) antagonist in the rostral anterior cingulate cortex (rACC) produced, respectively, an increase and a decrease in affective pain-related behaviour. When evaluating AMY neuronal discharge rate after the injection of the same reported drugs in the ACC, only glutamate had a significant effect, inducing an increase of AMY neuronal activity.

The set of studies performed for this thesis allowed the following conclusions: I) the activity of both RVM ON- and OFF-cells in neuropathic animals is altered towards promoting pronociception, but only ON-cell type appears to be a major role in the increased hypersensitivity observed; II) the structural plasticity observed in the AMY of nerve-injured animals - increased AMY volume as a result of local newborn neurons – is paralleled with the development of depressive-like behaviour; III) emotional pain-related behaviour in neuropathic animals is dependent, at least in part, on the activation of $mGluR_{1/5}$ in the AMY and of NMDAr in the ACC; IV) the activation of $mGluR_1$ in the AMY of neuropathic animals causes an increase in the activity of pronociceptive RVM ON-cells.

Resumo

A dor é uma experiência multidimensional desagradável, envolvendo não só uma componente sensorial mas também uma componente emocional. A dor neuropática resulta de uma lesão primária ou disfunção do tecido nervoso, que origina o envio incorrecto de sinais para os centros de modulação da dor. A dor neuropática origina hiperalgesia e alodínia prolongadas e dor espontânea, e resulta de um processo de sensitização periférica e central. A continuidade dos sintomas induzidos pela lesão nervosa depende de descargas anormais dos nervos periféricos e de alterações pronociceptivas nos mecanismos espinhais e supraspinhais de mediação e modulação da nocicepção. Os objectivos deste trabalho consistiram em: primeiro elucidar qual a contribuição relativa das células "ON" e "OFF" do bolbo rostral ventromedial (RVM) para a hipersensibilidade observada em animais com dor neuropática; segundo, avaliar se a indução de dor neuropática no rato resultava simultaneamente em distúrbios emocionais e alterações estruturais na amígdala (AMY), tal como no Homens; terceiro, determinar se a plasticidade neuronal da AMY, associada à lesão nervosa periférica influenciava o papel do RVM na regulação da dor; finalmente, avaliar as alterações nas respostas dos neurónios nociceptivos da AMY a estímulos periféricos induzidos pela neuropatia e, adicionalmente, avaliar a influência de estruturas corticais na dor neuropática.

Neste trabalho confirmámos a ideia já existente de que as células pronociceptivas do RVM, as células "ON", são as principais responsáveis pela hipersensibilidade observada em animais neuropáticos. Embora ambos os tipos de células "ON" e "OFF" tenham maior actividade espontânea no estado basal, a aplicação de estimulação nóxica e não-nóxica alterava apenas a resposta das células ON de modo significativo. Dado que há evidências clínicas indicativas de que a hipersensibilidade a estímulos mecânicos e de frio é comum depois de lesões nervosas traumáticas, e dado que há pouca informação sobre a influência da neuropatia somática em percepção visceral, os estímulos periféricos aplicados foram o beliscadura da cauda, o frio e a distensão colo-rectal. Como as células ON são consideradas pronociceptivas, podemos assumir, a partir dos nossos resultados, que estas células deverão ter um papel relevante na hipersensibilidade à estimulação periférica observada nos animais neuropáticos.

Aqui demonstrámos pela primeira vez, que animais com neuropatia periférica apresentam alterações estruturais na AMY associadas, simultaneamente, à expressão de um comportamento tipo depressivo. É de salientar que a plasticidade observada na AMY resultou, pelo menos em

parte, do número de neurónios, resultante de proliferação celular. Foi também observado um aumento do comportamento afectivo relativo à dor, avaliado através do teste aversivo de condicionamento-de-lugar. Este comportamento aumentou depois da administração, na AMY, de um agonista de receptores metabotrópicos do glutamato 1 e 5 ($mGluR_{1/5}$) e diminuiu depois da administração de um antagonista de receptores metabotrópicos do glutamato 1 ($mGluR_1$). A administração de um agonista de $mGluR_{1/5}$ provocou também um aumento na actividade das células "ON" do RVM dos animais neuropáticos, causado principalmente pela activação dos receptores metabotrópicos tipo 1 do glutamato ($mGluR_1$). O outro modo de plasticidade observada foi a alteração na taxa de actividade da AMY que, nos animais neuropáticos, aumentou nos níveis basais e diminuiu após estimulação periférica.

Na avaliação da influência cortical na neuropatia dos ratos, verificou-se que a injecção de glutamato (Glu) e de um antagonista do receptor NMDA (NMDAr) no cortex cingulado anterior rostral (rACC) provocou, respectivamente, um aumento e uma diminuição no comportamento afectivo relativo à dor nos animais neuropáticos, também avaliado pelo teste aversivo de condicionamento-de-lugar. Ao analisar a actividade neuronal da AMY depois da injecção dos mesmos fármacos no ACC, verificámos que apenas o glutamato teve um efeito significativo, provocando um aumento dessa actividade.

Depois de realizado este trabalho, podemos concluir que: I) a actividade das células "ON" e "OFF" do RVM está alterada nos animais neuropáticos no sentido de promover a pronocicepção, mas apenas as células ON aparentam ser as responsáveis pelo aumento de hipersensibilidade associada à neuropatia; II) há plasticidade estrutural na AMY dos animais neuropáticos – aumento de volume, resultante de neurónios recém-formados, que ocorre em paralelo com comportamento do tipo depressivo; III) nos animais neuropáticos, o comportamento afectivo relativo à dor depende, pelo menos, da activação dos $mGluR_{1/5}$ na AMY e dos NMDAr no ACC; IV) a activação dos $mGluR_1$ na AMY provoca um aumento da actividade das células ON nos animais neuropáticos.

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Abbreviations list

ACC - anterior cingulate cortex

AMPA - alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AMY - amygdala

BLA - basolateral nucleus of the amygdala

CeA - central nucleus of the amygdala

CGRP - calcitonin gene related peptide

CNS - central nervous system

CRD - colo-rectal distention

CONT - control animals

EMP - elevated-plus maze

FST - forced swimming test

Glu - glutamate

HPA - hypothalamus-pituitary-adrenal

LA - lateral nucleus of the amygdala

mGluR - metabotropic glutamate receptor

mPFC - medial prefrontal cortex

NK1 - neurokinin 1

NKA - neurokinin A

NMDA - *N*-methyl *D*-aspartate

OF - open field

PAF - peripheral afferent fibers

PAG - periaqueductal gray

PAP - place avoidance paradigm

RVM - rostroventromedial medulla

SDM - standard deviation of the mean

SNI - spared nerve injury

SP - substance P

Chapter 1

INTRODUCTION

1.1 Pain Control

1.1.1 Pain definition

Pain is usually defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage [International Association for the Study of Pain (IASP) - 1994 definition, reviewed in 2008]. Pain unpleasantness exists so the body can recognize that something is threatening it and leads to behaviour that will remove the organism from the source of potential injury (Landrieu et al., 1990). Individuals congenitally insensitive to pain are easily injured and most of them die at an early age (Nagazako et al., 2003). Pain is a key process for our nervous system to learn from and react to the environment (King et al., 1997). Actual pain, or the threat of it, usually leads to behavioural arousal, endocrine responses and sympathetic activation which, together with temporary antinociception, allows escaping from harmful situations that may potentially be life threatening (Millan, 1986; Wiertelak et al., 1994; Traub, 1997).

1.1.2 Pain pathways

Peripheral afferent fibers (PAF) can be classified in three main types on the basis of their diameter, structure and conduction velocity: 1) C, thin (0.4-1.2 μm in diameter), unmyelinated and slowly-conducting (0.5-2.0 m sec^{-1}); 2) Aδ, medium (2-6 μM), myelinated and of intermediate velocity (12-30 m sec^{-1}). 3) Aβ, large (>10 μM), myelinated and fast (30-100 m sec^{-1}) (Millan, 1999). Each of these classes of PAF encodes sensory information, but they are differentially sensitive to noxious and innocuous stimuli; although all these three types of fibers can transmit non-nociceptive information, only C and Aδ fibers transmit nociceptive information (nociceptors; Giordano, 2005).

Nociception is defined as the neural process that encodes and processes a noxious stimulus (Loeser and Treede, 2008) and that can be measured with electrophysiological techniques. (Schaible and Richter, 2004). Noxious stimuli activate primary nociceptive neurons with free nerve endings (Aδ and C nociceptors) at the periphery. Most nociceptors respond to noxious mechanical, thermal (heat or cold) and chemical stimuli (polymodal; Belmonte and Cervero 1996). Nociceptors can also have efferent functions in the injured tissue through the release of neuropeptides [substance P (SP), calcitonin gene-related peptide (CGRP)] from their endings,

inducing neurogenic inflammation (Lynn 1996; Schaible et al. 2005). Nociceptors project to the spinal cord and form synapses with second order neurons in the grey matter of the dorsal horn. A proportion of second-order neurons have ascending axons and project to the brainstem or to the thalamocortical system, triggering the production of the conscious pain response by relaying nociceptive input to the limbic system and sensory cortex (Millan, 1986).

There are two major ascending pathways that make different contributions to the various components of pain perception (Figure 1a): the lateral pain system, which projects through specific lateral thalamic nuclei to the somatosensory cortex and the medial pain system, which projects though medial thalamic nuclei to the anterior cingulate cortex and insula (Treede et al., 1999). The lateral system mediates the sensory-discriminative component of pain, while the medial system is responsible for processing the affective response to a painful stimulus (Carlsson et al., 2006). Loss of peripheral afferents would be expected to cause deficits in both the sensory discrimination of pain and in the affective response to it. However, a localized abnormality, such as a lesion in a specific brain region, might selectively impair only one component of pain processing and cause a more subtle deficit in pain perception (Ploghaus et al., 1999).

Descending tracts reduce or facilitate the spinal nociceptive processing, thus influencing pain processing. Descending projections are formed by pathways that originate from brainstem nuclei and descend mainly in the dorsolateral funiculus of the spinal cord (Willis and Westlund, 1997; Millan, 1999; Almeida et al., 2006). Pain control results, at least in part, from the balance between descending inhibitory and facilitatory modulation actions upon spinal nociceptive transmissions (Urban and Gebhart, 1999; Porreca et al., 2002; Vanegas and Schaible, 2004).

1.1.3 Pain modulation

In the present state-of the art, pain modulation is explained by two theories that complement each other: the "Gate Control" theory (Melzack and Wall, 1965) and the neuromatrix model of pain (Melzack, 1999).

The "Gate Control" theory was proposed by Melzack and Wall in 1965 and originally proposed that ramifications of the large sensory fibers (Aβ fibers) that transmit innocuous cutaneous sensory information activates spinal inhibitory interneurons, which will partially inhibit nociceptive transmission carried by nociceptors. Thus, nociception (and pain) is suppressed by non-noxious stimuli, which “close the gate” to nociceptors activated by noxious stimulation.

The endogenous pain modulatory system is a complex network of brain areas that control nociceptive transmission at the spinal cord by inhibitory and facilitatory actions. Impulses descend from brain stem nuclei to the spinal cord and modulate the transmission of nociceptive signals at the dorsal horn (Fields and Basbaum, 1978; Ossipov and Porreca, 2005; Figure 1b). The periaqueductal grey matter (PAG), a key region in descending inhibition, projects to the rostral ventromedial medulla (RVM), which includes the serotonin-rich nucleus raphe magnus (NRM), the nucleus reticularis gigantocellularis pars alpha and the nucleus paragigantocellularis lateralis (Fields et al. 1991), and receives inputs from the hypothalamus, cortical regions and the limbic system (involved in the processing of emotion, behaviour, cognition) (Ossipov and Porreca, 2005). Concerning their response to nociceptive stimuli, RVM cells are classified as ON-cells, if they give an excitatory response to noxious stimulation just prior to nociceptive withdrawal reflex (considered to have a pronociceptive role), OFF-cells, if give an inhibitory response to noxious stimulation (considered to have an antinociceptive role) (Heinricher et al., 1994; Fields et al., 2006) and NEUTRAL-cells, if give no response to noxious stimulation (Mason, 2006). RVM ON- and OFF-cells then project, through the spinal dorsolateral funiculus, to the dorsal horn (Field et al., 1995), where they cause facilitation and/or inhibition of nociceptive transmission (Fields et al., 1991). Therefore, the RVM is a brain area that is capable of causing antinociception and/or facilitation of spinal nociceptive transmission (Gebhart, 2004; Ossipov and Porreca, 2005), an effect that can be directly exerted by descending projections to the spinal dorsal horn, where the nociceptors synapse (D'Mello and Dickenson, 2008). The complexity of nociceptive modulation at dorsal horn level is further increased due to the multiple synaptic connections that descending fibers and nociceptors make with both excitatory and inhibitory local spinal interneurons (Millan, 1999).

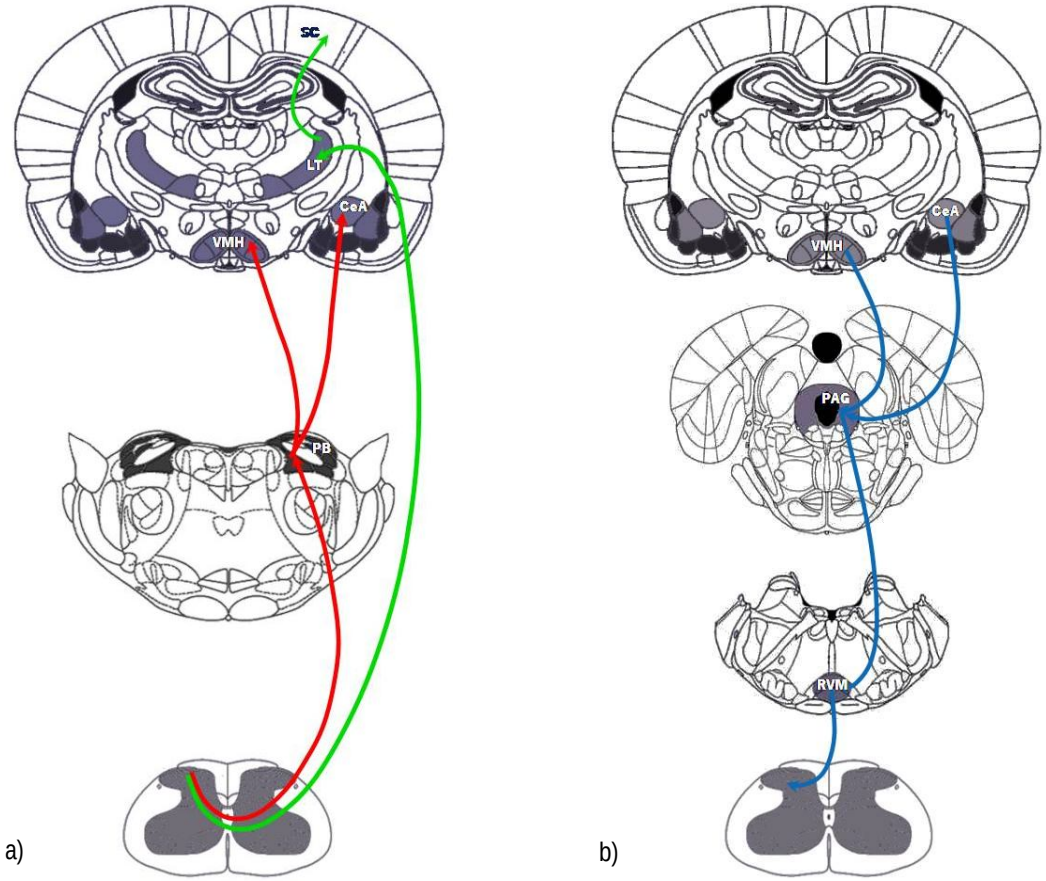

Figure 1 – Pathways of pain transmission and modulation. a) The two major ascending pain pathways: the lateral pain system (green arrow) projects through specific lateral thalamic nuclei (LT) to the somatosensory cortex (SC) and the medial pain system (red arrow) projects through the parabrachial nuclei (PB) to the central nuclei of amygdala (CeA) and ventromedial hypothalamic nuclei (VMH); b) The descending pain pathway: the CeA and the VMH project to the periaqueductal grey matter (PAG), which projects to the rostroventromedial medulla (RVM) ON and OFF cells, which in turn project to the spinal cord dorsal horn.

1.1.4 Types of pain

Pain can be primarily classified as acute and chronic. Although there are additional differences between these two types of pain than their time extent, acute pain is defined for having a short duration, whereas chronic pain must be present for more than three-six months to be classified as such (Russo and Brose 1998). Acute pain arises suddenly in response to a specific injury, has a predictable prognosis, and treatment usually includes analgesics (Viallona et al., 2008). It protects tissue from being (further) damaged because withdrawal reflexes are usually elicited and body protection is triggered. On the other hand, most chronic pain cases

have an unpredictable prognosis, a possibly unclear pathology, and treatment, when existent, should be multidisciplinary, since analgesics are inefficient, and pain control involves drugs like antidepressants and anticonvulsants (Buchner et al., 2007; Scascighini et al., 2008). In many chronic pain states there is no causal relation between nociception and pain, which persists beyond local recovery/healing and thus does not reflect tissue damage (Cervero and Laird, 1991). In contrast, emotional and cognitive factors seem to influence pain in acute pain states (Rhudy and Meagher, 2000) and mainly in complex cases of chronic pain (Kendall, 1999; Choi et al., 2007; Price, 2000), by neuroendocrine dysregulation, fatigue, dysphoria, and impaired physical and mental performance (Chapman and Gavrin 1999).

Regarding its origin, pain can be classified as nociceptive and neuropathic. Nociceptive pain arises from activation of nociceptors after noxious stimulation, following detection by specialized transducers inserted in the plasma membrane of Aδ and C fibers (Schaible, 2007). Therefore, nociceptive information is transmitted by normal physiologic pathways, and also during reversible inflammatory processing. Nociceptive pain can be somatic or visceral, the first originating from an irritation or damage to the soma (e.g., skin, muscle or joints), and the second being diffused, poorly localized, and often referred to a soma (Dunckley et al., 2005; Fairhurst et al., 2007 Schaible, 2007). The term, nociceptive pain, is applied when pain is perceived as proportional to tissue damage and chronic nociceptive pain is thought to be the result of continuous activation of nociceptors (Usunoff, 2006). On the other hand, when the tissue is inflamed or injured, pathophysiological nociceptive pain develops (Schaible and Richter, 2004). It may result in spontaneous pain (pain in the absence of any identifiable stimulation), hyperalgesia and/or allodynia. Hyperalgesia is the higher pain intensity felt upon an already painful noxious stimulation (Marchand et al., 2005), whereas allodynia is the sensation of pain elicited by stimuli that are normally below pain threshold (Wasner et al., 2007).

On the other hand, neuropathic pain arises as a direct consequence of a lesion or disease affecting the somatosensory system (IASP, 2008). While nociceptive pain is elicited by stimulation of the sensory endings in the tissue, neuropathic pain results from injury or disease of neurons in the peripheral or central nervous system (Schaible and Richter, 2004). This type of pain usually occurs within days, weeks, or months after the injury and tends to occur in waves of frequency and intensity (Biondi, 2006). The main signs of neuropathic pain are spontaneous and/or evoked pain (Rasmussen et al., 2004): spontaneous components include usually electric-

like and paroxysmal pain, burning pain (constant and superficial) and aching pain (Herr, 2004); evoked pain components include hyperalgesia and allodynia. As compared to acute pain, less is known about the etiology of chronic pain, as it often occurs with the presence of no illness or after its healing is completed.

1.1.5 Neuropathic pain

Neuropathic pain processing begins after nerve injury, with the degeneration of the axon distal to the site of transaction, the Wallerian degeneration. It consists in an initial reaction at the site of injury followed by progressive degeneration and phagocytosis of myelin and axons distal to the injury (Stoll et al., 2002). Wallerian degeneration is fundamental to neuropathology and it is tightly correlated with the development of neuropathic pain (Myers et al., 2006). Following Wallerian degeneration, nociceptors become increasingly sensitized to external mechanical and thermal stimuli (peripheral sensitization). Injured C-fiber nociceptors can also develop new adrenergic receptors and sensitivity, which helps to the sympathetic maintenance of pain. Additionally, there is neurogenic inflammation (vasodilation, plasma protein extravasation, release of vasoactive peptides), which leads to the release of several neurotransmitters [e.g., norepinephrine, serotonin, SP, neurokinin A (NKA), and CGRP] from nociceptive afferent fibers and resulting in chemosensitivity contributing to peripheral sensitization.

Peripheral nerve injury also results in central sensitization, which relates to changes within the spinal cord that increase the output of neurons within the nociceptive pathway (Woolf, 1983). The firing of C-fibers releases glutamate, activating alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), kainate and postsynaptic metabotropic glutamate (mGLU) receptors (Jones and Sorkin, 2003). The group I mGLU ($mGLU_1$ and $mGLU_5$; Conn and Pin, 1997) links to intracellular guanosine nucleotide proteins (G-proteins), potentiating the effects of AMPA/kainate receptor activation and contributing to the development of hyperalgesia (Meller et al., 1993; Meller and Gebhart, 1994). Aditionnally, higher stimulus intensities induce increased release of SP and NKA from primary afferent terminals (Duggan et al., 1990, 1995), which activate spinal neurokinin 1 (NK-1) receptors (McLean et al., 1993; Radhakrishnan and Henry, 1995, 1997; Hastrup and Schwartz, 1996) and mediate nociceptive transmission and spinal sensitization (Traub, 1996; Abbadie et al., 1997; Doyle and Hunt, 1999). Consequently, there is a polymodal depolarization of postsynaptic neurons, facilitating the removal of a voltage-dependent magnesium ion (Mg^{2+}) block from the glutamate ionotropic NMDA receptor (NMDAr).

This, in turn, results in additional neuronal depolarization by large calcium-ion (Ca^{2+}) influx through the NMDAr channel (Jones and Sorkin, 2003). Calcium then acts as an important secondary messenger, leading to immediate early gene expression (e.g., c-fos), and phosphorylation of numerous receptors at the level of the dorsal horn, including NMDA receptors, which leads to a decreased threshold of dorsal horn neurons (Ji, 2004; Zimmermann, 2001).

Neuropathic pain can have are numerous causes (Table 1), the most common having a peripheral origin. These include axotomy, nerve plexus damage, metabolic diseases such as diabetes mellitus, or herpes zoster. Damage to central neurons (e.g. in the thalamus) can result in central neuropathic pain.

Table 1– Summary and some examples of Neuropathic Pain causes

Neuropathic Pain causes	Examples
Traumas	Improper healing that involves peripheral/central sensitization/excitability
Metabolic and endocrinologic	Diabetes, alcoholism, renal dysfunction and hemodialysis, liver disease, hypothyroidism, acromegaly, B12, thiamine and other vitamin deficiencies
Ischemic	Toxic or drug induced: includes poisoning from substances such as arsenic and thalium, and drugs such as isoniazi, nitrofurantonin, vincrinstine, and cisplatinum
Paraneoplastic	Small-cell carcinomas sensory neuropathies
Hereditary	Sensory neuropathy type I, Charcot-Marie-Tooth Fabry's disease, familial amyloid polyneuropathy, porphyria
Infectious/ postinfectious	HIV related neuropathies, acute zoster, poste-herpetic neuralgia, hepatitis Band C, human T-cell lymphotrophic virus (HTLV-1), Lyme disease and Leprosy
Demyelinating disorders	Guillain-Barre' syndrome, multiple sclerosis, chronic inflammatory demyelinating polyneuropathy
Autoimmune and granulomatous disorders	Sjogren's syndrome, systemic lupus erythmatosus, rheumatoid arthritis, sarcoidosis, polyarteritis nodosa, Churg-Strauss vasculitis, Wegener's granulomatosis, giant cell or temporal arteritis
Immunoglobulinemias	Monoclonan (M) proteins, amyloidosis, cryoglobinemia
Toxic neuropathies	Heavy metals, chemotherapy
Crytogenic neuropathies	Idiopathic, complex regional pain syndromes, essential trigeminal and glossopharyngeal neuralgias

1.1.6 Animal models

As the mechanisms underlying neuropathic pain are still not entirely understood, the search for new animal models that reproduce, as close as possible, this painful condition in humans, still continues (Kim et al., 1997). These animal models must reproduce sensory deficits (allodynia, hyperalgesia, and spontaneous pain) over a sustained period of time to allow evaluation by sensory testing (Dowdall et al., 2005). There are several animal models using mechanical peripheral nerve injury. The first model was developed by Wall and colleagues

(1979). It involved complete transection (CT) of the sciatic nerve at midthigh level, and resulted in autotomy, used to quantify the degree of neuropathic pain. However, due to animal health care issues, this model is rarely used. The chronic constriction injury (CCI) model (Bennett and Xie, 1988), involves four ligatures loosely tied around the sciatic nerve proximal to the sciatic trifurcation. This constriction of the nerve leads to intraneural oedema, a focal ischemia, and Wallerian degeneration. This model results in chemical and heat-evoked hyperalgesia, cold and mechanical allodynia and some symptoms of spontaneous pain that extend for more than 2 months (Bennett and Xie, 1988; Attal et al., 1990). Seltzer et al. (1990) developed the partial sciatic nerve ligation (PSL) model by tightly ligating 1/3 to 1/2 of the sciatic nerve with a single ligature. The result is mechanical allodynia, heat-evoked hyperalgesia, and spontaneous pain, present for up to 7 months. The spinal nerve ligation (SNL) model, developed by Kim and Chung (1992), results from a tightly ligation of the L_5 and L_6 spinal nerves close to their respective ganglia. Mechanical and heat-evoked hyperalgesia together with spontaneous pain lasting at least 4 months are observed (Choi et al., 1994). Lee and colleagues (2000) developed a model where different combinations of the three branches of the sciatic nerve are transected (tibial, sural, and common peroneal), in order to investigate which combination produced the most robust and stable degrees of allodynia and hyperalgesia. Reportedly, sectioning the tibial and sural nerves resulted in the largest amount of mechanical allodynia, chemical hypperreactivity, and spontaneous pain. Almost simultaneously, Decosterd and Woolf (2000) developed the spared nerve injury model (SNI), which consists of an axotomy and ligation of the tibial and common peroneal nerves leaving the sural nerve intact. The SNI model produces mechanical allodynia, mechanical hyperalgesia, no change in thermal heat threshold, a hyper-responsiveness to a suprathreshold heat stimulus and cold allodynia, considered to be representative of many of the symptoms present in human patients with neuropathic pain (Woolf and Mannion, 1999).

1.2 Amygdala and pain

1.2.1 Limbic system

The limbic system was first defined by Paul Broca (1878), mainly to designate the areas included in this convolution: the great limbic lobe, the limbic fissure, the superior or inferior limbic arch. Later, functional studies by Klüver and Bucy (1939) started to show an association between the limbic lobe and complex emotions and motivational processes. In the early 1950s,

MacLean (1949, 1954) named these cortical and subcortical systems and their fibers as the ‘‘limbic system’’.

The limbic system is constituted by a group of brain structures considered to be evolutionarily primitive, located on top of the brainstem and basal to the cortex (Franks, 2006): amygdala, hippocampus, hypothalamus, thalamus, fornix, parahippocampal gyrus, cingulate gyrus, olfactory cortex, orbitofrontal and medialfrontal cortices (medial prefrontal cortex, mPFC), septal nucleus, ventral tegmental area, and some brainstem nuclei. These brain structures have major roles in the processing of the emotions, motivations and even pleasure, particularly when those are related to survival, like fear, anger, and emotions related to sexual behavior and feeding (Burgdorf and Panksepp, 2006). The limbic system also has important functions in memory processing: the amygdala is responsible for determining which memories are kept and where they are stored in the brain (this determination is based on how big an emotional response is invoked by an event; Markowitsch, 1995; Paz et al., 2006); the hippocampus sends memories out to the appropriate part of the cerebral hemisphere for long-term storage and retrieves them when necessary (damage to this area of the brain may result in an inability to form new memories; Dash and Moore, 2007). The hippocampus sends information through the fornix to the mamillary bodies, which then project to the anterior nucleus of the thalamus in the mamilothalamic tract, path that is classically known as the circuit of Papez (Amaral and Witter, 1995).

The limbic system also includes the diencephalon, which is located in the subcortical region and contains the thalamus and hypothalamus (Jacobson and Marcus, 2008). The thalamus is involved in sensory perception and regulation of motor functions (i.e., movement; Basso et al., 2005). The hypothalamus is also a component of the diencephalon and plays a major role in regulating hormones, the pituitary gland, body temperature, the adrenal glands, and many other vital activities (Butler and Hodos, 2005).

The septal nuclei are composed of medium-sized neurons and have a role in autonomic function and in emotional behavior (Panksepp, 1986). Lesions of the septal nuclei cause a variety of behavioral changes, like alterations in sexual behaviour, foraging behaviour and emotional behaviour (Date et al., 1998). The mPFC collects and integrates of behaviorally relevant information and has constant plasticity, allowing the adaptation to new tasks. Therefore, it has the properties needed to sustain cognitive processing. The mPFC contributes to the regulation of affective behaviors, namely through efferent projections to the AMY, which regulate AMY plasticity and responses to previously conditioned stimuli (Rosenkranz et al., 2003).

1.2.2. Amygdala

1.2.2.1 Anatomy and functional significance

It is known that, in humans, the experience of pain is enhanced by unpleasant emotional states (Chapman and Gavrin, 1993; Melzack and Chapman, 1975), and reduced by pleasant emotions (Chaves and Barber, 1974; Zillmann et al., 1996). The amygdala (AMY), as part of the limbic system, plays a key role in the affective and autonomic aspects of behavior, the evaluation of the emotional significance of sensory stimuli, emotional learning and memory, fear, anxiety and depression, and stress responses (Cahill, 1999; Davidson et al., 1999; Davis 1994, 1998; Gallagher and Chiba, 1996; Gallagher and Schoenbaum, 1999; Rasia-Filho et al., 2000; Rolls, 2000).

The AMY is includes approximately 12 different regions, which can be divided into several subregions (LeDoux, 2000). Different systems have been used to classify the amygdala areas, most of them are very similar. Amaral and colleagues (1992) classified AMY of the primate brain, and this classification has been widely used since then, and even applied to the rat brain (Pitkänen et al., 1997; LeDoux, 2000). In this classification, the most relevant areas are the lateral (La), basal (B), accessory basal (AB), and central (CeA) nuclei and the connections between them. In Paxinos and Watson classification (1998; Figure 2), Amaral's B is named basolateral nucleus (BL), and AB is the basomedial nucleus (BM). The term basolateral complex is sometimes used to refer the La and B (and sometimes AB) together. The CeA is the same in both classifications. Since the Paxinos and Watson classification (1998) was completely performed in adult rats, this was the classification used in all studies presented in this thesis.

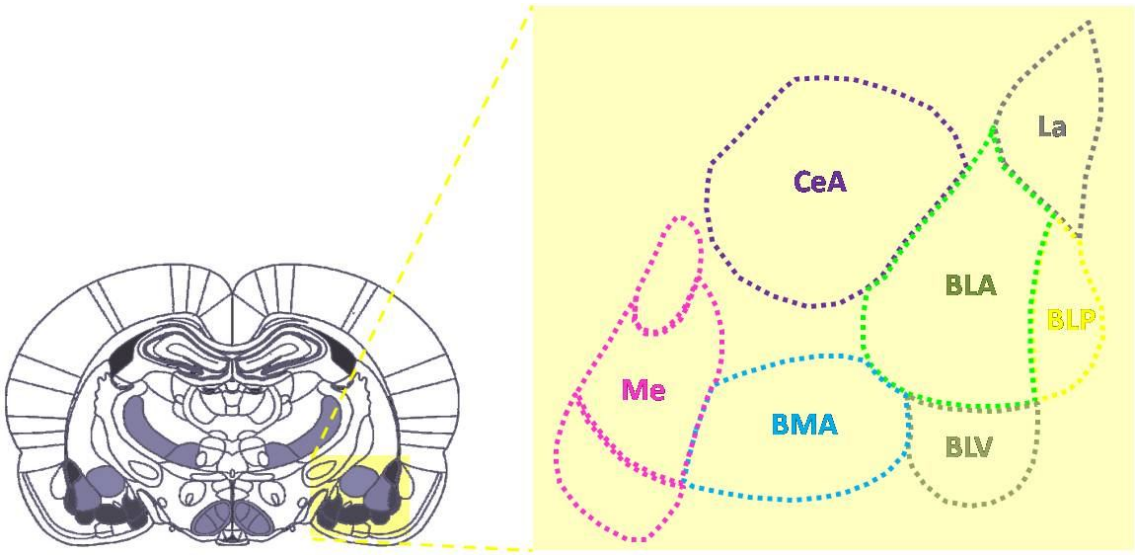

Figure 2 – AMY division as in Paxinos and Watson classification (1998). In this section, we can find the most relevant nuclei: central (CeA), lateral (La), basolateral anterior (BLA), posterior (BLP) and ventral (BLV), basomedial (in its anterior part, BMA) and medial (Me) nuclei.

The CeA modulates various effector systems involved in the expression of emotional responses through widespread connections with the forebrain and brainstem (Cassell et al. 1986; Krettek and Price 1979; LeDoux 2000; Price and Amaral 1981). The CeA receives a wide range of sensory information from descending cortical inputs and ascending thalamic and brainstem inputs (Fallon and Moore, 1978). More specifically, the CeA receives inputs from main and accessory olfactory systems (Pitkänen et al., 1997; Canteras et al., 1992; Petrovich et al., 1996), from mPFC, agranular insular and ventral subicular (Ottersen, 1982) cortical regions, from the solitary tract (Ricardo and Koh, 1978) and parabrachial nuclei (Bernard et al., 1993), and from the perigeniculate thalamus (probably transmitting somatosensory and auditory information; LeDoux et al., 1990; Yasui et al., 1991). Additionally, CeA receives inputs from the thalamic paraventricular nucleus (Moga et al., 1995) and from the LA and BLA. The latter also project to widespread, differentiated regions of the striatum, including the caudoputamen, nucleus accumbens and CeA (Swanson and Petrovich, 1998). These connections between La, BLA, and CeA were extensively studied and confirmed through studies in diferent species, including rats, cats, and primates (Pitkänen et al., 1997; Paré et al., 1995; Amaral et al., 1992; Cassell et al., 1999).

It is known that the BLA modulates memory consolidation by interacting with other brain areas (McGaugh, 2004). BLA has been described essential to memory-modulatory effects of drugs infused into other brain regions, including the hippocampus (Roozendaal and McGaugh, 1997; Roozendaal et al; 1999), entorhinal cortex (Roesler et al.; 2002), medial prefrontal cortex (Roozendaal et al.; 2004), insular cortex (Miranda & McGaugh, 2004) and nucleus accumbens (Roozendaal et al; 2001).

1.2.2.2 Role in pain modulation

The AMY is a major receiver of nociceptive information through the spino-parabrachio-amygdaloid pain pathway, which originates from lamina I neurons in the spinal cord and trigeminal nucleus caudalis and provides purely nociceptive input to the CeA (Bernard and Bandler, 1998; Bourgeais et al., 2001). Aditionally, spinal neurons in the deep dorsal horn and/or the area around the central canal form monosynaptic connections with AMY neurons and may provide sensory, including nociceptive, input to the AMY (Burstein and Potrebic, 1993; Newman et al., 1996; Wang et al., 1999). Finally, the CeA also receives nociceptive information from thalamic and cortical areas through connections with the lateral (La) and basolateral (BLA) amygdaloid nuclei (Bourgeais et al., 2001; Doron and LeDoux, 1999; LeDoux, 2000; Linke et al., 1999; Pitkanen et al., 1997; Savander et al., 1995; Shi and Cassell, 1998; Smith et al., 2000). The latero-capsular part of the CeA has been defined as the 'nociceptive amygdala' because of its high content of nociceptive neurons (Han and Neugebauer, 2004; Bernard et al., 1996; Neugebauer and Li, 2002). Lesion or inactivation of the AMY decreases emotional pain reactions (Borszcz, 1999; Calvino et al., 1982; Werka 1997), without affecting normal behavior or baseline nociceptive responses (Calvino et al., 1982; Fox and Sorenson, 1994; Helmstetter, 1992; Helmstetter and Bellgowan, 1993; Pavlovic et al., 1996a; Tershner and Helmstetter, 2000; Watkins et al., 1993, 1998). Thus, the AMY plays a major role in the processing of the motivational-affective component of pain. As the output nucleus for major amygdala functions, the CeA has important projections that terminate in several brainstem centers, which modulate pain through descending projections to the spinal dorsal horn and control of spinal transmission; these include the PAG (Vianna and Brandão, 2003), the RVM (Pavlovic et al., 1996b; Helmstetter et al., 1998), the dorsal reticular nucleus (DRt; Almeida et al., 1996, 1999, 2002) and the nucleus of the solitary tract (NST; Gamboa-Esteves et al., 2001).

Although it has not been entirely clarified, BLA seems also to have a role in pain modulation. Hasanein and colleagues suggested the existence of a cannabinoid-mediated inhibitory system in the BLA, which normally has no tonic effect on the response threshold to acute and tonic noxious stimuli, but that decreases the response to these stimuli when activated (2007). Additionally, it has been recently shown that opioid receptors are also involved in the CGRP-induced antinociception in the BLA (Li et al., 2008).

1.3 Affective disorders and persistent pain

1.3.1 Chronic pain and depression

It has been clearly demonstrated that pain has a negative impact upon the quality of life of a person (Hunfeld et al., 2001). Pain impairs the capacity to concentrate, work, socialize, perform daily tasks and sleep, which usually results in isolation, loss of self esteem and depression. Since chronic pain encloses not only its physical symptoms (like allodynia and hyperalgesia), but also mood disorders like depression, these should be carefully evaluated in all patients with chronic pain (Dworkin and Gitlin, 1991). In 1993, Magni and colleagues developed a study based on US household survey, where estimated rates of depression were of 16.4% in individuals with chronic pain, compared with 8% in individuals without chronic pain. A survey in the UK demonstrated that psychological distress is more frequent in persons with chronic low back pain than pain-free individuals (Croft et al., 1995). Additionally, data from a World Health Organization (WHO) study on psychological conditions in primary care (Sartorious et al., 1996) showed that 32% of patients with a somatosensory pain disorder also met criteria for a depressive disorder (Von Korff and Simon, 1996). These epidemiological studies clearly show that depression is more common in persons with chronic pain.

On the other hand, evidences have also shown that depressed patients are not only more prone to develop pain (Dickens et al., 2003), but also have significantly lower pain tolerance (Willoughby et al., 2002). It has been reported that depression symptoms are both emotional and physical (Stahl, 2002). The fact the depressed patients feel more pain is thought to be related with a common pathway between the two, which involves serotonin (5-HT) and norepinephrine (NE) neurotransmitter systems (Delgado, 2004). Ascending serotonergic and noradrenergic pathways mediate the emotional symptoms of depression and can be targets for serotonin and norepinephrine reuptake inhibitors to obtain relief of these symptoms (Stahl and Briley, 2004). Descending serotonergic and noradrenergic pathways may regulate the painful physical

symptoms of depression, and when targeted by serotonin and norepinephrine reuptake inhibitors (like antidepressants), these symptoms are also relieved (Stahl and Briley, 2004).

Drepressive-like behaviour has been previously observed associated with chronic mild stress (Bessa et al., 2008), acute stress (Simmons and Broderick, 2005), drug addiction (Cryan et al., 2003) and even maternal separation (Marais et al., 2008) in the rat. However, the simultaneous evaluation of pain and depressive like behaviour in rats is sparse, if not absent. Zeng and colleagues (2008) have recently reported that a subset of Wistar-Kyoto (WKY) rats, a genetic variation of the Wistar strain (Porsolt et al., 1978) that has been used as preclinical model of depression, showed an increase in mechanical allodynia in WKY rats with chronic pain (induced by CCI; 2008). In comparison with normal Wistar rats, WKY rats demonstrate hormonal, behavioural, and physiological changes similar to those found in patients with clinical depression.

1.3.2 Neuroplasticity induced by chronic pain

As mentioned before, peripheral and central sensitization contributes to chronic pain development. Previous experimental research has shown that long-term plastic changes occurring along sensory pathways after an insult are the main origin of chronic pain (Flor, 2008; Nichols et al., 1999; Svendsen et al., 1999). These plastic changes (e.g., increased activity, loss of grey matter) take place in peripheral nociceptors, spinal dorsal horn, subcortical areas (ex.: thalamus) and also in cortical areas (ex.: insular cortex, primary and secondary somatosensory cortex, mPFC, ACC) that are involved in the processing of painful information (Zhuo, 2008; Apkarian et al., 2004; Schmidt-Wilcke et al.; 2006). Prior studies have shown that in neuropathic pain, there is a decrease in the discriminative physiological function of pain (Hofbauer et al., 2006; Witting et al., 2006; Pukall et al., 2005; Kwan et al., 2005). One of the advantages of human studies is that they allow the determination of brain activity during spontaneous pain in patients. In one of these studies, Stern and colleagues (2006) showed that sustained high levels of spontaneous pain result in increases in activity within the mPFC, including the ACC. Phantom pain, a form of neuropathic pain physically caused by amputation, is believed to be due to cortical plasticity and reorganization (studies performed in animals; Wei and Zhuo, 2001; Kaas et al. 1999; Flor et al., 2006). It has been reported that chronic back pain is associated with the decrease of gray matter in the prefrontal, thalamus, brainstem, somatosensory cortex, ACC and mPFC (Apkarian et al. 2004; Schmidt-Wilcke et al. 2006, Kuchinad et al., 2007). Higher

activation is consistently reported in cortical areas (S1, parietal, hindlimb, cingulate and retrosplenial cortical areas) related to pain processing in patients with phantom pain or other forms of chronic pain (Paulson et al., 2000; Mao et al., 1993). However, it is still unclear if these structural changes are a consequence of chronic pain or, on the other hand, changes are due to affective disorders related to chronic pain, such as depression (Zhuo, 2008).

In the AMY, the first observation of synaptic plasticity was performed by Racine and colleagues (1983) in awake, behaving rats. In this study, tetanic long-term potentiation was induced in the amygdala though the application of high-frequency electrical stimuli to the pyriform cortex. Since then, different studies demonstrated that AMY exhibits a high degree of synaptic plasticity in models of tetanic and pharmacologically induced plasticity (McKernan and Shinnick-Gallagher, 1997; Maren, 1999; Wang and Gean, 1999; LeDoux, 2000; Bauer et al., 2001; Blair et al., 2001; Lin et al., 2001). Neugebauer and colleagues have also consistently reported that the AMY exhibits long-term modification of synaptic transmission in rats with kindling or knee-joint input (Neugebauer et al., 1997, 2000, 2003). Additionally, plasticity in AMY (changes in volumes) has also been reported in humans with affective disorders (Altshuler et al., 1998, Altshuler et al., 2000; Mervaala et al., 2000; Sheline et al., 1998; Strakowski et al., 1999; Tebartz van Elst et al., 2000). Increased amygdala volumes have been reported also in patients with temporal lobe epilepsy and comorbid depression (Tebartz van Elst et al 2000), and in patients with bipolar disorders (Altshuler et al., 1998; Strakowski et al., 1999). Furthermore, studies performed in humans have shown that the amygdalar volume was enlarged in patients with depressive disorders, as opposed to the controls (Frodl et al., 2002, 2003; Lange and Irle, 2004).

1.4 Aims and Methodology

Neuropathic pain development entails significant changes in pain pathways. These changes are translated in significant alterations in the pain modulation systems peripherally, spinally and supra-spinally. At the supraspinal level, RVM ON- and OFF-cells have pronociceptive and antinociceptive action, respectively; however, their precise contribution for neuropathy hypersensitivity is not clearly known (Burgess et al., 2002). Additionally, neuropathic pain is strongly related with mood disorders, like depression (Strouse, 2007). Plastic changes have been observed in the limbic system, namely in AMY, which is implicated in both emotion and pain

processing, both in neuropathic pain and depressive patients. Importantly, until now, no studies have analyzed these changes at the structural level in brains of patients (or animals) with peripheral neuropathy and depression. Moreover, it is yet to clarify how these changes in the AMY influence pain processing and modulation, namely at the level of the supraspinal pain control system.

In summary, this thesis aims to:

1. Assess the contribution of RVM ON- and OFF-cells to acute and prolonged neuropathy induced by the spared nerve injury (SNI) model, through an electrophysiological study (Chapter 2.1), in which four groups of animals were included: (i) Sham group tested 1 week after operation; (ii) sham group tested 8 weeks after operation; (iii) SNI group tested 1 week after operation; and (iv) SNI group tested 8 weeks after operation. The surgery consisted in the unilateral axotomy and ligation of the tibial and common peroneal nerves was performed under pentobarbitone anaesthesia (50 mg／kg i.p.) as described in detail earlier (Decosterd & Woolf, 2000). In each animal of these groups, the development of hypersensitivity was verified daily for 2–3 days after the surgery, through mechanical hyperalgesia and allodynia assessement. In order to perform the electrophysiological recordings, the animals were under anaesthesia, induced by pentobarbitone and then placed in a standard stereotaxic frame. The microelectrode was lowered to the RVM [according to the atlas of Paxinos & Watson (1998)] and, after a single cell has been found, its spontaneous activity was recorded for 2–3 min. Additionally, the activity was recorded after application of peripheral stimulati to the lateral side of the hind paw innervated by the sural nerve: cold stimulation (peak stimulus temperature, 4ºC; baseline temperature, 35ºC) and mechanical stimulation of the skin (applied through an a haemostatic clamp to the tail for 5 s), and noxious visceral stimulation [colorectal distension (CRD) at a noxious intensity (80 mmHg) (Ness et al., 1991)]. At the end of the experiment, an electrolytic lesion was made in the RVM recording site, the animals were given a lethal dose of pentobarbitone and the brains were removed for verification of recording sites.

2. Evaluate if chronic pain induces emotional disturbances that are associated with neuroplasticity of the amygdaloid complex (Chapter 2.2), through behavioural, structural and immunohistochemical analysis. The animals were subjected to the SNI model of chronic

neuropathic pain (Decosterd and Woolf, 2000), and tested for mechanical allodynia and hyperalgesia a day before and two days after the surgery procedure, followed by testing every two days then forward, during the two months of experimental period. Anxiety-like behaviour was evaluated by the elevated plus-maze (EPM) test, depression-like behaviour by the forced swimming test (FST), and motor activity and exploratory behaviour through the open field (OF) test. Structural studies performed were: a) stereological analysis of the AMY, which was subdivided in its nuclear components - CeA, La, BLA, BLP, BMA and BMP nuclei (Paxinos and Watson (1998); b) nuclei volume and cell number estimation of AMY, obtained through the Cavalieri's principle and optical fractionator method; c) 3D-morphologycal analysis of dendrites through the observation, at the optical microscope, of brain sections stained with the Golgi-Cox method, analysis of completely stained AMY neurons, and drawing of their dendrites and spines. In the immunohistochemical analysis of the AMY nuclei, all quantifications of markers for cell division and neuronal fate were performed in the AMY, positive controls were confirmed analyzing the subgranular zone (SGZ) of the hippocampus, and to achieve negative controls, the primary antibody was not included in the protocol of each reaction. The immunohistochemistry reactions were performed to reveal the following markers: (i) bromodeoxyuridine (BrdU; an analogue of thymidine, incorporated into the newly synthesized DNA of replicating cells), (ii) BrdU and GFAP (glial fibrillary acidic protein; a marker of astrocyte glial cells) double-labeling, (iii) BrdU and NeuN (protein expressed exclusively in mature neurons) double-labeling and (v) BrdU and Calb (Calbindin; a calcium binding protein present in functional mature neurons) double-labeling. The cells that stained positively to the referred markers were localized and counted in the AMY and nearby telencephalic areas.

3. Reproduce the behaviour results obtain in Chapter 2.2 and elucidate the actual origin of the newborn neurons observed in the AMY (Chapter 2.3). Eight weeks after SNI model induction to a group of animals, behavioural tests were performed to assess mechanical allodynia (von Frey filaments), hyperalgesia (pin-prick test), depressive-like behaviour (forced swimming test) and anxiety-like behaviour (elevated plus-maze). Immunohistochemical analysis were then performed in order to detect cells staining the following markers: doublecortin (DCX; protein expressed in migrating and differentiating neurons) + Ki-67 (nuclear protein expressed in proliferating cells in all phases of the active cell cycle); PSA-NCAM (specifically expressed in committed neuronal precursors present in regions that are undergoing some kind of structural

plasticity) + GFAP (glial fibrillary acidic protein); and nestin (protein markers for immature neural cells) + GFAP.

4. Determine if neural plasticity of the AMY induced by peripheral nerve injury (SNI model) influences RVM regulation of pain using an electrophysiological approach (Chapter 2.4). After SNI induction, the development of hypersensitivity was verified daily for 2–3 days after the surgery through mechanical hyperalgesia and allodynia assessment. In order to perform the electrophysiological recordings, anaesthetized animals were placed in a standard stereotaxic frame according to the atlas of Paxinos & Watson (1998) and had a guide cannula for drug administration inserted into the AMY ipsilateral to the SNI or sham-operated limb (left side), except for one group that had the cannula contralateral to the nerve injury (right side), and a control group that had the cannula in the hippocampus. Additionally, a group of animals tested in behavioral experiments only had a bilateral guide cannula for drug injections into the central nucleus of the amygdala. The microelectrode was lowered to the RVM and, after a single cell had been found, its spontaneous activity was recorded for 2–3 min. Then different drugs (a glutamatergic agonist or a glutamatergic antagonist) or saline (control) was then administered in a determined order to the amygdala and the spontaneous activity was recorded for up to 30 min. Finally, and in order to obtain a measure of affective pain induced by mechanical stimulation of the neuropathic hind paw, the place avoidance test was performed in three experimental groups of rats i) SNI animals with amygdaloid injections ipsilateral to the nerve injury, ii) SNI animals with bilateral amygdaloid injections, iii) sham-operated animals with amygdaloid injections ipsilateral to the sham operation.

5. Evaluate the response properties of amygdala nociceptive neurons in peripherally-evoked stimulation and what is the cortical influence upon their activity in the neuropathic rat (Chapter 2.5). The animals were subjected to SNI surgery and, under the same light anaesthesia, a cannula was placed in the rostral anterior cingulate cortex (rACC) of all animals, according to the atlas of Paxinos & Watson (1998). Nociceptive tests were performed a day before (baseline) and every two days after the surgery procedure, in order to verify the development of hypersensitivity. The place escape/avoidance testing was performed one or eight weeks after surgical implantation of the cannulas. Finally, the spontaneous activity and the peripherally-evoked

noxious stimulation response of BLA and CeA neurons was recorded using the electrophysiology technique described previously.

BIBLIOGRAPHY

References

Abbadie C, Trafton J, Liu H, Mantyh PW, Basbaum AI. Inflammation increases the distribution of dorsal horn neurons that internalize the neurokinin-1 receptor in response to noxious and non-noxious stimulation. J Neurosci. 1997;17:8049–8060.

Almeida A, Tjølsen A, Lima D, Coimbra A, Hole K. The medullary dorsal reticular nucleus facilitates acute nociception in the rat. Brain Res Bull. 1996;39:7-15.

Almeida A, Størkson R, Lima D, Hole K, Tjølsen A. The medullary dorsal reticular nucleus facilitates pain behaviour induced by formalin in the rat. Eur J Neurosci. 1999;11:110-22.

Almeida A, Cobos A, Tavares I, Lima D. Brain afferents to the medullary dorsal reticular nucleus: a retrograde and anterograde tracing study in the rat. Eur J Neurosci. 2002;16:81-95.

Almeida A, Leite-Almeida H, Tavares I. Medullary control of nociceptive transmission: Reciprocal dual communication with the spinal cord. Drug Discovery Today: Disease Mechanisms 2006;3:305-312.

Altshuler LL, Bartzokis G, Grieder T, Curran J, Mintz J. Amygdala enlargement in bipolar disorder and hippocampal reduction in schizophrenia: an MRI study demonstrating neuroanatomic specificity. Arch Gen Psychiatry. 1998;55:663-4.

Altshuler LL, Bartzokis G, Grieder T, Curran J, Jimenez T, Leight K, Wilkins J, Gerner R, Mintz J. An MRI study of temporal lobe structures in men with bipolar disorder or schizophrenia. Biol Psychiatry. 2000;48:147-62.

Amaral DG, Price JL, Pitkänen A, Carmichael ST. 1992. Anatomical organization of the primate amygdaloid complex. See Aggleton 1992, pp. 1–66.

Amaral DG, Witter MP. Hippocampal formation, in: G. Paxinos (Ed.), The Rat Nervous System, 2nd edn., Academic Press, London, 1995, pp. 443–493.

Apkarian AV, Sosa Y, Sonty S, Levy RM, Harden RN, Parrish TB, Gitelman DR. Chronic back pain is associated with decreased prefrontal and thalamic gray matter density. J. Neurosci. 2004;24:10410–10415.

Attal N, Jazat F, Kayser V, Guilbaud G. Further evidence for 'pain-related' behaviours in a model of unilateral peripheral mononeuropathy. Pain 1990; 41:235–251.

Basso MA, Uhlrich D, Bickford ME. Cortical Function: A View from the Thalamus. Neuron 2005;45:485-488.

Bauer EP, LeDoux JE, Nader K. Fear conditioning and LTP in the lateral amygdala are sensitive to the same stimulus contingencies. Nat Neurosci. 2001;4:687-8.

Belmonte C, Cervero E. Neurobiology of nociceptors. Oxford University Press, Oxford. 1996.

Bennett G, Xie Y. A peripheral mononeuropathy in rats that produces disorders of pain sensation like those in man. Pain 1988;33:87–107.

Bernard JF, Alden M, Besson JM. The organization of the efferent projections from the pontine parabrachial area to the amygdaloid complex: A *phaseolus vulgaris* leucoagglutinin (PHA-L) study in the rat. The Journal of Comparative Neurology 1993;329:201-229.

Bernard JF, Bester H, Besson JM, Involvement of the spinoparabrachio-amygdaloid and hypothalamic pathways in the autonomic and affective emotional aspects of pain, Prog. Brain Res. 1996;107:243–255.

Bernard JF, Bandler R. Parallel circuits for emotional coping behaviour: new pieces in the puzzle. J Comp Neurol. 1998;401(4):429-36

Bessa JM, Ferreira D, Melo I, Marques F, Cerqueira JJ, Palha JA, Almeida OF, Sousa N. The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. Mol Psychiatry. 2008 [Epub ahead of print].

Biondi DM. Is migraine a neuropathic pain syndrome? Curr Pain Headache Rep. 2006;10(3):167-78.

Blair HT, Schafe GE, Bauer EP, Rodrigues SM, LeDoux JE. Synaptic plasticity in the lateral amygdala: a cellular hypothesis of fear conditioning. Learn Mem. 2001;8:229-42. Review.

Borszcz GS. Differencial contributions of medullary, thalamic, and Amygdaloid serotonin to the antinociceptive action of morphine administered into the periaqueductal gray: a model of morphine analgesia. Behav Neurosci. 1999;113:612–631.

Bourgeais L, Gauriau C, Bernard JF. Projections from the nociceptive area of the central nucleus of the amygdala to the forebrain: a PHA-L study in the rat. Eur J Neurosci. 2001;14(2):229-55.

Broca P. Anatomie comparée des circonvolutions cérébrales: le grand lobe limbique. Rev. Anthropol. 1878;1:385–498.

Buchner M, Neubauer E, Zahlten-Hinguranage A, Schiltenwolf M. The influence of the grade of chronicity on the outcome of multidisciplinary therapy for chronic low back pain. Spine 2007;32(26):3060-6.

Burgdorf J, Panksepp J. The neurobiology of positive emotions. Neuroscience and Biobehavioral Reviews 2006;30:173–187.

Burgess SE, Gardell LR, Ossipov MH, Malan TP Jr, Vanderah TW, Lai J, Porreca F. Time-dependent descending facilitation from the rostral ventromedial medulla maintains, but does not initiate, neuropathic pain. J Neurosci 2002;22:5129–5136.

Burstein R and Potrebic S. Retrograde labeling of neurons in the spinal cord that project directly to the amygdala or the orbital cortex in the rat. J Comp Neurol. 1993;335:469–485.

Butler AB, Hodos W. The Visceral Brain: The Hypothalamus and the Autonomic Nervous System. Comparative Vertebrate Neuroanatomy (Second Edition) 2005;Chapter 23:445-467.

Cahill L. A neurobiological perspective on emotionally influenced, long-term memory. *Semin Clin* Neuropsych. 1999;4:266–273.

Calvino B, Levesque G, and Besson JM. Possible involvement of the amydaloid complex in morphine analgesia as studied by electrolytic lesions in rats. Brain Res. 1982;233:221–226.

Canteras NS, Simerly RB, Swanson LW. Connections of the posterior nucleus of the amygdala. J. Comp. Neurol. 1992;324:143–179.

Carlsson K, Andersson J, Petrovic P, Petersson KM, Ohman A, Ingvar M. Predictability modulates the affective and sensory-discriminative neural processing of pain. Neuroimage 2006;32(4):1804-14.

Cassell MD, Gray TS, Kiss JZ. Neuronal architecture in the rat central nucleus of the amygdala: a cytological, hodological, and immunocytochemical study. J Comp Neurol. 1986;246:478-99.

Cassell MD, Freedman LL, Shi C. The intrinsic organization of the central extended amygdala. Ann. NY Acad. Sci. 1999;877:217–41.

Cervero F, Laird JMA. One pain or many pains? A new look at pain mechanisms. News Physiol Sci. 1991;6:268–273.

Chapman CR, Gavrin. Suffering and its relationship to pain. Am J Palliat Care 1993;9(2):5-13.

Chaves JF, Barber TX. Cognitive strategies, experimenter modeling, and expectation in the attenuation of pain. J Abnorm Psychol. 1974;83:356–63.

Choi DS, Choi DY, Whittington RA, Nedeljkovic SS. Sudden amnesia resulting in pain relief: The relationship between memory and pain. Pain 2007;132:206–210.

Choi J, Yoon HS, Na HS, Kim SH, Chung JM. Behavioral signs of ongoing pain and cold allodynia in a rat model of neuropathic pain. Pain 1994;59:369–376.

Conn PJ, Pin JP. Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol 1997;37:205–237.

Croft PR, Papageorgiou AC, Ferry S, Thomas E, Jayson MI, Silman AJ. Psychologic distress and low back pain. Evidence from a prospective study in the general population. Spine 1995;20:2731–7.

Cryan JF, Hoyer D, Markou A. Withdrawal from chronic amphetamine induces depressive-like behavioral effects in rodents. Biol Psychiatry 2003;54(1):49-58.

Dash P, Moore AN. Neurochemistry and Molecular Neurobiology of Memory. Springer-Verlag Berlin Heidelberg 2007; 19:709-728.

Date Y, Ueta Y, Yamashita H, Yamaguchi H, Matsukura S, Kangawa K, Sakurai T, Yanagisawa M, Nakazato M. Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. PNAS 1999;96:748-753.

Davis M. The role of the amygdala in emotional learning. Int Rev Neurobiol. 1994;36:225–266.

Davis M. Anatomic and physiologic substrates of emotion in an animal model. J Clin Neurophysiol. 1998;15:378–387.

Davidson RJ, Abercrombie H, Nitschke JB, Putnam K. Regional brain function, emotion and disorders of emotion. Curr Opin Neurobiol. 1999;9:228–334.

Decosterd I, Woolf CJ. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain 2000;87:149-158

Delgado PL. Common pathways of depression and pain. J Clin Psychiatry. 2004;12:16-9.

Dickens C, McGowan L, Dale S. Impact of depression on experimental pain perception: a systematic review of the literature with meta-analysis. Psychosomatic Medicine 2003;65:369–375.

D'Mello R, Dickenson AH. Spinal cord mechanisms of pain. British Journal of Anaesthesia 2008 101(1):8-16.

Doron NN and LeDoux JE. Organization of projections to the lateral amygdala from auditory and visual areas of the thalamus in the rat. J Comp Neurol. 1999;412:383–409.

Doyle CA, Hunt SP. Substance P receptor (neurokinin-1)-expressing neurons in lamina I of the spinal cord encode for the intensity of noxious stimulation: a c-Fos study in rat. Neuroscience 1999;89:17–28.

Dunckley P, Wise RG, Fairhurst M, Hobden P, Aziz Q, Chang L, Tracey I. A comparison of visceral and somatic pain processing in the human brainstem using functional magnetic resonance imaging. J Neurosci. 2005;25(32):7333-41.

Dowdall T, Ian Robinson I, Meert TF. Comparison of five different rat models of peripheral nerve injury. Pharmacology Biochemistry and Behavior 2005;80:193-108.

Duggan AW, Hope PJ, Jarrott B Schaible HG, Fleetwood-Walker SM. Release, spread and persistence of immunoreactive neurokinin A in the dorsal horn of the cat following noxious cutaneous stimulation. Studies with antibody microprobes. Neuroscience 35;1990:195–202.

Duggan AW, Riley RC, Mark MA MacMillan SJ, Schaible HG. Afferent volley patterns and the spinal release of immunoreactive substance P in the dorsal horn of the anaesthetized spinal cat. Neuroscience 1995;65:849–858.

Dworkin SF, Gitlin MJ. Clinical aspects of depression in chronic pain patients. Clin J Pain 1991;7:79–94.

Fairhurst M, Wiech K, Dunckley P, Tracey I. Anticipatory brainstem activity predicts neural processing of pain in humans. Pain. 2007;128(1-2):101-10.

Fallon JH, Moore RY. Catecholamine innervation of the basal forebrain. IV. Topography of the dopamine projection to the basal forebrain and neostriatum. J. Comp. Neurol. 1978;180:545–580.

Fields HL, Basbaum AI. Brainstem control of spinal pain-transmission neurons. Annu Rev Physiol. 1978;40:217-48. Review.

Fields HL, Heinricher MM, Mason P. Neurotransmitters in nociceptive modulatory circuits. Annu Rev Neurosci. 1991;14:219–245

Fields HL, Malick A, Burstein R. Dorsal horn projection targets of ON and OFF cells in the rostral ventromedial medulla. J Neurophysiol. 1995;74:1742-59.

Fields HL, Basbaum AI, Heinricher M.M. Central nervous system mechanisms of pain modulation. In McMahon, S.B. & Koltzenburg, M. (Eds), Wall and Melzack's Textbook of Pain, 5th edn. Elsevier, Location, China, 2006; pp. 125–142.

Fishbain DA, Cutler R, Rosomoff HL, Rosomoff RS. Chronic pain associated depression: antecedent or consequence of chronic pain? A review. Clin J Pain 1997;13:116–37.

Flor H, Nikolajsen L, Staehelin Jensen T. Phantom limb pain: a case of maladaptive CNS plasticity? Nat. Rev. Neurosci. 2006;7:873–881.

Flor H. Maladaptive plasticity, memory for pain and phantom limb pain: review and suggestions for new therapies. Expert Rev Neurother. 2008;8(5):809-18. Review.

Fox RJ, Sorenson CA. Bilateral lesions of the amygdala attenuate analgesia induced by diverse environmental challenges. Brain Res. 1994;648:215–221.

Franks DD. The Neuroscience of Emotions. Handbook of the Sociology of Emotions, Springer US 2006; Section I, 38-62.

Frodl T, Meisenzahl EA, Zetzsche T, Bottlender R, Born C, Groll C, Jäger M, Leinsinger G, Hahn K, Möller H-J. Enlargement of the amygdala in patients with a first episode of major depression. Biol Psychiatry 2002;51:708-714.

Frodl T, Meisenzahl EA, Zetzsche T, Born C, Jäger M, Groll C, Bottlender R, Leinsinger G, Möller H-J. Larger amygdala volumes in first depressive episode as compared to recurrent major depression and healthy control subjects. Biol Psychiatry 2003;53:338–344.

Gallagher M, Chiba AA. The amygdala and emotion. Curr Opin Neurobiol. 1996;6:221–227.

Gallagher M, Schoenbaum G. Functions of the amygdala and related forebrain areas in attention. Ann N Y Acad Sci. 1999;877:397-411. Review.

Gamboa-Esteves FO, Lima D, Batten TFC. Neurochemistry of superficial spinal neurons projecting to nucleus of the solitary tract that express c-fos on chemical somatic and visceral nociceptive input in the rat. Metabolic Brain Disease 2001;16:151-164.

Gebhart GF. Descending modulation of pain. Neurosci Biobehav Rev. 2004.27:729–737

Giordano J. The neurobiology of nociceptive and anti-nociceptive systems. Pain Physician 2005;8:277-290.

Han JS, Neugebauer V. Synaptic plasticity in the amygdala in a visceral pain model in rats. Neuroscience Letters 2004;361:254–257.

Hasanein P, Parviz M, Keshavarz M, Javanmardi K. CB1 receptor activation in the basolateral amygdala produces antinociception in animal models of acute and tonic nociception. Clin Exp Pharmacol Physiol. 2007;34:439-49.

Hastrup H, Schwartz TW. Septide and neurokinin A are high-affinity ligands on the NK-1 receptor: evidence from homologous versus heterologous binding analysis. FEBS Lett 1996;399:264–266.

Heinricher MM, Morgan MM, Tortorici V, Fields HL. Disinhibition of off-cells and antinociception produced by an opioid action within the rostral ventromedial medulla. Neuroscience 1994;63(1):279-88.

Helmstetter FJ. The amygdala is essential for the expression of conditional hypoalgesia. *Behav* Neuroscience 1992;106:518–528.

Helmstetter FJ, Bellgowan PS. Lesions of the amygdala block conditional hypoalgesia on the tail flick test. Brain Res. 1993;612:253–257.

Helmstetter FJ, Tershner SA, Poore LH, Bellgowan PS, Antinociception following opioid stimulation of the basolateral amygdala is expressed through the periaqueductal gray and rostral ventromedial medulla. Brain Res. 1998;779:104-118.

Herr K. Neuropathic Pain: A Guide to Comprehensive Assessment. Pain Management Nursing 2004; 5:9-18.

Hofbauer RK, Olausson HW, Bushnell MC. Thermal and tactile sensory deficits and allodynia in a nerve-injured patient: a multimodal psychophysical and functional magnetic resonance imaging study. Clin. J. Pain 2006;22:104–108

Hunfeld JA, Perquin CW, Duivenvoorden HJ, Hazebroek-Kampschreur AA, Passchier J, van Suijlekom-Smit LW, van der Wouden JC. Chronic pain and its impact on quality of life in adolescents and their families. J Pediatr Psychol. 2001;26(3):145-53.

Jacobson S, Marcus EM. Diencephalon. Neuroanatomy for the Neuroscientist, Springer US 2008; Part1:147-164.

Ji RR. Peripheral and central mechanisms of inflammatory pain, with emphasis on MAP kinases. Curr Drug Targets Inflamm Allergy. 2004;3:299-303.

Jones TL, Sorkin LS. Basic neurochemistry of central sensitization. Seminars in Pain Medicine 2003;1:184-194.

Kaas JH, Florence SL, Jain N. Subcortical contributions to massive cortical reorganizations. Neuron 1999;22:657–660.

Kendall NA. Psychosocial approaches to the prevention of chronic pain: the low back paradigm. Baillieres Best Pract Res Clin Rheumatol. 1999;13:545-54. Review.

Kerr BJ, Bradbury EJ, Bennett DLH, Trivedi PM, Dassan P, French J, Shelton DB, McMahon SB, Thompson SWN. Brain-Derived Neurotrophic Factor Modulates Nociceptive Sensory Inputs and NMDA-Evoked Responses in the Rat Spinal Cord. J Neurosci. 1999;19:5138-5148.

Kim S, Chung J. An experimental model of peripheral neuropathy produced by segmental spinal nerve ligation. Pain 1992;50:355–363.

Kim KJ, Yoon YW, Chung JM. Comparison of three rodent neuropathic pain models. Exp Brain Res. 1997;113:200-206.

King TE, Joynes RL, Grau JW. Tail-flick test: II. The role of supraspinal systems and avoidance learning. Behav Neurosci. 1997;4:754-67.

Klüver H, Bucy PC. Preliminary analysis of functions of the temporal lobes in monkeys. 1939. J Neuropsychiatry Clin Neurosci. 1997;9:606-20.

Kötter R, Meyer N. The limbic system: a review of its empirical foundation. Behav Brain Res. 1992;52:105-27.

Krettek JE, Price JL. Amygdaloid projections to subcortical structures within the basal forebrain and brainstem in the rat and cat. J Comp Neurol. 1979;178:225–254.

Kuchinad A, Schweinhardt P, Seminowicz DA, Wood PB, Chizh BA, Bushnell MC. (2007) Accelerated brain gray matter loss in fibromyalgia patients: premature aging of the brain? J. Neurosci. 2007;27:4004–4007

Kwan CL, Diamant NE, Pope G, Mikula K, Mikulis DJ, Davis KD. Abnormal forebrain activity in functional bowel disorder patients with chronic pain. Neurology 2005;65:1268–1277.

Kyrozis A, Goldstein PA, Heath MJ, MacDermott AB. Calcium entry through a subpopulation of AMPA receptors desensitized neighbouring NMDA receptors in rat dorsal horn neurons. Physiol. 1995; 485:373–381.

Lange C, Irle E. Enlarged amygdala volume and reduced hippocampal volume in young women with major depression. Psychol Med. 2004;34(6):1059-64.

Lao LJ, Song B, Marvizón JC. Neurokinin release produced by capsaicin acting on the central terminals and axons of primary afferents: relationship with N-methyl-D-aspartate and GABA(B) receptors. Neuroscience. 2003;121:667-80.

Landrieu P, Said G, Allaire C. Dominantly transmitted congenital indifference to pain. Ann Neurol. 1990;27(5):574-8.

LeDoux JE, Farb C, Ruggiero DA. Topographic organization of neurons in the acoustic thalamus that project to the amygdala. Journal of Neuroscience 1990;10:1043-1054.

LeDoux JE. Emotion circuits in the brain. Annu Rev Neurosci. 2000;23:155–184.

Lee BH, Won R, Baik EJ, Lee SH, C.H. Moon CH. An animal model of neuropathic pain employing injury to the sciatic nerve branches. NeuroReport 2000;11:657–661.

Leite-Almeida H, Valle-Fernandes A, Almeida A. Brain projections from the medullary dorsal reticular nucleus: An anterograde and retrograde tracing study in the rat. Neuroscience 2006; 140:577-95.

Li N, Liang J, Fang C-Y, Han H-R, Ma M-S, Zhang G-X. Involvement of CGRP and CGRPl receptor in nociception in the basolateral nucleus of amygdala of rats. Neuroscience Letters 2008.443:184–187.

Linke R, De Lima AD, Schwegler H, and Pape HC. Direct synaptic connections of axons from superior colliculus with identified thalamo-amygdaloid projection neurons in the rat: possible substrates of a subcortical visual pathway to the amygdala. J Comp Neurol. 1999;403:158–170.

Loeser JD, Treede RD. The Kyoto protocol of IASP Basic Pain Terminology. Pain 2008;137: 473-7.

Lynn B. Neurogenic inflammation caused by cutaneous polymodal receptors. Prog Brain Res.1996;113:361-8. Review.

MacLean PD. Psychosomatic disease and the ‘‘visceral brain’’: recent developments bearing on the Papez theory of emotion. Psychosom. Med. 1949;11:338–353.

MacLean PD. The limbic system and its hippocampal formation. J. Neurosurg. 1954;11:29–44.

Magni G, Caldieron C, Rigatti-Luchini S, Merskey H. Chronic musculoskeletal pain and depressive symptoms in the general population. An analysis of the 1st National Health and Nutrition Examination Survey data. Pain 1990;43:299–307.

Mao J, Mayer DJ, Price DD. Patterns of increased brain activity indicative of pain in a rat model of peripheral mononeuropathy. J Neurosci. 1993;13(6):2689-702.

Marais L, van Rensburg SJ, van Zyl JM, Stein DJ, Daniels WM. Maternal separation of rat pups increases the risk of developing depressive-like behavior after subsequent chronic stress by altering corticosterone and neurotrophin levels in the hippocampus. Neurosci Res. 2008;61:106-12.

Marchand F, Perretti M, McMahon SB. Role of the immune system in chronic pain. Nature Reviews Neuroscience 2005;6:521-532.

Maren S. Long-term potentiation in the amygdala: a mechanism for emotional learning and memory. Trends Neurosci. 1999;22:561-7. Review.

Markowitsch HJ. Which brain regions are critically involved in the retrieval of old episodic memory? Brain Research Reviews 1995;21:117-127.

Mason P. Chapter 15 Descending pain modulation as a component of homeostasis.Handb Clin Neurol. 2006;81:211-8.

McGaugh JL. The amygdala modulates the consolidation of memories of emotionally arousing experiences. Annual Review of Neuroscience 2004;27:1–28.

McKernan MG, Shinnick-Gallagher P. Fear conditioning induces a lasting potentiation of synaptic currents in vitro. Nature. 1997;390:607-11.

McLean S, Ganong A, Seymour PA Snider RM, Desai MC, Rosen T, Bryce DK, Longo KP, Reynolds LS, Robinson G, et al. Pharmacology of CP-99,994; a nonpeptide antagonist of the tachykinin neurokinin-1 receptor. J Pharmacol Exp Ther. 1993;267:472–479.

Melzack R, Wall PD. Pain mechanisms: a new theory. Science 1965;150:971-9.

Melzack R, Chapman CR. Psychological aspects of pain. Postgrad Med. 1973;53:69–75.

Melzack R. From the gate to the neuromatrix. Pain. 1999;6:S121-6.

Meller ST, Dykstra CL, Gebhart GF. Acute mechanical hyperalgesia is produced by coactivation of AMPA and metabotropic glutamate receptors. Neuroreport 1993;4:879–882.

Meller ST, Gebhart GF. Spinal mediators of hyperalgesia. Drugs 1994;47**:**10–20.

Millan MJ. Multiple opioid systems and pain. Pain 1986;27(3):303-47. Review.

Millan MJ. The Induction of pain: an integrative review. Progress in Neurobiology 1999;57:1-164. Review.

Miranda MI, aMcGaugh JL. Enhancement of inhibitory avoidance and conditioned taste aversion memory with insular cortex infusions of 8-Br-cAMP: involvement of the basolateral amygdala. Learning and Memory 2004;11:312–317.

Moga MM, Weis RP, Moore RY. Efferent projections of the paraventricular thalamic nucleus in the rat. J. Comp. Neurol. 1995;359:221–238.

Myers RR, Campana WM, Shubayev VI. The role of neuroinflammation in neuropathic pain: mechanisms and therapeutic targets. Drug Discov Today. 2006;11(1-2):8-20.

Nagasako EM, Oaklander AL, Dworkin RH. Congenital insensitivity to pain: an update. Pain. 2003;101(3):213-9. Review.

Ness TJ, Randich A, Gebhart GF. Further behavioral evidence that colorectal distension is a 'noxious' visceral stimulus in rats. Neurosci. Lett. 1991;131:113–116.

Neugebauer V, Bradley KN, Shinnick-Gallagher P. Epileptogenesis *In Vivo* Enhances the Sensitivity of Inhibitory Presynaptic Metabotropic Glutamate Receptors in Basolateral Amygdala Neurons *In Vitro*. The Journal of Neuroscience 1997;17(3):983–995.

Neugebauer V, Zinebi F, Russell R, Gallagher JP, Shinnick-Gallagher P. Cocaine and Kindling Alter the Sensitivity of Group II and III Metabotropic Glutamate Receptors in the Central Amygdala. J Neurophysiol 2000;84:759-770.

Neugebauer V, Li W, Processing of nociceptive mechanical and thermal information in central amygdala neurons with knee-joint input, J. Neurophysiol. 2002;87:103–112.

Neugebauer V, Li W, Differential sensitization of amygdala neurons to afferent inputs in a model of arthritic pain, J. Neurophysiol. 2003;89:716–727.

Neugebauer V, Li W, Bird GC, Bhave G, Gereau RW IV. Synaptic Plasticity in the Amygdala in a Model of Arthritic Pain: Differential Roles of Metabotropic Glutamate Receptors 1 and 5. The Journal of Neuroscience 2003;23(1):52– 63.

Newman HM, Stevens RT, and Apkarian AV. Direct spinal projections to limbic and striatal areas: anterograde transport studies from the upper cervical spinal cord and the cervical enlargement in squirrel monkey and rat. J Comp Neurol. 1996;365:640–658.

Nichols ML, Allen BJ, Rogers SD, Ghilardi JR, Honore P, Luger NM, Finke MP, Li J, Lappi DA, Simone DA, Mantyh PW. Transmission of chronic nociception by spinal neurons expressing the substance P receptor. Science 1999;286(5444):1558-61.

Ossipov MH, Porreca F. Descending modulation of pain. In: Merskey H, Loeser JD, Dubner R (eds) The paths of pain 1975–2005. IASP Press, Seattle, 2005; pp 117–130.

Ottersen OP. Connections of the amygdala of the rat. IV: Corticoamygdaloid and intraamygdaloid connections as studied with axonal transport of horseradish peroxidase. J. Comp. Neurol. 1982;205:30–48.

Panksepp J. The Neurochemistry of Behavior. Annual Review of Psychology 1986;37:77-107.

Paré D, Smith Y, Paré JF. Intra-amygdaloid projections of the basolateral and basomedial nuclei in the cat: *Phaseolus vulgaris*–leucoagglutinin anterograde tracing at the light and electron microscopic level. Neuroscience 1995;69:567–83.

Paulson PE, Morrow TJ, Casey KL. Bilateral behavioral and regional cerebral blood flow changes during painful peripheral mononeuropathy in the rat. Pain 2000;84(2-3):233-45.

Paulson PE, Casey KL, Morrow TJ. Long-term changes in behavior and regional cerebral blood flow associated with painful peripheral mononeuropathy in the rat. Pain 2002;95:31-40.

Pavlovic ZW, Cooper ML, Bodnar RJ. Enhancements in swim stressinduced hypothermia, but not analgesia, following amygdala lesions in rats. Physiol and Behav. 1996a;59:77–82.

Pavlovic ZW, Cooper ML, Bodnar RJ. Opioid antagonists in the periaqueductal gray inhibit morphine and beta-endorphin analgesia elicited from the amygdala of rats. Brain Res. 1996b;741:13–26.

Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. Academic Press, Sydney 1998.

Paz R, Pelletier JG, Bauer EP, Paré D. Emotional enhancement of memory via amygdala-driven facilitation of rhinal interactions. Nat Neurosci. 2006;9:1321-1329.

Petrovich GD, Risold PY, Swanson LW. Organization of projections from the basomedial nucleus of the amygdala: a PHAL study in the rat. J. Comp. Neurol. 1996;374**:**387–420.

Pitkänen A, Savander V, and LeDoux JE. Organization of intra-amygdaloid circuitries in the rat: an emerging framework for understanding functions of the amygdala. Trends Neurosci. 1997;20:517-523.

Ploghaus A, Tracey I, Gati JS, Clare S, Menon RS, Matthews PM, Rawlins JN. Dissociating pain from its anticipation in the human brain. Science. 1999;284:1979-81.

Porreca F, Ossipov MH, Gebhart GF. Chronic pain and medullary descending facilitation, Trends Neurosci. 2002;25:319–325.

Porsolt RD, Anton G, Blavet N, Jalfre M. Behavioral despair in rats: a new model sensitive to antidepressant treatment. Eur. J. Pharmacol. 1978;47:379–391.

Price DD. Psychological and neural mechanisms of the affective dimension of pain. Science 2000;288:1769–72.

Price JL, Amaral DG. An autoradiographic study of the projections of the central nucleus of the monkey amygdala. J Neurosci. 1981;1:1242–1259.

Pukall CF, Strigo IA, Binik YM, Amsel R, Khalifé S, Bushnell MC. Neural correlates of painful genital touch in women with vulvar vestibulitis syndrome. Pain 2005;115:118–127.

Radhakrishnan V, Henry JL. Electrophysiology of neuropeptides in the sensory spinal cord. Prog Brain Res. 1995;104:175–195.

Radhakrishnan V, Henry JL. Electrophysiological evidence that neurokinin A acts via NK-1 receptors in the cat dorsal horn. Eur J Neurosci. 1997;9:1977–1985.

Rasia-Filho AA, Londero RG, Achaval M. Functional activities of the amygdala: an overview. J Psychiat Neurosci. 2000;25:14–23.

Racine RJ, Milgram NW, Hafner S. Long-term potentiation phenomena in the rat limbic forebrain. Brain Res. 1983;260(2):217-31.

Rasmussen PV, Sindrup SH, Jensen TS, Bach FW. Symptoms and signs in patients with suspected neuropathic pain. Pain 2004;110:461–46.

Rhudy JL, Meagher MW. Fear and anxiety: divergent effects on human pain thresholds. Pain. 2000;84:65-75.

Ricardo JA, Koh ET. Anatomical evidence of direct projections from the nucleus of the solitary tract to the hypothalamus, amygdala, and other forebrain structures in the rat. Brain Res. 1978;153:1–26.

Roesler R, Roozendaal B, McGaugh JL. Basolateral amygdala lesions block the memory-enhancing effect of 8-Br-cAMP infused into the entorhinal cortex of rats after training. The European Journal of Neuroscience 2002;15:905–910.

Rolls ET. Memory systems in the brain. Annu Rev Psychol. 2000;51:599–630.

Roozendaal B, McGaugh JL. Glucocorticoid receptor agonist and antagonist administration into the basolateral but not central amygdala modulates memory storage. Neurobiol Learn Mem. 1997;67:176-179.

Roozendaal B, Nguyen BT, Power AE, McGaugh JL. Basolateral amygdala noradrenergic influence enables enhancement of memory consolidation induced by hippocampal glucocorticoid receptor activation. PNAS USA 1999;96:11642–11647.

Roozendaal B, de Quervain DJ, Ferry B, Setlow B, McGaugh JL. Basolateral amygdala-nucleus accumbens interactions in mediating glucocorticoid enhancement of memory consolidation. The Journal of Neuroscience 2001;21:2518–2525.

Roozendaal B, McReynolds JR, McGaugh JL. The basolateral amygdala interacts with the medial prefrontal cortex in regulating glucocorticoid effects on working memory impairment. The Journal of Neuroscience 2004;24:1385–1392.

Rosenkranz JA, Moore H, Grace AA. The prefrontal cortex regulates lateral amygdala neuronal plasticity. The Journal of Neuroscience. 2003;23:11054-11064.

Ru-Rong Ji. Peripheral and Central Mechanisms of Inflammatory Pain, with Emphasis on MAP Kinases. Current Drug Targets - Inflammation & Allergy 2004;3:299-303.

Russo CM, Brose WG. Chronic pain. Annu Rev Med. 1998;49:123-33.

Sartorious N, Usten TB, Lecrubier Y, Wittchen HU. Depression comorbid with anxiety: results from the WHO study of psychological disorders in primary health care. Br J Psychiatry 1996;168:138–43.

Savander V, Go CG, LeDoux JE, Pitkanen A. Intrinsic connections of the rat amygdaloid complex: projections originating in the basal nucleus. J Comp Neurol. 1995;361:345–368.

Scascighini L, Toma V, Dober-Spielmann S, Sprott H. Multidisciplinary treatment for chronic pain: a systematic review of interventions and outcomes. Rheumatology 2008;47(5):670-678.

Schaible HG, Richter F. Pathophysiology of pain. Langenbecks Arch Surg. 2004;389(4):237-43.

Schaible HG, Del Rosso A, Matucci-Cerinic M. Neurogenic aspects of inflammation. Rheum Dis Clin North Am. 2005;31:77-101.

Schaible HG. Peripheral and Central Mechanisms of Pain Generation. Peripheral and central mechanisms of pain generation. Handb Exp Pharmacol. 2007;177:3-28.

Schmidt-Wilcke T, Leinisch E, Gänssbauer S, Draganski B, Bogdahn U, Altmeppen J, May A. Affective components and intensity of pain correlate with structural differences in gray matter in chronic back pain patients. Pain 2006;125:89–97.

Seltzer Z, Dubner R, Shir Y. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. Pain 1990;43:205–218.

Shi CJ and Cassell MD. Cascade projections from somatosensory cortex to the rat basolateral amygdala via the parietal insular cortex. J Comp Neurol 1998;399:469–491.

Simmons DA, Broderick PA. Cytokines, stressors, and clinical depression: augmented adaptation responses underlie depression pathogenesis. Prog Neuropsychopharmacol Biol Psychiatry. 2005;29:793-807. Review.

Smith Y, Paré JF, and Pare D. Differential innervation of parvalbumin-immunoreactive interneurons of the basolateral amygdaloid complex by cortical and intrinsic inputs. J Comp Neurol. 2000;416:496–508.

Stahl SM. Does depression hurt? J Clin Psychiatry. 2002;63:273-4.

Stahl S, Briley M. Understanding pain in depression. Hum Psychopharmacol Clin Exp 2004;19: S9–S13.

Stern J, Jeanmonod D, Sarnthein J. Persistent EEG overactivation in the cortical pain matrix of neurogenic pain patients. Neuroimage 2006;31:721–731.

Stoll G, Jander S, Myers RR. Degeneration and regeneration of the peripheral nervous system: From Augustus Waller's observations to neuroinflammation. J. Peripher. Nerv. Syst. 2002;7:13–27.

Strakowski SM, DelBello MP, Sax KW, Zimmerman ME, Shear PK, Hawkins JM, Larson ER. Brain magnetic resonance imaging of structural abnormalities in bipolar disorder. Arch Gen Psychiatry. 1999;56:254-60.

Strong J, Unruh AM, Wright A, Baxter GD, Wall PD. Pain: A Textbook for Therapists; Contributor Patrick D. Wall. Published by Elsevier Health Sciences, 2002; 461 p.

Strouse TB. The relationship between cytokines and pain/depression: A review and current status. Current Pain and Headache Reports. 2007;11:108-113.

Svendsen F, Rygh LJ, Gjerstad J, Fiskå A, Hole K, Tjølsen A. Recording of long-term potentiation in single dorsal horn neurons in vivo in the rat. Brain Res Brain Res Protoc. 1999;4:165-72.

Swanson LW, Petrovich GD. What is the amygdala? Trends in Neurosciences 1998;21:323-331.

Tershner SA, Helmstetter FJ. Antinociceptioon produced by mu opioid receptor activation in the amygdala is partly dependent on activation of mu opioid and neurotensin receptors in the ventral periaqueductal gray. Brain Res. 2000;865:17–26.

Traub RJ. The spinal contribution of substance P to the generation and maintenance of inflammatory hyperalgesia in the rat. Pain 1996;67:151–161.

Traub RJ. Spinal modulation of the induction of central sensitization. Brain Res. 1997;778:34-42.

Treede RD, Kenshalo DR, Gracely RH, Jones AK. The cortical representation of pain. Pain. 1999;79:105-11. Review.

Urban MO, Gebhart GF. Supraspinal contributions to hyperalgesia, Proc. Nat. Acad. Sci. U. S. A. 1999;96:7687–7692.

Usunoff KG, Popratiloff A, Schmitt O, Wree A. Functional Neuroanatomy of Pain (Advances in Anatomy, Embryology and Cell Biology) (Paperback). Springer 2006; 1 edition.

Vanegas H, Schaible HG. Descending control of persistent pain: inhibitory or facilitatory? Brain Res Brain Res Rev. 2004;46:295-309. Review.

Viallona A, Marjolleta O, Guyomarcha P, Roberta F, Bergera C, Guyomarcha S, Navezb ML, Bertranda J-C. Acute pain in emergency departments - Analgesic efficacy of orodispersible paracetamol in patients admitted to the emergency department with na osteoarticular injury. Acute Pain 2008;10:55-56.

Vianna DML, Brandão ML. Anatomical connections of the periaqueductal gray: specific neural substrates for different kinds of fear. Brazilian Journal of Medical and Biological Research 2003;36:557-566.

Von Korff M, Simon G. The relationship between pain and depression. Br J Psychiatry 1996;168: 101–8.

Wall PD, Devor M, Inbal R, Scadding JW, Schonfeld D, Seltzer Z, Tomkiewicz MM. Autotomy following peripheral nerve lesions: experimental anesthesia dolorosa, Pain 1979;7:103–113.

Wang CC, Willis WD, and Westlund KN. Ascending projections from the area around the spinal cord central canal: a *Phaseolus vulgaris* leucoagglutinin study in rats. J Comp Neurol. 1999:415:341-367.

Wang SJ, Gean PW. Long-term depression of excitatory synaptic transmission in the rat amygdala. J Neurosci. 1999;19:10656-63.

Wasner G, Naleschinski D, Baron R. A role for peripheral afferents in the pathophysiology and treatment of at-level neuropathic pain in spinal cord injury? A case report. Pain 2007;131:219–225.

Watkins LR, Wiertelak EP, and Maier SF. The amygdala is necessary for the expression of conditioned but not unconditioned analgesia. Behav Neurosci. 1993;107:402–405.

Watkins LR, Wiertelak EP, McGorry M, Martinez J, Schwartz B, Sisk D, and Maier SF. Neurocircuitry of conditioned inhibition of analgesia: effects of amygdala, dorsal raphe, ventral medullary, and spinal cord lesions on antianalgesia in the rat. Behav Neurosci. 1998;112:360–378.

Wei F, Zhuo M. Potentiation of sensory responses in the anterior cingulate cortex following digit amputation in the anaesthetised rat. J. Physiol. 2001;532,823–833.

Werka T. The effects of the medial and cortical amygdala lesions on poststress analgesia in rats. Behav Brain Res. 1997;86:59–65.

Wiertelak EP, Smith KP, Furness L, Mooney-Heiberger K, Mayr T, Maier SF, Watkins LR. Acute and conditioned hyperalgesic responses to illness. Pain. 1994;56(2):227-234.

Willis WD, Westlund KN. Neuroanatomy of the pain system and of the pathways that modulate pain. J Clin Neurophysiol. 1997;14:2-31.

Willoughby SG, Hailey BJ, Mulkana S, Rowe J. The effect of laboratoryinduced depressed mood state on responses to pain. Behav. Med. 2002;28:23–31.

Wilson LB, Hand GA. Segmental effect of spinal NK-1 receptor blockade on the pressor reflex. Am J Physiol. 1998;275:789-796.

Witting N, Kupers RC, Svensson P, Jensen TS. A PET activation study of brush-evoked allodynia in patients with nerve injury pain. Pain 2006;120:145–154.

Woolf CJ. Evidence for a central component of post-injury pain hypersensitivity. Nature 1983; 306:15-21.

Woolf CJ, Mannion RJ. Neuropathic pain: aetiology, symptoms, mechanisms, and management. Lancet 1999;353:1959–1964.

Yasui Y, Saper CB, Cechetto DF. Calcitonin gene-related peptide (CGRP) immunoreactive projections from the thalamus to the striatum and amygdala in the rat. J. Comp. Neurol. 1991;308**:**293–310.

Zeng Q, Wang S, Lim G, Yang L, Mao J, Sung B, Chang Y, Lim JA, Guo G, Mao J. Exacerbated mechanical allodynia in rats with depression-like behavior. Brain Research 2008;1200:27-38.

Zhuo M. Cortical excitation and chronic pain. Trends in Neurosciences 2008;31:199-207.

Zillmann D, de Wied M, King-Jablonski C, Jenzowsky MA. Drama-induced affect and pain sensitivity. Psychosom Med. 1996;58:333–41.

Zimmermann M. Pathobiology of neuropathic pain. Eur J Pharmacol. 2001;429:23-37.

Chapter 2

RESULTS

Chapter 2.1

Gonçalves L, Almeida A, Pertovaara A

Pronociceptive changes in response properties of rostroventromedial medullary neurons in a rat model of peripheral neuropathy

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Pronociceptive changes in response properties of rostroventromedial medullary neurons in a rat model of peripheral neuropathy

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Keywords: cold hypersensitivity, descending facilitation of nociception, descending inhibition of nociception, mechanical hypersensitivity

Abstract

The spared nerve injury (SNI) model of peripheral neuropathy produces a robust and long-lasting hypersensitivity. Previous behavioural studies suggest that brainstem–spinal pathways originating in or relaying through the rostroventromedial medulla (RVM) contribute to neuropathic hypersensitivity. We determined whether SNI induces changes in response properties of RVM neurons that might influence descending modulation of nociception. RVM neurons included in the study were classified into presumably pronociceptive ON-cells and antinociceptive OFF-cells (giving excitatory or inhibitory responses to noxious stimulation, respectively). Spontaneous activity and the response to cold, pinch and colorectal distension were assessed under light anaesthesia in the rat, 1 week and 8 weeks following nerve injury or sham operation. Spontaneous activity was increased 1 week but not 8 weeks after nerve injury in ON-cells but decreased in OFF-cells at both time points. In the SNI group, cold-evoked responses were enhanced particularly in ON-cells, independent of the postoperative time point. Responses of ON-cells to pinch and visceral stimulation were enhanced 8 weeks but not 1 week following nerve injury, whereas OFF-cell responses to pinch or colorectal distension were not changed. The results indicate that SNI induces pronociceptive changes in spontaneous activities of ON-cells and OFF-cells and peripherally evoked responses of ON-cells that vary with the postoperative time point. Increased ON-cell activity and decreased OFF-cell activity in the RVM are likely to enhance spinal nociception in a tonic fashion, whereas increased responses of ON-cells to peripheral stimulation are likely to enhance ascending nociceptive signals by a positive feedback following peripheral noxious stimulation.

Introduction

Peripheral nerve injuries may produce long-lasting neuropathic pain and hypersensitivity that is particularly prominent with mechanical and cold stimulation of the skin (Scadding & Koltzenburg, 2006). The maintenance of these nerve injury-induced symptoms depends on abnormal discharge from peripheral nerves (Devor, 2006) and pronociceptive changes in the spinal segmental mechanisms mediating and modulating pain-related signals (Woolf & Salter, 2006). Additionally, changes in descending pain modulation contribute to neuropathic symptoms (Pertovaara, 2000; Porreca *et al*., 2002; Ossipov *et al*., 2006). The rostroventromedial medulla (RVM), consisting of the raphe magnus and its adjacent reticular nuclei, is a final common pathway for many descending pathways and is involved in descending facilitation as well as inhibition of pain-related spinal responses (Gebhart, 2004; Vanegas & Schaible, 2004). Among the various cell types of the RVM, one giving an excitatory response to noxious stimulation just prior to nociceptive withdrawal reflex (ON-cell) is considered to have a pronociceptive role, whereas one giving an inhibitory response to noxious stimulation (OFF-cell) is considered to have an antinociceptive role (Fields *et al*., 2006). A third cell type of the RVM giving no response to noxious stimulation (NEUTRAL-cell) has a less clear role, although it has been proposed to contribute to tonic modulation of various spinal processes (Mason, 2006), possibly including nociception. Whereas there is accumulating behavioural evidence indicating that the RVM contributes to neuropathic hypersensitivity through descending pathways, the contribution of various cell types of the RVM to hypersensitivity during an early vs. a later phase of neuropathy is only partly known (see Discussion for references).

In this study, we assessed the contribution of presumably pro- and antinociceptive RVM cells to acute and prolonged neuropathy by determining their response properties at two different postoperative time points in sham-operated controls vs. in animals with a spared nerve injury (SNI) model of peripheral neuropathy (Decosterd & Woolf, 2000). This model of neuropathy provides stable and long-lasting symptoms mimicking those observed in clinical conditions.

Materials and methods

The experiments were performed in adult, male Hannover-Wistar rats weighing 180–190 g at the beginning of the experiment (Harlan, Horst, The Netherlands). The experimental protocol was accepted by the Institutional Ethics Committee and the experiments were

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performed according to the guidelines of European Communities Council Directive of 24 November 1986 (86/609/EEC).

Techniques for producing neuropathy

The unilateral axotomy and ligation of the tibial and common peroneal nerves was performed under pentobarbitone anaesthesia (50 mg/kg i.p.) as described in detail earlier (Decosterd & Woolf, 2000). Briefly, the skin of the lateral surface of the thigh was incised and a section made directly through the biceps femoris muscle exposing the sciatic nerve and its three terminal branches. Following ligation and removal of 2–4 mm of the distal nerve stumps of the tibial and common peroneal nerves, muscle and skin were closed in two layers. In sham-operated animals, the surgical procedure was identical, except that the tibial and common peroneal nerves were not ligated or sectioned.

Behavioural verification of neuropathy

Development of hypersensitivity was verified behaviourally in animals habituated to the experimental conditions 1–2 h daily for 2–3 days. For assessment of tactile allodynia, the hind limb withdrawal threshold was determined by stimulating the sural nerve area in the hind paw of the operated limb with monofilaments. The calibrated series of monofilaments used in this study produced forces ranging from 0.16 to 15 g (North Coast Medical, Inc. Morgan Hill, CA, USA). The monofilaments were applied to the foot pad with increasing force until the rat withdrew its hind limb. The lowest force producing a withdrawal response was considered the threshold. The threshold for each hind paw of each rat was based on three separate measurements and the median of these values was considered to represent the threshold. Threshold values ≤1 g were considered to represent hypersensitivity. It should be noted that the currently used strain of rats delivered by Harlan (Horst, The Netherlands) has an exceptionally low withdrawal threshold to monofilament stimulation in the baseline (unoperated) condition: in 10 unoperated control animals, the lowest withdrawal threshold was only 4 g, and therefore the criterion for hypersensitivity was set to as low as ≤1 g in this study.

Electrophysiological recordings

For electrophysiological recordings, the anaesthesia was induced by pentobarbitone at a dose of 50 mg/kg i.p. and the animal was placed in a standard stereotaxic frame according to the atlas of Paxinos & Watson (1998). Anaesthesia was maintained by infusing pentobarbitone (15–20 mg/kg/h). The level of anaesthesia was frequently monitored by observing the size of the pupils and by assessing withdrawal responses to noxious stimulation. When necessary, the infusion rate of pentobarbitone was increased. The rats were spontaneously breathing. A warming blanket was used to maintain body temperature within the physiological range. Peripheral perfusion was checked by evaluating the colour of ears and extremities. The skull was exposed and a hole drilled for placement of a recording electrode in the RVM. The desired recording site in the RVM was 1.8–2.3 mm posterior from the ear bar, 0.0–0.9 mm lateral from the midline, and 8.9–10.7 mm ventral from the dura mater.

Single neuron activity was recorded extracellularly with lacquer-coated tungsten electrodes (tip impedance 3–10 MΩ at 1 kHz) and then 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).

Actual recordings did not start until the animal was under light anaesthesia; that is, the animals gave a brief withdrawal response to a noxious pinch, but the pinch did not produce any longer-lasting motor activity, and nor did the animals have spontaneous limb movements. Neurons were classified on the basis of their response to noxious pinch of the tail with a haemostatic clamp. This stimulus was painful when applied to the finger of the experimenters. Neurons giving excitatory responses to pinch were considered ON-cells, those giving inhibitory responses were considered OFF-cells and neurons showing no or only a negligible (<10%) change in their discharge rates as a response to pinch were considered NEUTRAL-cells. This classification scheme of medullary neurons was modified from that described by Fields *et al*. (2006). A noteworthy difference is that we did not verify whether pinch-evoked responses of RVM neurons were associated with spinal reflex responses as in the original classification scheme (Fields *et al*., 2006). Therefore, the populations of ON- and OFF-cells in this study may not be identical with those in a study in which cells are classified strictly according to the classification scheme of Fields *et al*. (2006). Our previous results suggest, however, that there is only little difference in the classification of RVM neurons whether or not spinal reflex responses are concurrently measured in lightly anaesthetized animals (Pertovaara *et al*., 2001).

Peripheral test stimuli

In electrophysiological experiments, cold stimuli (peak stimulus temperature, 4 °C; baseline temperature, 35 °C; rate of stimulus temperature decrease, 4 °C/s; duration of the peak temperature of 4 °C, 10 s) were applied with a feedback-controlled Peltier device (LTS-3 Stimulator; Thermal Devices Inc., Golden Valley, MN, USA) to the lateral side of the hind paw innervated by the sural nerve. Whereas some models of neuropathy may be associated with significant skin temperature changes that provide a confounding factor to assessment of thermal responses, particularly when using radiant heat for stimulation (Luukko *et al*., 1994), applying thermal stimuli with a contact thermostimulator and starting from a standard adapting temperature reduces the possibility that a neuropathy-associated change in skin temperature will influence the results.

ON- and OFF-neurons in the RVM typically have very large receptive fields covering almost the whole body and allowing testing of not only responses evoked by stimulating the operated limb but also responses evoked by the nonoperated region within the same neurons. In this study, responses evoked by stimulating the tail and the viscera were assessed to observe potential nerve injury-induced changes outside of the injured region. Mechanical stimulation of the skin consisted of applying a haemostatic clamp to the tail for 5 s. When applied to the experimenter's hand, this stimulus produced a painful pinch sensation. Noxious visceral stimulation consisted of colorectal distension (CRD) at a noxious intensity (80 mmHg) (Ness *et al*., 1991). CRD was applied for 10 s by inflating with air a 7–8-cm flexible latex balloon inserted transanally into the descending colon and rectum. The pressure in the balloon was controlled by an electronic device (Anderson *et al*., 1987).

When the stimulus-evoked responses were analysed, the baseline discharge frequency recorded during a corresponding period just before the stimulation was subtracted from the discharge frequencies determined during stimulation; that is, positive values represent excitatory responses evoked by peripheral stimulation and negative ones inhibitory responses.

Course of the study

Four groups of animals were included in the electrophysiological study: (i) sham group tested 1 week after operation; (ii) sham group tested 8 weeks after operation; (iii) SNI group tested 1 week after operation; and (iv) SNI group tested 8 weeks after operation. In each of these groups, behavioural assessment of sensitivity to monofilament stimulation was performed before the start of the electrophysiological experiment.

After induction of anaesthesia, the microelectrode was lowered to the RVM. After a single cell had been found, its spontaneous activity was recorded for 2–3 min. Then, cold stimulation was applied to the sural nerve area in the operated limb followed by pinch of the tail and CRD at 1-min intervals. The testing procedure, including the order of testing different submodalities of nociception, was the same in all experimental groups. To minimize serial effects, every other animal tested in this series belonged to the sham group and every other to the SNI group.

At the completion of the study, an electrolytic lesion was made in the recording site, the animals were given a lethal dose of pentobarbitone and the brains were removed for verification of recording and microinjection sites.

Statistics

Data are presented as mean ± SEM. For assessment of differences in incidence of various types of neurons in different experimental conditions, the chi-squared test was used. Two-way ANOVA followed by the Student–Newman–Keuls test was used for assessing differences in neuronal responses between the experimental conditions. $P < 0.05$ was considered to represent a significant difference.

Results

All animals with SNI showed mechanical hypersensitivity both 1 week and 8 weeks following surgery, as indicated by hind limb withdrawal thresholds that were ≤1.0 g ipsilateral to the nerve injury. In contrast, sham-operated animals did not develop mechanical hypersensitivity and their hind limb withdrawal thresholds were significantly higher than those in the SNI group ($F_{1,28} = 40$, $P < 0.0001$), independent of the postoperative time point (1 or 8 weeks, Fig. 1).

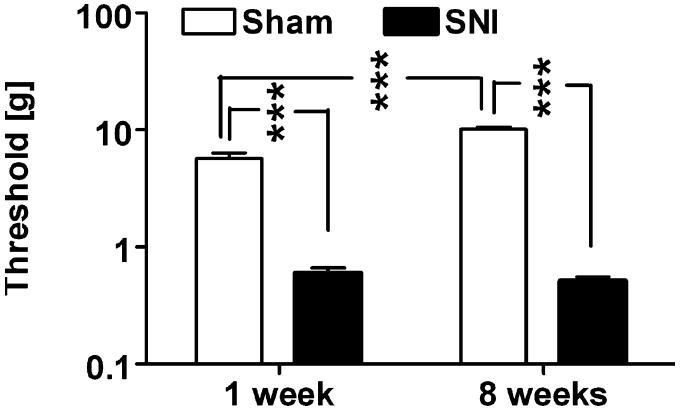

FIG. 1. Hind limb withdrawal thresholds evoked by monofilament stimulation of the operated limb determined in nerve-injured (SNI) and sham-operated (Sham) animals 1 week and 8 weeks following the operation. Thresholds were determined prior to induction of anaesthesia. The error bars represent SEM ($n = 7$–9). ***$P < 0.005$ (Student–Newman–Keuls test).

TABLE 1. Numbers of different types of rostroventromedial medullary neurons studied in different experimental groups

	ON-cells	OFF-cells	Neutral-cells
Sham			
1 week	17	11	6
8 weeks	27	18	10
SNI			
1 week	18	12	5
8 weeks	29	16	8

SNI, spared nerve injury; Sham, sham operation; 1 week and 8 weeks refer to the postoperative time point of the study.

In general, ON-cells gave excitatory and OFF-cells inhibitory responses to noxious stimulation. Their receptive fields covered bilaterally wide areas of the body and all extremities. NEUTRAL-cells, in contrast, gave no marked responses to noxious stimulation of the extremities, and they were not further studied here. Distributions of RVM cells with different types of response properties were not significantly different between SNI and sham groups at either 1 week or 8 weeks following surgery (chi-square test; Table 1).

Spontaneous activity

The spontaneous discharge rate of ON-cells was significantly changed from 1 week to 8 weeks postoperatively ($F_{1,69} = 8.9$, $P < 0.005$; Fig. 2A), and the change in the discharge rate varied with the experimental group (Sham vs. SNI animals; $F_{1,69} = 4.8$, $P < 0.04$). One week following the operation, the spontaneous discharge rate of ON-cells was significantly higher in the SNI than in the Sham group. By the 8-week postoperative time point, the spontaneous discharge rate of ON-cells was significantly decreased in the SNI group only. Consequently, the spontaneous discharge rate of ON-cells 8 weeks following the operation was no longer higher in the SNI than in the Sham group, but was, if anything, the opposite.

The spontaneous discharge of the OFF-cells tended to increase from the 1-week to the 8-week postoperative time point ($F_{1,69} = 14.8$, $P < 0.0005$; Fig. 2B). The spontaneous discharge rate of OFF-cells was significantly lower in the SNI group than in the Sham group ($F_{1,69} = 8.5$, $P < 0.005$; Fig. 2B), independent of the postoperative time point (1 week vs. 8 weeks; $F_{1,69} = 14.8$, $P < 0.0005$).

Peripherally evoked responses

Application of cold (4 °C) to the sural nerve area in the operated hind limb evoked markedly stronger excitatory responses in ON-cells of the SNI than of the Sham group ($F_{1,87} = 116$, $P < 0.0001$; Figs 3 and 4A), independent of the postoperative time point ($F_{1,87} = 0.67$). Cold-evoked responses of ON-cells did not vary with elapsed time from the surgery (1 week vs. 8 weeks; $F_{1,87} = 0.9$). Cold stimulation induced stronger inhibitory responses in the OFF-cells of the SNI than in the Sham group ($F_{1,53} = 64.6$, $P < 0.0001$; Fig. 4B). Postoperative time (1 week vs. 8 weeks) did not influence the magnitude of the cold-evoked inhibitory response in OFF-cells ($F_{1,53} = 0.8$).

Noxious tail pinch produced significantly stronger excitatory responses in ON-cells of the SNI than of the sham group ($F_{1,87} = 54$, $P < 0.0001$; Figs 5 and 6A), and this difference in the response magnitude was significantly larger at 8 weeks than at 1 week after the operation ($F_{1,87} = 17.4$, $P < 0.0001$). There were no significant differences in pinch-evoked inhibitory responses of

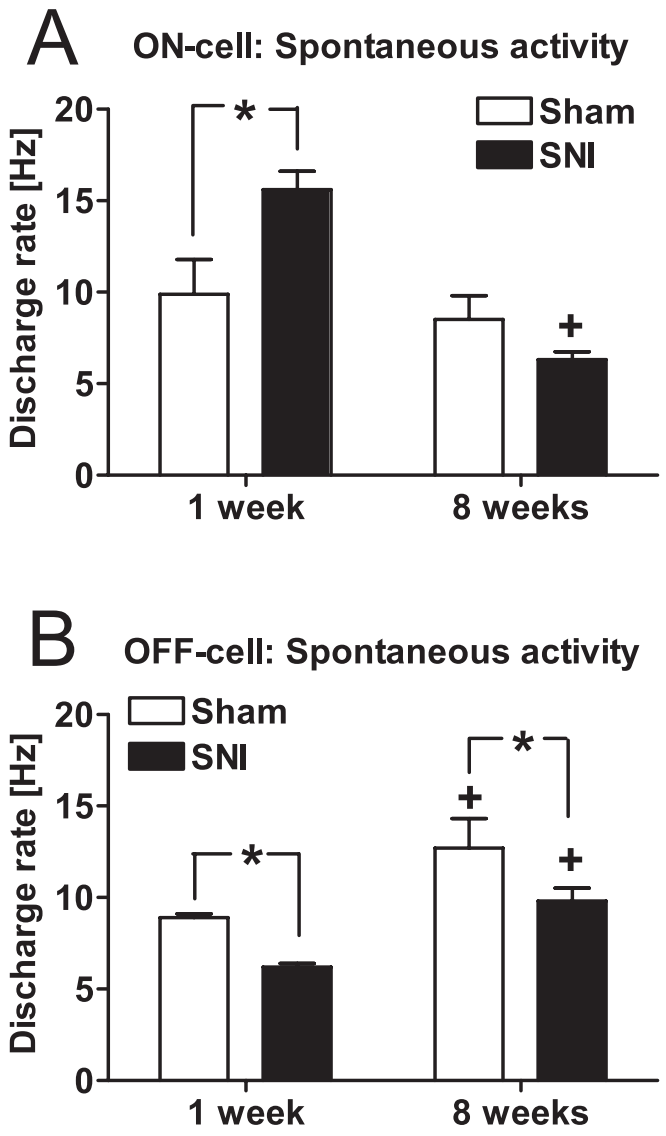

FIG. 2. Mean spontaneous activities of ON-cells (A) and OFF-cells (B) in the RVM of sham-operated (Sham) and nerve-injured (SNI) animals 1 week and 8 weeks following sham operation or nerve injury. The error bars represent SEM ($n = 11$–29). $^{*}P < 0.05$ (Student–Newman–Keuls test; reference, the corresponding value in the Sham group), $^{+}P < 0.05$ (Student–Newman–Keuls test; reference, the corresponding value in the 1-week group).

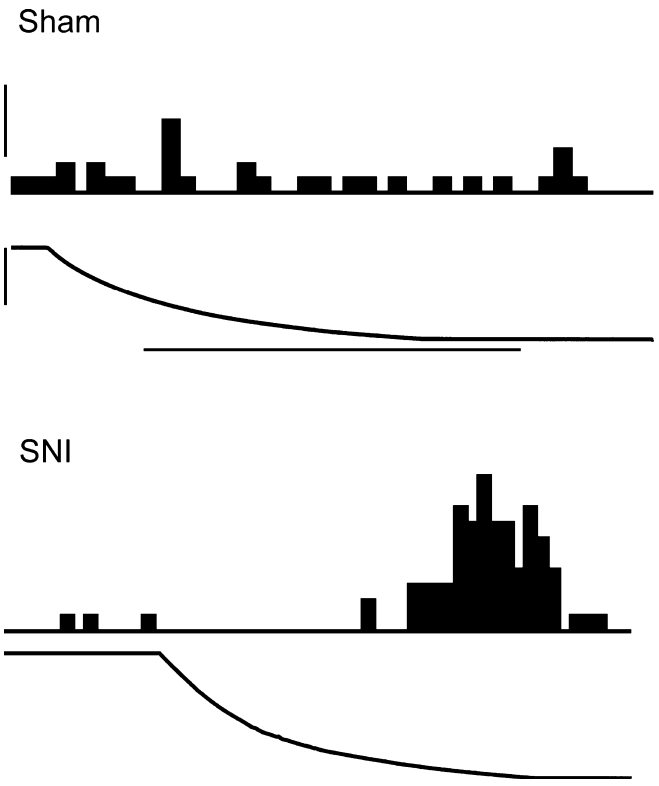

FIG. 3. Examples of original recordings of cold-evoked responses of ON-cells in a sham-operated animal and a nerve-injured (SNI) animal 8 weeks following the operation. Cold stimulation was applied to the skin of the sural nerve area in the operated limb. The peristimulus time histogram of the neuronal response (above) and the change of stimulus temperature from 35 °C to 4 °C (below) are shown for each neuron. The calibration bar for the peristimulus time histogram represents 10 impulses/s and the horizontal calibration bar represents 25 s for the Sham condition and 20 s for the SNI condition.

OFF-cells between the SNI and the Sham groups ($F_{1,53} = 3.2$; Fig. 6B). Pinch-evoked inhibitory responses in OFF-cells, however, were stronger at 8 weeks than at 1 week after the operation ($F_{1,53} = 6.7$, $P < 0.02$), independent of nerve injury ($F_{1,53} = 0.4$).

CRD at a noxious intensity (80 mmHg) evoked significantly stronger excitatory responses in ON-cells of the SNI than of the Sham group ($F_{1,87} = 7.2$, $P < 0.01$; Figs 6C and 7), and this difference in the response magnitudes was significantly larger at 8 weeks than at 1 week following the operation ($F_{1,87} = 10.7$, $P < 0.005$). One week following the operation, ON-cell responses evoked by CRD were minor ones and not different between the experimental groups, whereas at 8 weeks following operation, the CRD-evoked ON-cell response was significantly increased in the SNI group only (see Fig. 6C for detailed results of *post hoc* tests). In OFF-cells, the inhibitory response evoked by CRD was not significantly different between the SNI and Sham groups ($F_{1,53} = 2.4$; Fig. 6D), and the magnitude of the response evoked by CRD in OFF-cells was not significantly different when assessed 1 week vs. 8 weeks postoperatively ($F_{1,53} = 1.8$).

Recording sites

Figure 8 shows the recording sites in the medulla.

Discussion

The results indicate that the SNI model of peripheral neuropathy induces marked pronociceptive changes in response properties of cells in the RVM, a nucleus with an important role in descending modulation of pain (Pertovaara & Almeida, 2006). These plastic changes vary with elapsed postoperative time and they are likely to contribute to maintenance of neuropathic hypersensitivity.

Spontaneous activity and peripherally evoked responses

Spontaneous discharge rate of RVM ON-cells, which are known to have an excitatory effect on nociceptive transmission (Fields *et al.*, 2006), was significantly increased 1 week but not 8 weeks after nerve injury. The spontaneous discharge rate of RVM OFF-cells, which have an inhibitory effect on nociceptive transmission (Fields *et al.*, 2006), was decreased both 1 week and 8 weeks following nerve injury. Taken together, these findings suggest that both ON- and OFF-cell

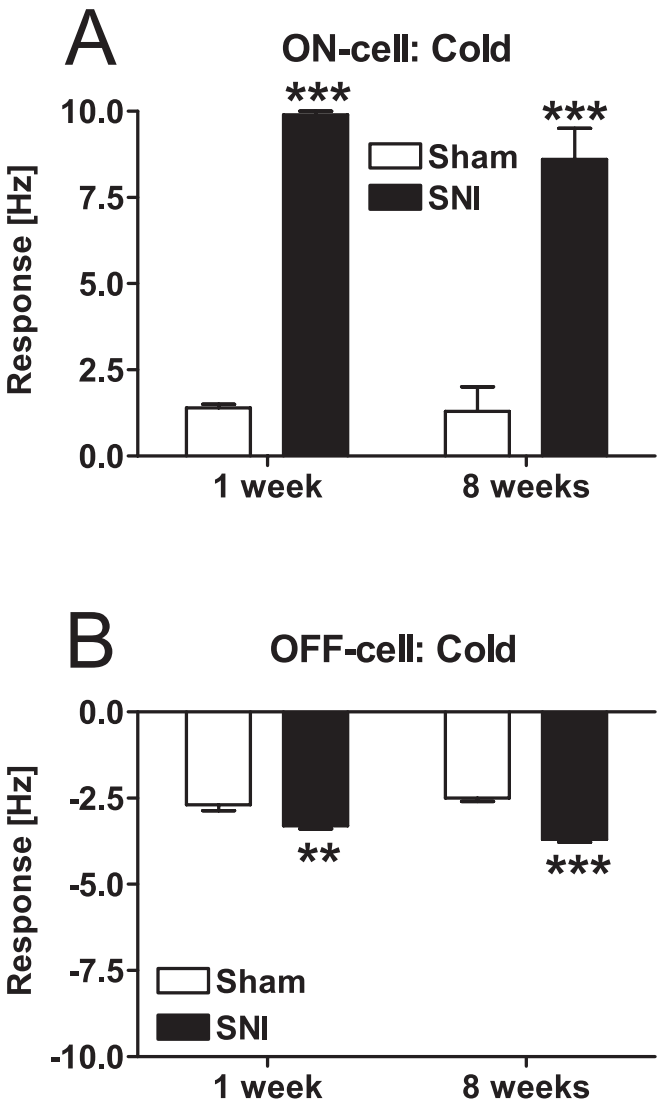

FIG. 4. Mean changes of ON-cell (A) and OFF-cell (B) discharge rates induced by cold applied to the sural nerve area in the operated limb 1 week and 89 weeks following the operation. SNI, spared nerve-injury group; Sham, sham-operated group. $^{**}P < 0.01$, $^{***}P < 0.005$ (Student–Newman–Keuls test; reference, the corresponding Sham group). The error bars represent SEM ($n = 11$–29).

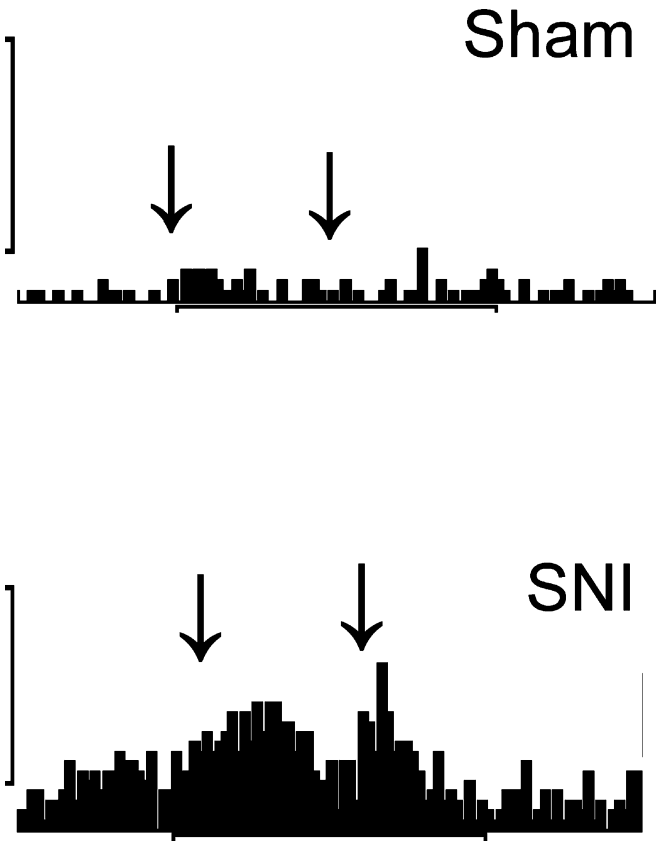

FIG. 5. Examples of original recordings of tail pinch-evoked responses of ON-cells in a sham-operated animal and a nerve-injured (SNI) animal 8 weeks following the operation. The arrows show the duration of stimulation (5 s). The vertical calibration bars represent 20 impulses/s.

activities in the RVM promote neuropathic hypersensitivity in a tonic fashion during the first week following nerve injury. At a later phase of neuropathy, however, only the OFF-cell activity has a tonic pronociceptive effect, as a result of its decreased activity.

When assessing responses of RVM cells to peripheral stimulation, we focused on mechanical and cold stimulation, because clinical studies indicate that hypersensitivity to mechanical stimulation and cold are frequent and prominent symptoms after traumatic nerve injuries, whereas hyperalgesia to heat occurs only occasionally under neuropathic conditions (Scadding & Koltzenburg, 2006). Importantly, hypersensitivity to cold and mechanical stimulation is also a prominent finding in rats with the SNI model of neuropathy (Decosterd & Woolf, 2000). Because very little is known about the possible influence of somatic neuropathy on visceral sensations, we also determined responses of RVM cells to CRD.

The results indicate that SNI produced a significant hypersensitivity to cold in both ON- and OFF-cells throughout the observation period of 8 weeks. It should be noted, however, that the increase of excitatory responses to cold was considerably stronger in ON-cells than the increase of inhibitory ones in OFF-cells. Possibly, the SNI-induced reduction in the spontaneous activity of OFF-cells limited the amount of a further stimulus-evoked inhibition of OFF-cells. Responses to cutaneous pinch and visceral stimulation were markedly enhanced in ON-cells 8 weeks but not 1 week following nerve injury, whereas OFF-cell responses to pinch or visceral stimulation were not influenced by SNI. The present finding that hypersensitivity to peripheral stimulation was observed predominantly in presumably pronociceptive ON-cells of the RVM is in line with the hypothesis that a positive feedback loop involving ON-cells in the RVM is involved in promoting neuropathic hypersensitivity to peripheral stimulation. This hypothesis is further supported by the earlier behavioural findings indicating that nerve injury-induced hypersensitivity to mechanical stimulation (Pertovaara, 2000; Porreca *et al.*, 2002) and cold (Urban *et al.*, 2003) is dependent on descending facilitatory influence from the RVM. Moreover, it is noteworthy that although behaviourally assessed mechanical hypersensitivity develops within 24 h in the SNI model of neuropathy, it may not be fully developed until the second postoperative week (Decosterd & Woolf, 2000), whereas hypersensitivity to cold is fully developed within a week (Allchorne *et al.*, 2005). These behavioural findings parallel the present neurophysiological results with ON-cells. However, when considering the potential behavioural significance of SNI-induced changes in stimulus-evoked responses of RVM neurons in the present study, one should note that assessments of neuronal responses to noxious peripheral stimulation were not

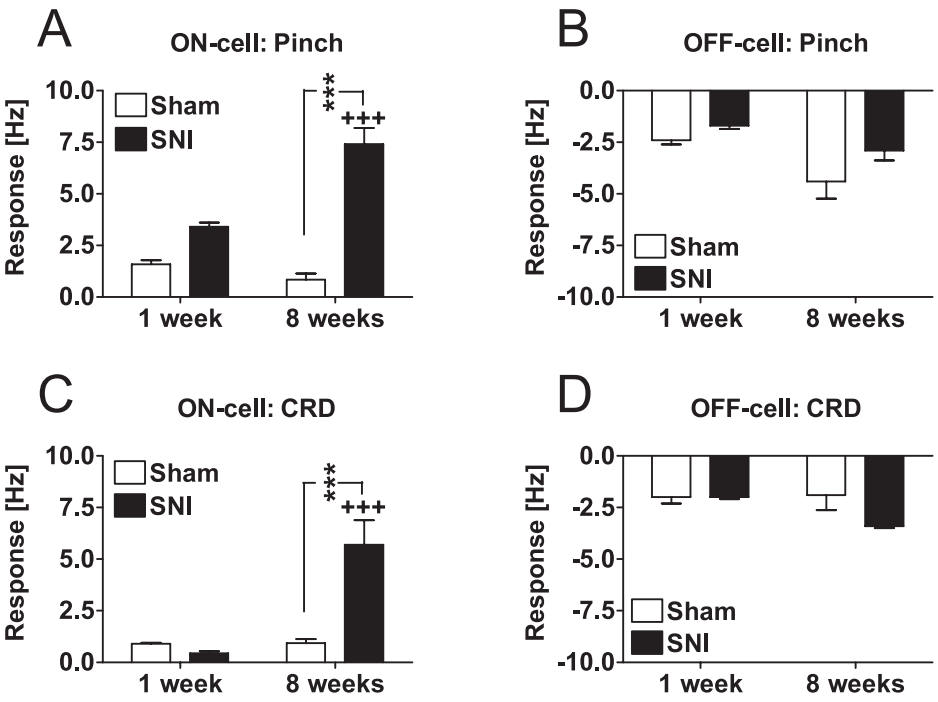

FIG. 6. Mean changes of ON-cell (A and C) and OFF-cell (B and D) discharge rates induced by noxious pinch of the tail or CRD at a noxious intensity (80 mmHg) 1 week and 8 weeks following sham operation or nerve injury. SNI, spared nerve-injury group; Sham, sham-operated group. $^{***}P < 0.005$ (Student–Newman–Keuls test; reference, the corresponding Sham group). $^{+++}P < 0.005$ (Student–Newman–Keuls test; reference, the corresponding group 1 week following operation). The error bars represent SEM ($n = 11$–29).

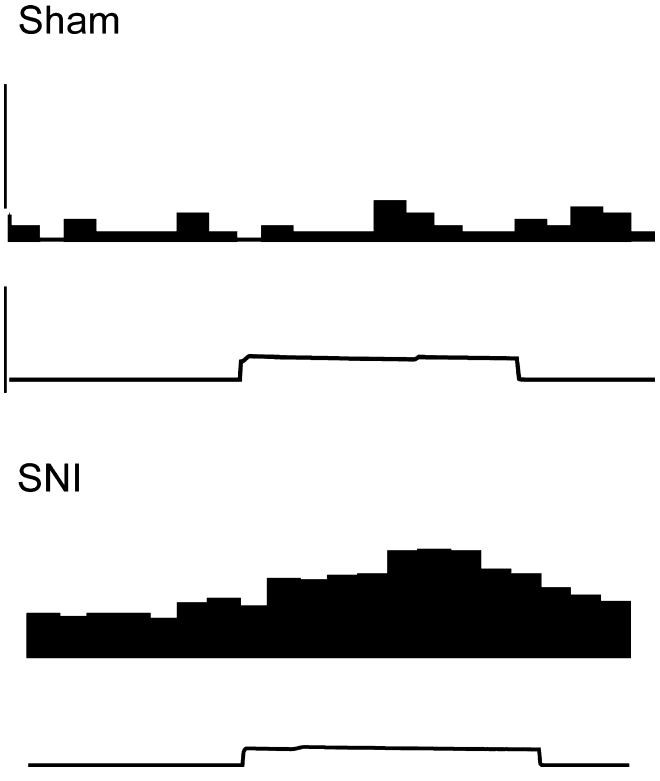

FIG. 7. Examples of original recordings of CRD-induced responses of ON-cells in a sham-operated animal and a nerve-injured (SNI) animal 8 weeks following the operation. The peristimulus time histogram of the neuronal response (above) and the change of stimulus intensity from 0 mmHg to 80 mmHg and back (below) are shown for each neuron. The calibration bar for the peristimulus time histogram represents 30 impulses/s. The duration of the CRD is 10 s.

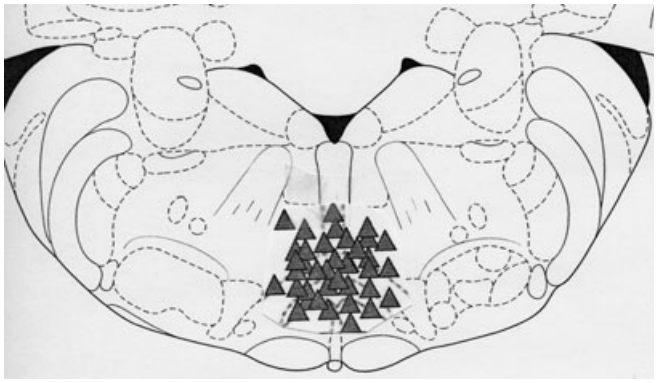

FIG. 8. Schematic diagram showing recording sites in the RVM.

accompanied by simultaneous assessments of corresponding behavioural responses. Moreover, behavioural responses of SNI animals to two of the currently used stimuli (pinch of an uninjured tail and CRD) have not been assessed in any previous study.

Comparison with previous studies

In line with the present findings, it has been shown earlier that ON-cells in the RVM have a pronociceptive role in acute inflammation as indicated by the association of their discharge rate with heat hypersensitivity induced by mustard oil (Kincaid *et al.*, 2006; Xu *et al.*, 2007). Previous studies addressing the contribution of RVM cells to neuropathic hypersensitivity have given partly contradictory results. It has been reported that chemical ablation of RVM cells expressing the μ-opioid receptor both prevents and reverses

spinal nerve ligation-induced experimental neuropathy (Porreca *et al*., 2001). Because ON-cells are the only RVM neurons responding directly to μ-opioid agonists (Heinricher *et al*., 1992), the selective reversal of neuropathic hypersensitivity by ablation of medullary cells expressing μ-opioid receptors suggests, in line with the present results, that ON-cells promote neuropathic hypersensitivity. On the other hand, the results of our previous electrophysiological study (Pertovaara *et al*., 2001) suggested that spinal nerve ligation-induced neuropathy of 2–3 weeks' duration may not produce as marked pronociceptive changes in response properties of RVM cells as the SNI model of neuropathy in the present study or acute inflammation in other studies (Kincaid *et al*., 2006; Xu *et al*., 2007). Among possible explanations of the difference between the results of our present and earlier study (Pertovaara *et al*., 2001) are the differences in the genetic background of the animals, model of neuropathy, and postoperative time point of study. Interestingly, the sciatic constriction-induced model of neuropathy had no significant influence on response properties of RVM cells giving excitatory responses to noxious stimulation (Luukko & Pertovaara, 1993), whereas complete denervation of the sciatic area, as opposed to incomplete denervation in the present study, produced an enhancement of excitatory responses of RVM cells to peripheral stimulation of the adjacent intact skin area (Pertovaara & Kauppila, 1989). Together, these findings support the hypothesis that the magnitude or time course of the change in the response properties of RVM cells may vary with the model of peripheral neuropathy, with models involving sectioning of the peripheral nervous system resulting in strong neuropathic changes of the supraspinal pain control system. As anatomical feedback loops implicated in the crosstalk between the brainstem and spinal cord are essential for pain modulation (Almeida *et al*., 2006), the drastic changes associated with the afferent barrage of nociceptive messages after nerve fibre sectioning may have a role in the differences observed between models. In the specific case of the RVM, a clear anatomical basis for reciprocal interaction exists, as it receives direct projections from the spinal cord (Chaouch *et al*., 1983) and ON- and OFF-cells are known to project directly to dorsal horn laminae I–II and V (Fields *et al*., 1995) and modulate spinal nociceptive transmission (Fields *et al*., 2006).

Various models of peripheral neuropathy may increase spontaneous discharge rates and responses to somatic stimulation in nociceptive spinal dorsal horn neurons (e.g. Palecek *et al*., 1992; Laird & Bennett, 1993; Takaishi *et al*., 1996; Pertovaara *et al*., 1997), whereas their responses to visceral stimulation were not influenced 2–3 weeks after spinal nerve injury (Kalmari *et al*., 2001). It remains to be studied whether the SNI-induced changes in response properties of RVM cells observed in the present study reflect corresponding changes in response properties of spinal dorsal horn neurons, changes in supraspinal processing of nociceptive signals, or both.

Conclusions

The SNI model of peripheral neuropathy induced pronociceptive changes in response properties of RVM neurons that are considered to have an important role in descending regulation of pain. These pronociceptive changes included an increased baseline discharge rate in presumably pronociceptive ON-cells and a decreased baseline discharge rate in presumably antinociceptive OFF-cells. Moreover, responses to mechanical stimulation and cold were markedly enhanced in the pronociceptive cell type. It is proposed that the SNI-induced changes in spontaneous discharge rates of ON- and OFF-neurons of the RVM make a tonic contribution to maintenance of neuropathic hypersensitivity, whereas the enhanced responses of pronociceptive ON-cells to peripheral stimulation promote hypersensitivity via a positive feedback loop.

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Abbreviations

CRD, colorectal distension; RVM, rostroventromedial medulla; SNI, spared nerve injury.

References

Allchorne, A.J., Broom, D.C. & Woolf, C.J. (2005) Detection of cold pain, cold allodynia and cold hyperalgesia in freely behaving rats. *Mol. Pain*, **1**, Art no. 36.

Almeida, A., Leite-Almeida, H. & Tavares, I. (2006) Medullary control of nociceptive transmission: reciprocal dual communication with the spinal cord. *Drug Discov. Today: Dis. Mech.*, **3**, 305–312.

Anderson, R.H., Ness, T.J. & Gebhart, G.F. (1987) A distension control device useful for quantitative studies of hollow organ sensation. *Physiol. Behav.*, **41**, 635–638.

Chaouch, A., Menetrey, D., Binder, D. & Besson, J.M. (1983) Neurons at the origin of the medial component of the bulbopontine spinoreticular tract in the rat: an anatomical study using horseradish peroxidase retrograde transport. *J. Comp. Neurol.*, **214**, 309–320.

Decosterd, I. & Woolf, C.J. (2000) Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain*, **87**, 149–158.

Devor, M. (2006) Response of nerves to injury in relation to neuropathic pain. In McMahon, S.B. & Koltzenburg, M. (Eds), *Wall and Melzack's Textbook of Pain*, 5th edn. Elsevier, Location, China, pp. 905–927.

Fields, H.L., Basbaum, A.I. & Heinricher, M.M. (2006) Central nervous system mechanisms of pain modulation. In McMahon, S.B. & Koltzenburg, M. (Eds), *Wall and Melzack's Textbook of Pain*, 5th edn. Elsevier, Location, China, pp. 125–142.

Fields, H.F., Mallick, A. & Burstein, R. (1995) Dorsal horn projection targets of on and off cells in the rostral ventromedial medulla. *J. Neurophysiol.*, **74**, 1742–1759.

Gebhart, G.F. (2004) Descending modulation of pain. *Neurosci. Biobehav. Rev.*, **27**, 729–737.

Heinricher, M.M., Morgan, M.M. & Fields, H.L. (1992) Direct and indirect actions of morphine on medullary neurons that modulate nociception. *Neuroscience*, **48**, 533–543.

Kalmari, J., Niissalo, S., Konttinen, Y.T. & Pertovaara, A. (2001) Modulation of visceral nociceptive responses of rat spinal dorsal horn neurons by sympathectomy. *Neuroreport*, **12**, 797–801.

Kincaid, W., Neubert, M.J., Xu, M., Kim, C.J. & Heinricher, M.M. (2006) Role for medullary pain facilitating neurons in secondary thermal hyperalgesia. *J. Neurophysiol.*, **95**, 33–41.

Laird, J.M. & Bennett, G.J. (1993) An electrophysiological study of dorsal horn neurons in the spinal cord of rats with an experimental peripheral neuropathy. *J. Neurophysiol.*, **69**, 2072–2085.

Luukko, M. & Pertovaara, A. (1993) Influence of an experimental peripheral mononeuropathy on the responses of medial bulboreticular neurons to noxious skin stimulation and the modulation of the responses by an α_2-adrenoceptor agonist in the rat. *Exp. Neurol.*, **124**, 390–394.

Luukko, M., Konttinen, Y., Kemppinen, P. & Pertovaara, A. (1994) Influence of various experimental parameters on the incidence of mechanical and thermal hyperalgesia induced by a constriction mononeuropathy of the sciatic nerve in rats. *Exp. Neurol.*, **128**, 143–154.

Mason, P. (2006) Descending pain modulation as a component of homeostasis. In Cervero, F. & Jensen, T.S. (Eds), *Handbook of Clinical Neurology*, **Vol. 81**. Elsevier, Amsterdam, pp. 211–218.

Ness, T.J., Randich, A. & Gebhart, G.F. (1991) Further behavioral evidence that colorectal distension is a 'noxious' visceral stimulus in rats. *Neurosci. Lett.*, **131**, 113–116.

Ossipov, M.H., Lai, J. & Porreca, F. (2006) Mechanisms of experimental neuropathic pain: integration from animal models. In McMahon, S.B. & Koltzenburg, M. (Eds), *Wall and Melzack's Textbook of Pain*, 5th edn. Elsevier, Location, China, pp. 929–946.

Palecek, J., Paleckova, V., Dougherty, P.M., Carlton, S.M. & Willis, W.D. (1992) Responses of spinothalamic tract cells to mechanical and thermal stimulation of skin in rats with experimental peripheral neuropathy. *J. Neurophysiol.*, **67**, 1562–1573.

Paxinos, G. & Watson, C. (1998) *The Rat Brain in Stereotaxic Coordinates*. Academic Press, Sydney.

Pertovaara, A. & Kauppila, T. (1989) Effect of chronic sciatic nerve section on saphenous nerve input to midline bulboreticular formation in the rat. *Neurosci. Lett.*, **105**, 68–72.

Pertovaara, A., Kontinen, V.K. & Kalso, E.A. (1997) Chronic spinal nerve ligation induces changes in response characteristics of nociceptive spinal dorsal horn neurons and in their descending regulation originating in the periaqueductal gray in the rat. *Exp. Neurol.*, **147**, 428–436.

Pertovaara, A. (2000) Plasticity in descending pain modulatory systems. *Prog. Brain Res.*, **129**, 231–242.

Pertovaara, A., Keski-Vakkuri, U., Kalmari, J., Wei, H. & Panula, P. (2001) Response properties of neurons in the rostroventromedial medulla of neuropathic rats: attempted modulation of responses by [1DMe]NPYF, a neuropeptide FF analogue. *Neuroscience*, **105**, 457–468.

Pertovaara, A. & Almeida, A. (2006) Endogenous pain modulation. Descending inhibitory systems. In Cervero, F. & Jensen, T.S. (Eds), *Handbook of Clinical Neurology*, **Vol. 81**. Elsevier, Amsterdam, pp. 179–192.

Porreca, F., Burgess, S.E., Gardell, L.R., Vanderah, T.W., Malan, T.P. Jr, Ossipov, M.H., Lappi, D.A. & Lai, J. (2001) Inhibition of neuropathic pain by selective ablation of brainstem medullary cells expressing the μ-opioid receptor. *J. Neurosci.*, **21**, 5281–5288.

Porreca, F., Ossipov, M.H. & Gebhart, G.H. (2002) Chronic pain and medullary descending facilitation. *Trends Neurosci.*, **25**, 319–325.

Scadding, J.W. & Koltzenburg, M. (2006) Painful peripheral neuropathies. In McMahon, S.B. & Koltzenburg, M. (Eds), *Wall and Melzack's Textbook of Pain*, 5th edn. Elsevier, Location, China, pp. 973–999.

Takaishi, K., Eisele, J.H. Jr & Carstens, E. (1996) Behavioral and electrophysiological assessment of hyperalgesia and changes in dorsal horn responses following partial sciatic nerve ligation in rats. *Pain*, **66**, 297–306.

Urban, M.O., Hama, A.T., Bradbury, M., Anderson, J., Varney, M.A. & Bristow, L. (2003) Role of metabotropic glutamate receptor subtype 5 (mGluR5) in the maintenance of cold hypersensitivity following a peripheral mononeuropathy in the rat. *Neuropharmacology*, **44**, 983–993.

Vanegas, H. & Schaible, H.G. (2004) Descending control of persistent pain: inhibitory or facilitatory? *Brain Res. Rev.*, **46**, 295–309.

Woolf, C.J. & Salter, M.W. (2006) Plasticity and pain: role of the dorsal horn. In McMahon, S.B. & Koltzenburg, M. (Eds), *Wall and Melzack's Textbook of Pain*, 5th edn. Elsevier, Location, China, pp. 91–105.

Xu, M., Kim, C.J., Neubert, M.J. & Heinricher, M.M. (2007) NMDA receptor-mediated activation of medullary pronociceptive neurons is required for secondary thermal hyperalgesia. *Pain*, **127**, 253–262.

Chapter 2.2

Gonçalves L, Silva R, Pinto-Ribeiro F, Pêgo JM, Bessa JM, Pertovaara A, Sousa N, Almeida A

Neuropathic pain is associated with depressive behaviour and induces neuroplasticity in the amygdala of the rat

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Neuropathic pain is associated with depressive behaviour and induces neuroplasticity in the amygdala of the rat

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ABSTRACT

Chronic pain is associated with the development of affective disorders but the underlying mechanisms are not fully understood. Changes in brain centres implicated in both emotional and pain processing are likely to be critical in the interplay of pain control and affective emotional behaviour. In the present study, we assessed emotional behaviour and performed a structural analysis of the amygdala (AMY) in neuropathic rats after two months of hyperalgesia and allodynia, induced by the spared nerve injury model (SNI). When compared with Sham-controls, SNI animals displayed signs of depressive-like behaviour. In addition, we found an increased amygdalar volume in SNI rats. No alterations were found in the dendritic arborizations of AMY neurons but, surprisingly, the amygdalar hypertrophy was associated with an increased cell proliferation [bromodeoxyuridine (BrdU)-positive cells] in the central (CeA) and basolateral (BLA) amygdaloid nuclei. The phenotypic analysis of the newly-acquired cells revealed that they co-label for neuronal markers (BrdU+NeuN and BrdU+Calbindin), but not for differentiated glial cells (BrdU+glial fibrillary acidic protein).
We demonstrate that neuropathic pain promotes generation of new neurons in the AMY. Given the established role of the AMY in emotional behaviour, we propose that these neuroplastic changes might contribute for the development of depressive-like symptoms that are usually present in prolonged pain syndromes in humans.

Introduction

Pain is a multidimensional experience with sensitive–discriminative and motivational-affective dimensions (Anand and Craig, 1996). Persistent pain, including chronic pain syndromes (Tal and Bennett, 1994), is a common condition associated to a wide spectrum of disorders including cancer, inflammation and neuropathic pain. Neuropathic pain (NP) is caused by a primary lesion or dysfunction of the nervous tissue (Merskey and Bogduk, 1994) and results in prolonged hyperalgesia, allodynia and spontaneous pain (Devor, 2006). NP results from a process of peripheral and central sensitization that generates an enhanced transmission of nociceptive input to the brain (Gao et al., 2005; Ren and Dubner, 1996), which may impair the endogenous supraspinal pain control system (Danziger et al., 2001; Kauppila et al., 1998; Pertovaara, 2000; Rasmussen et al., 2004; Tal and Bennett, 1994).

The amygdala (AMY) is a central component of the limbic system and plays a crucial role in behavioural responses to emotional stimuli (Davis and Whalen, 2001; Han and Neugebauer, 2004; Neugebauer and Li, 1992). Moreover, the AMY is deeply involved in processing the emotional component of pain, probably through a modulatory role upon major supraspinal pain control centres (SPCC) (Manning and Mayer, 1995; Manning, 1998; Manning et al., 2001). On the other hand, it is possible that neuroplasticity in higher centres controlling SPCC may contribute to alterations in the fine control of pain. In fact, an imbalance between inhibiting and facilitating descending modulation of nociceptive transmission may underlie, at least in part, the development of chronic pain (Almeida et al., 2006; Lima and Almeida, 2002; Pertovaara, 2000; Porreca et al., 2002; Schaible et al., 1991). Accordingly, arthritic and neuropathic pain enhance synaptic transmission of nociceptive-specific input to the AMY (Han and Neugebauer, 2004; Neugebauer and Li, 1992; Neugebauer et al., 2003), which reinforces the potential role of AMY in SPCC alterations resulting from prolonged pain syndromes.

Chronic pain induces mood disorders, including depression and anxiety (Rasmussen and Rummans, 2002). In addition, the adverseness of pain is amplified or reduced depending on the emotional environment (Merskey, 1965), and conditions of increased anxiety (Rhudy and Meagher, 2000) and depression (Merskey, 1965; Willoughby et al., 2002; Zelman et al., 1991) are usually associated with decreased pain tolerance. This vicious circle may trigger, or even result from, neuronal changes in the limbic system. Accordingly, imaging studies indicate that gross structural changes may occur in the AMY in situations of major depression (Altshuler et al., 2005; Bremner et al., 2000; Frodl et al., 2002; Strakowski et al., 1999; Tebartz van Elst et al., 2000).

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As a rationale for the present study, we hypothesized that chronic pain induces emotional disturbances that are associated with neuroplasticity of the amygdaloid complex. To assess this hypothesis, we performed behavioural, stereological and immunocytochemical analysis during or after the induction of a two month neuropathy following the model of Decosterd and Woolf (2000). Part of the present results have already been published in abstract form (Gonçalves et al., 2006).

Materials and methods

Animals

All procedures were performed on adult (200–250 g, 55–65 days) male Wistar–Han rats. Animals were housed under standard laboratory conditions (12 h light cycle; 22 °C, 55% humidity; food and water available *ad libitum*). Experiments were conducted in accordance with local regulations, European Union Directive 86/609/EEC, NIH guidelines on animal care experimentation and IASP ethical guidelines for pain experimentation on awaken animals (Zimmermann, 1983). Sixty animals were divided in two main experimental groups of 30 rats each: spared nerve injury (SNI) and sham operated (Sham). A set of rats (n=18 each group) received one injection of the cell proliferation marker bromodeoxyuridine (BrdU; Miller and Nowakowski, 1988), 50 mg/kg body weight, i.p. (Sigma, St. Louis, MO) for three consecutive days before their death (see below), two months after SNI induction or Sham surgery.

Spared nerve injury surgery

The SNI model of chronic neuropathic pain included an axotomy and ligation of two of the three peripheral ramifications of the sciatic nerve, the tibial and common peroneal nerves and leaving the sural nerve intact, as described elsewhere (Decosterd and Woolf, 2000). The animals were lightly anesthetized with pentobarbital 0.5% (Eutasil, Ceva Saúde Animal, Portugal). The common peroneal and tibial nerves were tight-ligated with 5.0 silk and sectioned distal to the ligation, removing 2–4 mm of the distal nerve stump. Great care was taken to avoid any contact with or stretching of the intact sural nerve. Muscle and skin were closed in two layers. Sham-controls involved exposure of the sciatic nerve and its branches without performing any manipulation.

Nociceptive tests

Nociceptive tests were performed in all animals a day before and two days after the surgery procedure, followed by testing every two days then forward, during the two months of experimental period. Both the ipsilateral (right hind paw) and the contralateral hind paw were tested in order to evaluate the presence of "mirror pain", described elsewhere as present in neuropathic pain pathologies (Tal and Bennett, 1994).

Mechanical allodynia

Animals were placed on an elevated wire grid and the lateral plantar surface of the paw stimulated with a series of ascending force von Frey monofilaments. The nociceptive threshold was taken as the lowest force that evoked a brisk withdrawal response to one of five repetitive stimuli (Tal and Bennett, 1994).

Mechanical hyperalgesia

With the animals on the elevated grid, a pin-prick test was performed using a safety pin. The lateral part of the plantar surface of the paw was briefly stimulated at intensity sufficient to touch but not penetrate the skin (Decosterd et al., 1998). The duration of paw withdrawal was measured, with an arbitrary minimal time of 0.5 seconds (s) (for the brief normal response) and maximal cut-off of 20 s (Tal and Bennett, 1994).

Assessment of emotional behaviour

All behavioural tests were performed five days preceding animal sacrifice during light period (9am to 6pm) in a restricted group of animals (n=18 each group).

Anxiety-like behaviour — elevated plus-maze test (EPM)

Anxiety-like behaviour was evaluated in the EPM test through an apparatus consisting of two open and two closed arms (50.8×10.2×40.6 cm each arm) (MedAssociates Inc., St. Albans, Vermont, USA). Each rat was placed in the centre of the elevated plus-maze facing one of the open arms, and the time spent (s) in the open or closed arms was recorded during a 5-min test period (Mesquita et al., 2006; Sousa et al., 2006). The elevated plus-maze was carefully cleaned with 10% ethanol before each animal was placed on the equipment.

Depressive-like behaviour — forced-swimming test (FST)

The test was performed as in the original method described elsewhere (Porsolt et al., 1977, 1978). On day 1 (conditioning, pre-test session), rats were individually placed in a clear Plexiglass cylinder (29 cm in diameter and 50 cm in height) containing 30 cm of water (25+0.5 °C) and left to swim for 5 min. The rats were then

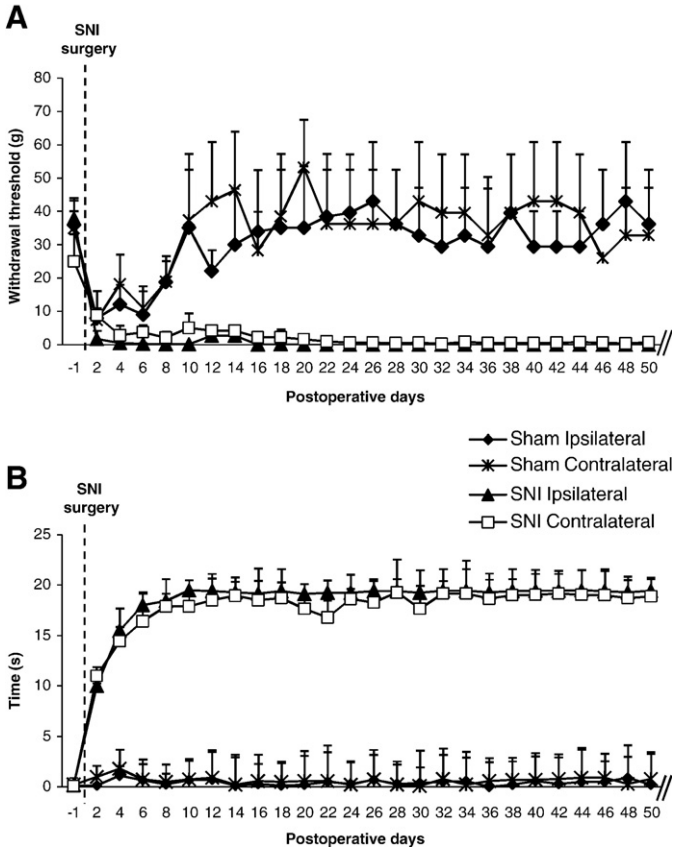

Fig. 1. Mechanical allodynia assessed by von Frey filaments (A) and mechanical hyperalgesia assessed by the pin-prick test (B) before and after surgery in SNI and Sham groups (dotted line indicates the day of SNI surgery). (A) Note that the pre-surgery threshold to von Frey filaments was similar in both SNI and Sham groups and in both hind paws; after surgery, the withdrawal threshold of the SNI group decreased within 24 h and remained low until the end of the 2 month experimental period. In Sham animals, the withdrawal threshold to von Frey filaments was decreased during the first postoperative days but returned to baseline values. (B) In the pin-prick test, SNI animals showed a strong hyperalgesia from the first postoperative day onwards, whereas Sham animals showed no hyperalgesia. The symbols and error bars represent mean+S.D.

removed from water and towel-dried, placed under a heating lamp for 5 min, and finally returned to their home cage. Twenty-four hours later, the rats were tested under the same conditions for 5 min (test session). Rats were judged to be immobile when both hind legs were not moving, and the rat was slightly bent forward (Mesquita et al., 2006).

Locomotion and exploratory behaviour — open field test (OF)

Motor activity and exploratory behaviour were evaluated by placing the rat into an infrared photobeam controlled open field activity test chamber in a brightly illuminated (white light) room. Animals were tested for 10 min in an arena (43.2 cm×43.2 cm transparent acrylic walls and white floor) (MedAssociates Inc., St. Albans, Vermont) that was divided into a central and a peripheral zone. The time spent by each animal in the central and peripheral (residual) zone and its vertical activity (rearings) were the parameters evaluated in this test (Mesquita et al., 2006). Environmental odours were removed with 10% ethanol solution.

Tissue preparation

Both the SNI and Sham groups were divided as follows: i) in the first group (n=6 each), designated to stereological analysis, the animals were anaesthetized with pentobarbital and perfused with 4% paraformaldehyde (PFA), the brains were removed, embedded in 2-hydroxyethyl glycol methacrylate, serially sectioned in a microtome at 30 μm and stained with Giemsa; ii) in the second group (n=6 each), designated to 3D-morphologycal analyses of dendritic arborization of AMY neurons, the animals were anesthetized with pentobarbital, perfused with saline and the brains were removed and processed for posterior staining following the Golgi-Cox method (Gibb and Kolb, 1998) and slicing in a vibratome at 100 μm; iii) in the third group (n=18 each), processed for immunocytochemistry for detection of BrdU, GFAP (glial fibrillary acidic protein), NeuN (neuronal nuclei) and Calb (Calbindin), the animals were decapitated, the brains dissected, frozen in liquid nitrogen and sectioned in a cryostat (−14 °C).

Stereological procedures

The amygdaloid complex was subdivided in its nuclear components as in Paxinos and Watson (1998): central (CeA), lateral (La), basolateral anterior (BLA) and posterior (BLP), basomedial anterior (BMA) and posterior (BMP) nuclei. The nuclei volume and cell number estimation in AMY nuclei in every 8th section stained with Giemsa was obtained through the Cavalieri's principle and optical fractionator methods using the Stereoinvestigator software (MicroBrightField, Inc., Williston, VT, USA).

3D-morphologycal analysis of dendrites

The brain sections stained with the Golgi-Cox method were observed at the optical microscope and multipolar and bipolar AMY neurons completely and perfectly stained (Cerqueira et al., 2007) were considered for further analysis using the Neurolucida software (MicroBrightField, Inc., Williston, VT, USA). The dendrites and spines of 6 AMY neurons per animal were drawn.

Immunohistochemical procedures

All quantifications of markers for cell division and neuronal fate were performed in the AMY. Positive controls for histochemical reactions were confirmed by analysing the subgranular zone (SGZ) of the hippocampus, since neuronal proliferation is known to occur in this area (Gould et al., 1999a). As negative controls of immunocytochemical reactions, the primary antibody was not included in the protocol of each reaction; no specific immunoreaction was observed following negative controls.

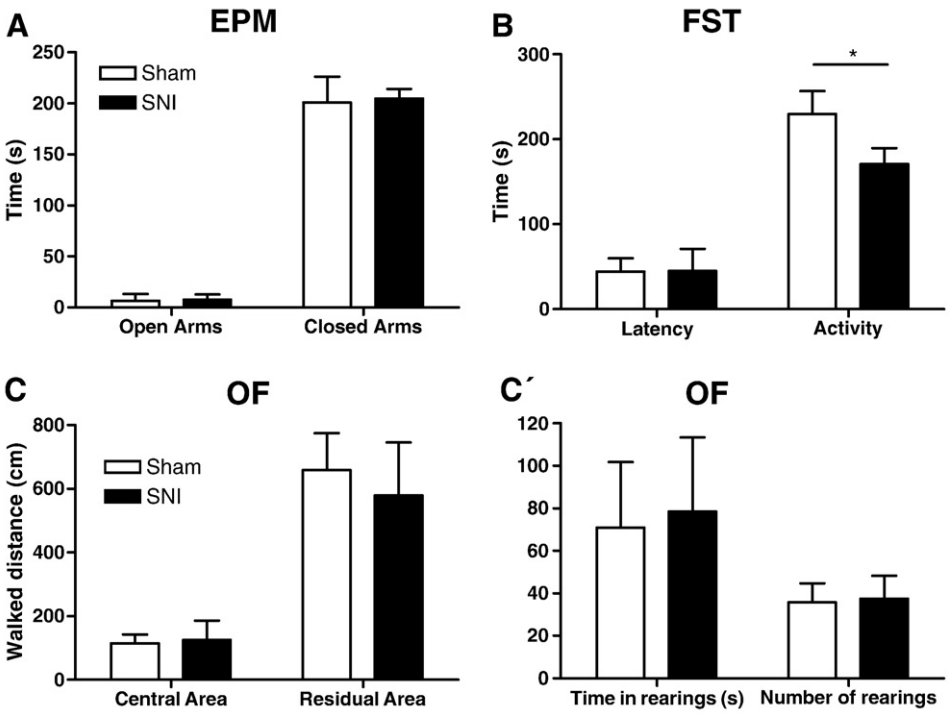

Fig. 2. Performance of SNI and Sham groups during behavioural tests. No differences were observed between the two groups in the EPM test (A), neither in the time spent in the open or closed arms. In the FST (B), the time of activity was lower in SNI animals, which indicates the presence of depressive-like behaviour. No differences were observed for the OF test (C, C′) in any of the parameters evaluated.

BrdU immunohistochemistry and quantification of BrdU-labelled cells

Bromodeoxyuridine (BrdU; an analogue of thymidine, incorporated into the newly synthesized DNA of replicating cells) incorporation was detected by immunocytochemistry on every 8th serial brain section containing the amygdaloid complex. Briefly, sections were fixed in 4% PFA for 30 minutes (min), permeabilized for 10 min in a solution containing 0.2% Triton X-100 in Tris buffer saline (TBS) after a 3×3 min wash in TBS, heated during 20 min in citrate buffer 0.1 M following a 3×3 min wash and acidified in HCl 2 M for 30 min after rinsing in distillated water. Endogenous peroxidase activity was blocked with 3% H2O2 in TBS for 10 min after a 3×3 min wash in TBS, followed by immersion in 4% bovine serum albumin (BSA) in TBS for 30 min (to block non-specific staining) after a 3×3 min wash. After another 3×3 min wash in TBS, the tissue was incubated overnight with a primary monoclonal anti-BrdU antibody raised in mouse (1:50, Dako, Glostrup, DK) and stained cells were detected using a universal detection system (BioGenex, San Ramon, CA, USA) and diaminobenzidine (DAB 0.025% and H2O2 0.5% in Tris–HCl 0.05M pH 7.2), after a 3×2 min wash in TBS and a 1×3 min wash in Tris–HCl, followed by counterstaining with haematoxylin. BrdU-positive cells were counted throughout the entire AMY area.

Immunofluorescence and quantification of double-labelled cells

Double-staining immunofluorescent reactions were performed in order to reveal three different groups: (i) BrdU and GFAP (glial fibrillary acidic protein; a marker of astrocyte glial cells; Reeves et al., 1989), (ii) BrdU and NeuN (protein expressed exclusively in mature neurons; Mullen et al., 1992) and (iii) BrdU and Calb (Calbindin; a calcium-binding protein present in functional mature neurons; Meguro et al., 2004). The following primary antibody dilutions were used: rat anti-BrdU (1:500, Accurrate, Westbury, MA), mouse anti-GFAP (1:500, Dako Glostrup, Denmark), mouse anti-NeuN (1:500, Chemicon International, Temecula, CA, USA) and rabbit anti-Calb (1:200, Chemicon International, Temecula, CA, USA). The initial protocol procedure (until the primary antibody incubation) was the same in the first three groups and similar to that described above for revealing BrdU. The following specific procedures for each double-staining method are explained briefly and separately for each group.

Brain sections were mounted in slides with Vectashield (Vector Laboratories, Burlingame, CA, USA) to delay fluorescence decay, and observed two days later in a fluorescence microscope. Data were confirmed posteriorly using confocal microscopy (Olympus FluoViewTM FV1000, OLYMPUS).

i) BrdU and GFAP

After overnight incubation with the primary antibody anti-BrdU raised in rat, sections were washed 3×2 min in TBS and then incubated with a fluorescent Alexa 568 secondary antibody (goat anti-rat, 1:200; Molecular Probes, Eugene, OR, USA) for 1 h. Following a 3×3 min wash in TBS, sections were incubated during 3 h with the primary antibody mouse anti-GFAP, followed by the fluorescent Alexa 488 secondary antibody (goat anti-mouse, 1:100, Molecular Probes, Eugene, OR, USA) for 1 h. The sections were finally washed 2×2 min in TBS and 2 min in distillate water before being mounted in slides.

ii) BrdU and NeuN

Sections were incubated overnight with the primary antibody anti-BrdU raised in rat followed by the fluorescent Alexa 568 secondary antibody (goat anti-rat, 1:200; Molecular Probes, Eugene, OR, USA) for 1 h, after a 3×3 min wash in TBS. Then, sections were immersed for 3 h with the primary antibody anti-NeuN raised in mouse (1:500) and washed 3×3 min. Subsequently, they were incubated with biotinylated secondary antibody anti-mouse (1:200) for 1 h and, after a 3×3 min wash, incubated with Alexa Streptavidine 488 (1:100, Molecular Probes, Eugene, OR, USA) for one final hour. The sections were washed in TBS and distillate water as above and mounted in slides.

iii) BrdU and Calb

Sections were incubated overnight with the rat anti-BrdU and mouse anti-Calb primary antibodies. In the next day, after a 3×3 min wash in TBS sections were firstly incubated with fluorescent Alexa 568 (goat anti-mouse, 1:200) secondary antibody for 1 h and then with fluorescent Alexa 488 (goat anti-rat, 1:200; Molecular Probes, Eugene, OR, USA) secondary antibody, after a 3×3 min wash. The sections were washed in TBS and distillate water and mounted in slides.

Statistic analysis

For the analysis of baseline thresholds of SNI/Sham and ipsi/contralateral hind paws in the von Frey and pin-prick tests, one-way analysis of variance (ANOVA) was performed. Considering that in the

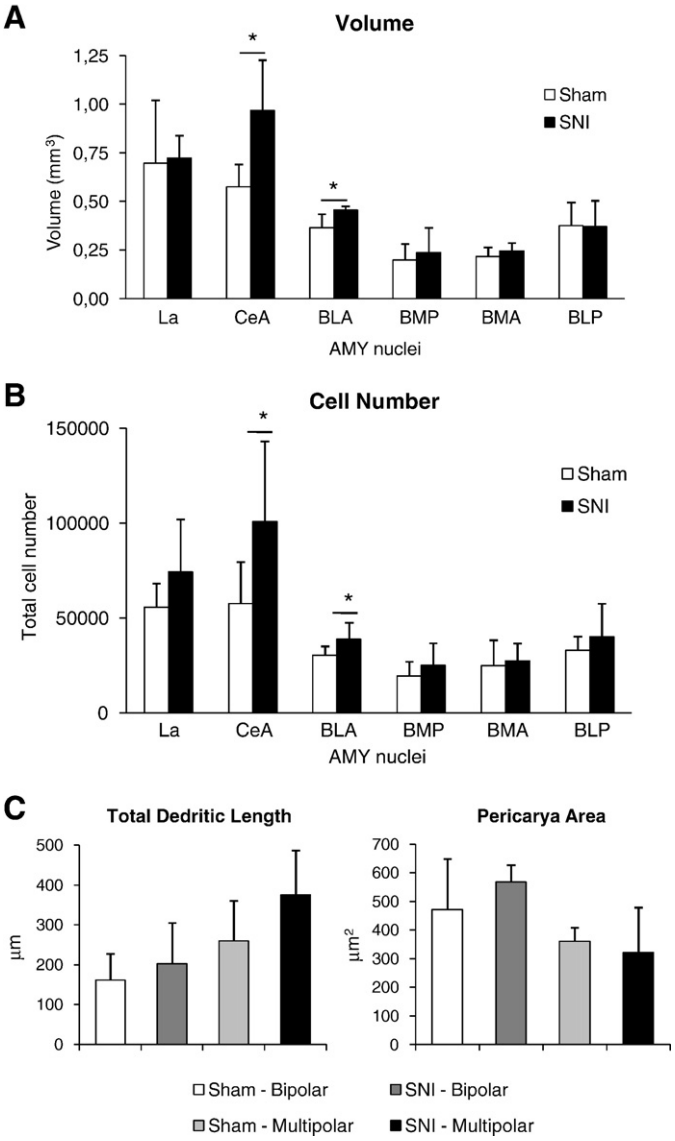

Fig. 3. Morphological analysis of AMY nuclei. (A) Volumes of different AMY nuclei were higher in neuropathic animals when compared to Sham, with differences being statistically significant for the CeA and BLA nuclei. (B) Cell number is also higher in all amygdalar nuclei of SNI animals, with differences being significant again in the CeA and BLA nuclei. (C) Structural analysis through Golgi-Cox method showed no differences in cell body volume and dendrite length of AMY neurons between SNI and Sham groups.

rest of this study only comparisons between two groups were performed, the Student's *t* test was used to analyse the results of all tests and procedures. The results were considered to be statistically different when $p<0.05$. Data are presented as mean± standard deviation (SD).

Results

The spared nerve injury model induces hypersensitivity for at least 2 months

Assessment of mechanical allodynia and hyperalgesia using, respectively, von Frey filament and pin-prick tests, were performed twice before the SNI surgery (baseline measurements) and every two days afterwards (during a two month period). Both neuropathic (SNI group) and sham-control (Sham group) animals presented a similar baseline withdrawal threshold measured by von Frey filaments (SNI: ipsilateral 38±6.1 g, contralateral 25±8.2 g; Sham: ipsilateral 36±7.3 g, contralateral 35±5.1 g; Fig. 1A). A bilateral decrease in nociceptive threshold was observed in neuropathic animals within 24 h after surgery. This threshold decrease reached the level of 0–5 g four days after the surgery, a value that remained constant until the end of the two month experimental period. These data showed that the SNI group developed and maintained a strong mechanical allodynia in both hind paws, as a consequence of the surgery. On the contrary, nociceptive threshold in Sham animals decreased slightly with the sham surgery, returning to baseline values within a week, never reaching thresholds as low as those presented by SNI animals (Fig. 1A). In what concerns the pin-prick test, the baseline duration of hyperalgesic behaviour was less than 1 s in all animals, and there were no differences between groups (SNI: ipsilateral 0.17±0.17 s, contralateral 0.11±0.8 s; Sham: ipsilateral 0.13±1.11 s, contralateral 0.2±0.2; Fig. 1B). Within 24 h from the surgery, SNI animals reached the maximal duration of hyperalgesic behaviour in both hind paws (20 s) whereas no changes were observed in Sham animals (Fig. 1B). These data showed that the SNI group developed and maintained a clear hyperalgesic state during virtually the entire experimental period. In summary, data on pain-related behaviour demonstrated that SNI animals developed a clear neuropathy that extended throughout the complete experimental period.

Neuropathic animals develop a depressive-like behaviour but do not display signs of increased anxiety

Emotional behaviour was assessed seven weeks after the surgery. EPM was performed to evaluate anxious behaviour, FST to assess depressive-like behaviour and the OF test to determine locomotion and exploratory behaviour (Mesquita et al., 2006). In the EPM, no differences were found in the behavioural responses between SNI and

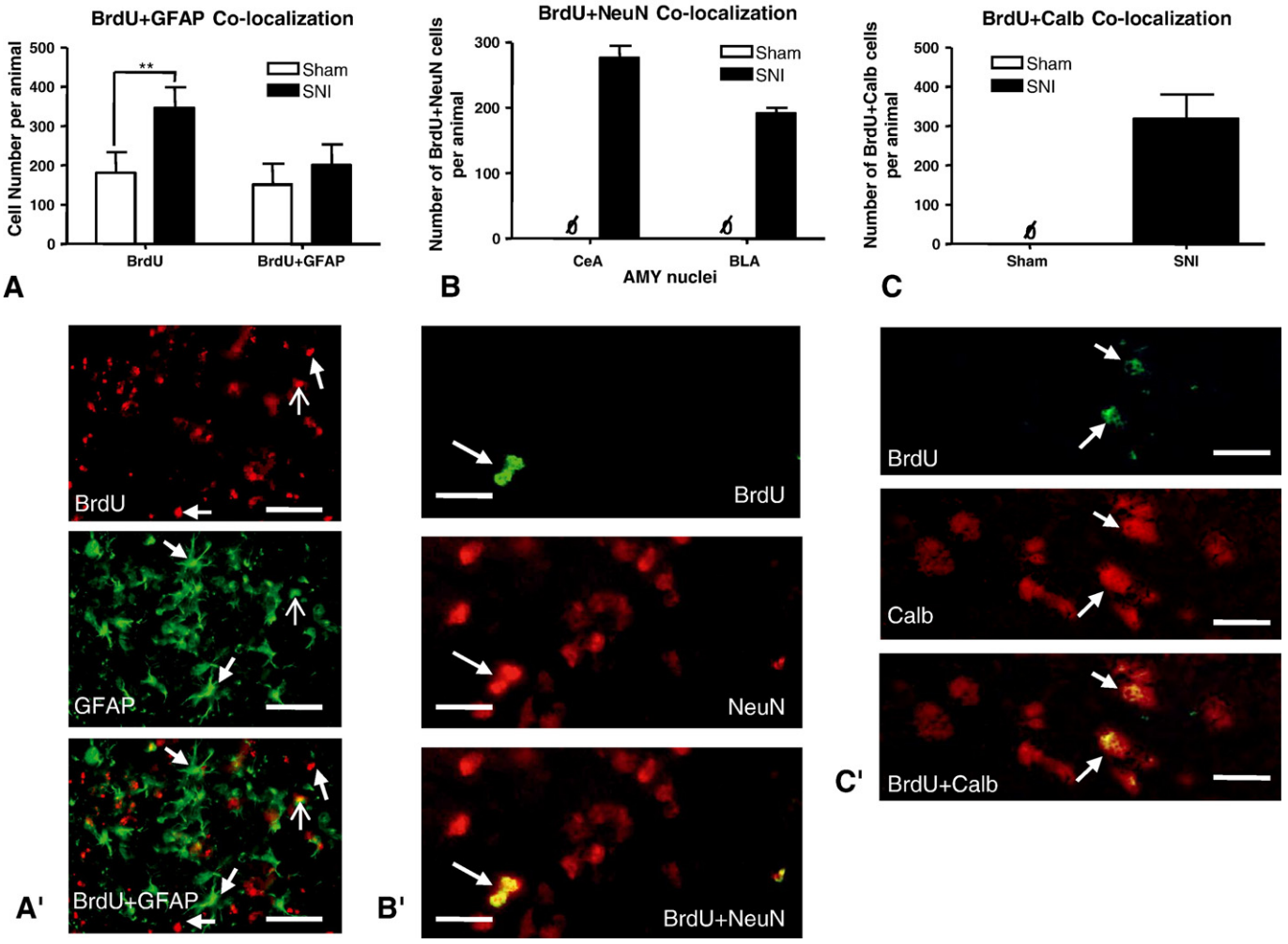

Fig. 4. Cell fate resulting from amygdalar neuroplasticity. (A) The number of cells that were BrdU-positive was significantly superior in SNI animals, but no differences were observed in the number of BrdU+GFAP double-labelled cells between SNI and Sham groups. (A′) Representative images of GFAP, BrdU and GFAP+BrdU (double-stained)-positive cells. (B) BrdU+NeuN double-labelled cells were present only in AMY nuclei, being absent in Sham animals. (B′) Representative images of BrdU, NeuN and NeuN+BrdU double-stained cells. (C) Calb+BrdU double-stained cells were also present only in neuropathic animals. (C′) Representative images of BrdU, Calb and Calb+BrdU double-stained cells. Magnification bar: 60 μm (A′), 20 μm (B′, C′).

Sham groups (Fig. 2A), thereby showing that the anxiety levels were unaltered by induction of SNI. On the other hand, the FST revealed significant differences between experimental groups (Fig. 2B): while Sham animals were active for 230±27 s, SNI animals only tried to escape/swim for 180±38 s (p=0.012), which indicates the presence of a learned helplessness (depressive-like) behaviour in neuropathic animals. Since FST test includes movement of the paws and neuropathic animals are hyperalgesic and allodynic in both ipsilateral and contralateral hind paws, the OF test was performed in order to validate the FST test. This test revealed that the SNI group had no differences in the locomotion ability when compared with Sham group and it also revealed that the number of rearings (an indicator of exploratory behaviour) did not differ between experimental groups (Figs. 2C,C′). The absence of differences in the time spent in central vs. peripheral part of the OF arena also indicates the absence of altered anxiety behaviour in neuropathic animals. In summary, these behavioural studies demonstrate that a 2 month neuropathy induced a depressive-like, but not anxious-like, behaviour.

Volume and cell number are increased in amygdaloid nuclei

After animal perfusion, 6 brains of each experimental group were prepared for stereological analysis and other 6 SNI and Sham brains were processed for tri-dimensional morphological analysis. For stereological analysis the AMY was divided in 6 nuclei (Paxinos and Watson, 1998): central (CeA), lateral (La), basolateral anterior (BLA) and posterior (BLP), basomedial anterior (BMA) and posterior (BMP). We found a general increase in the volume of all these nuclei in SNI neuropathic animals, with a significant increase being observed in CeA (p=0.02) and BLA (p=0.019) nuclei (Fig. 3A). In order to determine the causes for these structural changes of AMY, we analysed potential alterations in cell numbers and cellular volumes. SNI neuropathic animals showed a general increase in the number of cells in all AMY nuclei, with a significant difference being present again in CeA (p=0.015) and BLA (p=0.016) nuclei (Fig. 3B). On the contrary, 3D-morphological analysis revealed no significant differences in dendritic lengths (Fig. 3C) or perikarya areas (Fig. 3D) between neuropathic and Sham animals, both in bipolar and multipolar AMY neurons. Taken together, these results indicate that the significant increase observed in CeA and BLA nuclear volumes of SNI animals was due, at least in part, to an increase in cell numbers.

Newborn neurons contribute to increased cell numbers in AMY

Rats received one injection of the cell proliferation marker bromodeoxyuridine (BrdU) in the three consecutive days before their sacrifice. The aim of this procedure was to determine if cell proliferation was responsible for the higher number of cells observed in the CeA and BLA nuclei in SNI animals. Immunohistochemistry revealed the presence of BrdU-positive cells in the AMY of both SNI and Sham groups, but with significantly higher numbers in neuropathic animals (p=0.001; Fig. 4A). In order to identify the phenotype of these newly-acquired cells, two different double-staining immunohistochemistry reactions were performed: BrdU+

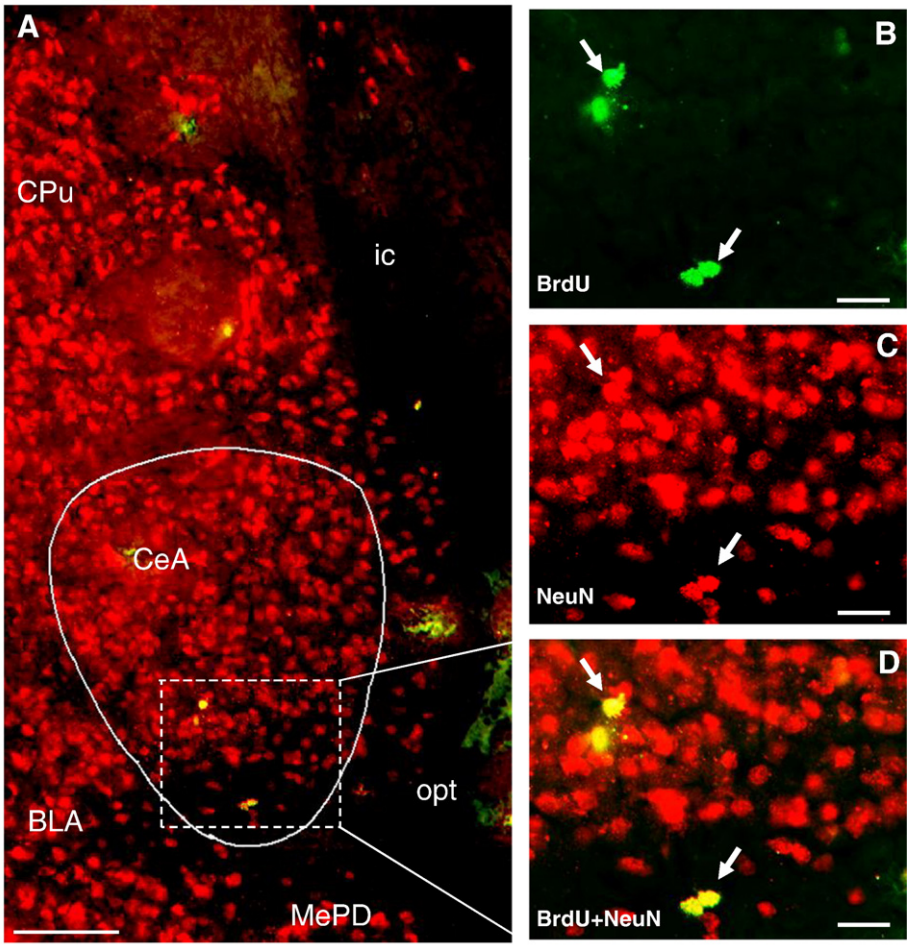

Fig. 5. (A–D) Microphotograph showing examples of BrdU+NeuN double-labelled cells in the CeA. The rectangle in micrograph A is magnified in figures B–D; the border of CeA nucleus is outlined by a continuous line. CPu — caudate putamen (striatum); MePD — medial amygdaloid nucleus, posterodorsal part; ic — internal capsule; opt — optic tract. Magnification bar: 100 μm (A), 20 μm (B–F).

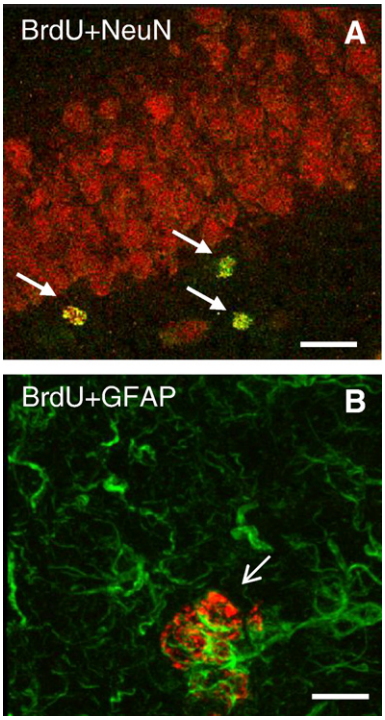

Fig. 6. Examples of BrdU+NeuN (A) and BrdU+GFAP (B) double-labelled cells (arrows) obtained in positive-control sections from the subgranular zone of the dentate gyrus of the hippocampus. Magnification bar: 20 μm (A), 10 μm (B).

glial fibrillary acidic protein marker (GFAP) and BrdU+post-mitotic neuronal marker (NeuN). The number of BrdU+GFAP-positive cells was similar between the SNI and Sham groups. On the other hand, BrdU+NeuN double-labelled cells were observed only in the SNI group; interestingly, they were mainly located in the CeA and BLA nuclei (Figs. 4B, B′, 5). These findings indicate the presence of newly proliferating neurons in the AMY after prolonged SNI, as further demonstrated by the presence of BrdU+Calbindin-positive cells in the AMY of neuropathic animals (Fig. 4C,C′). Positive control sections obtained from the subgranular zone of the hippocampal dentate gyrus showed the presence of both BrdU+NeuN and BrdU+GFAP double-labelled cells (Fig. 6).

In summary, data demonstrate not only that recently-divided newborn neurons are formed in the AMY of chronic pain animals, but also that these neurons reach a physiologically mature (i.e., functional) state.

Discussion

After two months of neuropathic pain, SNI animals exhibited signs of sustained persistent pain associated with a significant depressive-like behaviour. At the CNS level, a structural reorganization of the amygdaloid complex was observed that was associated with a significant increase in the volume of the basolateral (BLA) and central (CeA) AMY nuclei. The volume increase was due to an increased number of AMY cells, and not to hypertrophy of dendrites or perikarya of amygdalar neurons. The present study is the first demonstrating cell proliferation in a limbic area, as a result of chronic neuropathic pain. Earlier, only electrophysiological studies have shown chronic pain-related neuroplasticity of AMY neurons in persistent arthritis, visceral pain (Han and Neugebauer, 2004) or neuropathy (Ikeda et al., 2007). Moreover, this is the first study demonstrating that chronic pain results in depressive-like behaviour associated with neuroplasticity in a major brain centre implicated in the control of both emotions and pain.

Changes in emotional behaviour and neuroplasticity in the AMY

Morphological plasticity in the AMY was previously suggested in cases of prolonged emotional disturbance, as shown by increased AMY volumes measured by structural magnetic resonance in patients with depression and anxiety (Frodl et al., 2002; Tebartz van Elst et al., 2000). Clinical data also reveal that prolonged pain conditions are associated with a high incidence of emotional disorders, including anxiety and depression (Rasmussen et al., 2004). Herein, we show that in the rat, a two month neuropathy resulted also in a depressive-like behaviour measured by the forced-swimming test (FST), but no alterations in anxiety levels were detected in the elevated plus-maze and open field tests. We also show that increased immobility time in the FST should not be ascribed to motor impairments as there were no changes in locomotor activity and exploratory behavior. As in humans, SNI neuropathy associated with emotional alterations may result from, or contribute to, the structural changes observed in the AMY. It has been proposed that the increase in AMY volume observed in depressive patients was a consequence of the continuous prolonged activation of this area (Frodl et al., 2002). Following the same rationale, the present increase in AMY volume may result from the continuous flow of nociceptive information into AMY regions receiving sensory information (including the BLA) and the consequent prolonged activity of AMY neurons triggering the appropriate response action (CeA is the main effector of AMY). Especially relevant is the increase in the CeA volume, as its latero-capsular part is defined as the 'nociceptive amygdala' due to its high content in neurons implicated in nociceptive processing (Bernard et al., 1996; Neugebauer and Li, 1992; Neugebauer et al., 2004).

The volume increase in the AMY after two months of neuropathic pain may have resulted from one or various different processes: cell size (soma and dendritic size) increase, cell number (neurons or glial cells) increase, or increased extracellular volume. However, subsequent analysis revealed that the increased volume of the AMY in SNI animals could not be ascribed to cell size variations, but rather to an increase in cell number. Interestingly, such increase in cell numbers was confirmed by the observation of newly proliferating cells in AMY nuclei of SNI animals. Although the presence of newborn neurons in the adult brain of mammals is considered to be restricted to two areas, the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) (Doetsch et al., 1997; Gould et al., 1999b; Kempermann and Gage, 2000), the possibility of neurogenesis in the AMY has already been raised in a study showing evidence for the presence of newly generated neurons in the AMY of adult primates, at basal conditions (Bernier et al., 2002). The results of double-immunoreactions (BrdU+NeuN) performed in the present study demonstrate that a significant number of these newly-born cells undergo a neuronal phenotype. Thus, the genesis of newborn neurons is responsible, at least in part, for the increase in cell number underlying the increase of volume observed in the AMY of SNI animals. In contrast, the number of cells stained simultaneously for markers of cell proliferation (BrdU) and glia (GFAP) revealed no additional glial cell proliferation in the AMY following SNI induction; this indicates that SNI results only in additional neuronal proliferation, with a similar basal rate of astrocyte cell division being common to both Sham and SNI animals.

Neurogenesis and the AMY

Our observation of NeuN and BrdU co-localization in AMY cells indicate that newly generated cells reached neuronal maturation in the amygdaloid complex. This is in accordance with the time points for expression of neuronal differentiation markers described by Kempermann et al. (2004) and Steiner et al. (2004): in the hippocampus of adult mice NeuN expression becomes higher than immature-neuron markers 3 days after cell division. Additionally, the presence of BrdU+Calb double-labelled neurons in the AMY confirms the maturation and

phenotypical differentiation of newborn neurons in definitive AMY of SNI animals.

Whether these newly-born cells observed in the AMY of SNI rats result from local progenitor cells or migrate from adjacent neurogenic regions is still not known. However, several studies have shown that besides the normal migration of proliferative cells from the SVZ to the olfactory bulb (through the rostral migratory stream, RMS) or from the SGZ to other areas of the DG, they can migrate from the SVZ to injured areas of the brain (Iwai et al., 2003; Parent et al., 2002; Van Kampen et al., 2004). Therefore, it is possible that the new neurons here observed have their origin in SVZ progenitor cells that, through migration, reached the amygdaloid complex following the prolonged pain syndrome induced by the SNI model. Supporting this hypothesis, post-natal neurogenesis in the SVZ and SGZ can be regulated positively through the enhancement of the survival of newly generated cells and negatively through the down regulation of cell proliferation (Gould and Gross, 2002) following different stimuli (Jin et al., 2001). On the other hand, a growing amount of evidence supports the notion that the CNS itself is not as static as once believed: BrdU-positive cells were shown to be present in several regions of the adult CNS currently thought to be mitotically quiescent (Rietze et al., 2000); studies report that neurogenesis is prone to occur in other areas of adult mammals, like the neocortex (Gould et al., 1999a; Takemura, 2005), the striatum (Van Kampen et al., 2004; Bedard et al., 2006), the substantia nigra (Yoshimi et al., 2005) and the amygdala itself (Bernier et al., 2002). Taking into account these data, it should not be excluded the possibility that neural stem cells could be present in the AMY and proliferate following the prolonged neuropathy resulting from the SNI model. Further experimental procedures must be performed to elucidate this issue.

Roles of AMY in pain and emotional processing

Several data implicate the AMY in pain modulation, as shown by changes in pain tolerance induced by AMY manipulation (Manning, 1998). Moreover, the AMY has a role in both pain inhibition and pain facilitation (Manning and Mayer, 1995; Manning et al., 2001; Tershner and Helmstetter, 2000). This dual effect may result from direct AMY projections to brainstem areas implicated in both descending antinociception and pronociception (Almeida et al., 1999; Bouhassira et al., 1992; Porreca et al., 2002). As a balance between descending inhibiting (antinociceptive) and facilitating (pronociceptive) actions upon spinal nociceptive transmission can contribute to the normal control of pain perception (Lima and Almeida, 2002; Pertovaara, 2000; Porreca et al., 2002; Ren and Dubner, 1996; Schaible et al., 1991), the AMY may have a crucial role as a higher centre modulating the brainstem pain centres responsible for the fine regulation of the spinal nociceptive transmission. Thus, it is possible that the here observed amygdalar neuroplasticity may contribute not only to emotional changes but also to alterations in nociception. In support of this hypothesis, volume changes of AMY were already shown in imaging studies of patients with a major depression (Drevets, 2000) and changes in synaptic function of nociceptive AMY neurons have been described in sustained pain conditions (Han and Neugebauer, 2004; Ikeda et al., 2007). Additionally, the neuronal proliferation observed in AMY areas involved in afferent (BLA) and efferent (CeA) nociceptive processing may disrupt fine neuronal networks between high brain centres, which provide a structural basis for deregulation of emotional behaviour.

Conclusion

In conclusion, this study shows that besides mechanical hyperalgesia and allodynia, animals subjected to the SNI model of neuropathic pain during a two month period developed a depressive-like behaviour associated with an increased volume of AMY nuclei that results from cell proliferation. Importantly, this is the first study providing evidence for the presence of newly-born cells in the amygdaloid complex as a consequence of a sustained chronic (neuropathic) pain condition. We hypothesize that these neuroplastic changes of the AMY could be associated with the development of depressive-like behaviour in neuropathic animals. Nonetheless, future studies on the origin of newborn neurons and their integration in the pre-existing synaptic network should be performed in order to determine the relevance of this phenomenon.

Acknowledgments

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References

Almeida, A., Størkson, R., Lima, D., Hole, K., Tjølsen, A., 1999. The medullary dorsal reticular nucleus facilitates pain behaviour induced by formalin in the rat. Eur. J. Neurosci. 11, 110–122.
Almeida, A., Leite-Almeida, H., Tavares, I., 2006. Medullary control of nociceptive transmission: reciprocal dual communication with the spinal cord drug discovery today: disease mechanisms, 3, pp. 305–312.
Altshuler, L., Bookheimer, S., Proenza, M.A., Townsend, J., Sabb, F., Firestine, A., Bartzokis, G., Mintz, J., Mazziotta, J., Cohen, M.S., 2005. Increased amygdala activation during mania: a functional magnetic resonance magnetic resonance imaging study. Am. J. Psychiatry 162, 1211–1213.
Anand, K.J., Craig, K.D., 1996. New perspectives on the definition of pain. Pain 70, 209–211.
Bedard, A., Gravel, C., Parent, A., 2006. Chemical characterization of newly generated neurons in the striatum of adult primates. Exp. Brain Res. 170, 501–512.
Bernard, J.F., Bester, H., Besson, J.M., 1996. Involvement of the spino-parabrachio-amygdaloid and -hypothalamic pathways in the autonomic and affective emotional aspects of pain. Prog. Brain Res. 107, 243–255.
Bernier, P.J., Bedard, A., Vinet, J., Levesque, M., Parent, A., 2002. Newly generated neurons in the amygdala and adjoining cortex of adult primates. Proc. Natl. Acad. Sci. U. S. A. 99, 11464–11469.
Bouhassira, D., Villanueva, L., Le Bars, D., 1992. Effects of systemic morphine on diffuse noxious inhibitory controls: role of the periaqueductal grey. Eur. J. Pharmacol. 216, 149–156.
Bremner, J.D., Narayan, M., Anderson, E.R., Eric, R., Staib, L.H., Miller, H.L., Charney, D.S., 2000. Hippocampal volume reduction in major depression. Am. J. Psychiatry 157, 115–117.
Cerqueira, J.J., Taipa, R., Uylings, H.B., Almeida, O.F., Sousa, N., 2007. Specific configuration of dendritic degeneration in pyramidal neurons of the medial prefrontal cortex induced by differing corticosteroid regimens. Cereb. Cortex 17, 1998–2006.
Danziger, N., Weil-Fugazza, J., Le Bars, D., Bouhassira, D., 2001. Stage-dependent changes in the modulation of spinal nociceptive neuronal activity during the course of inflammation. Eur. J. Neurosci. 13, 230–240.
Davis, M., Whalen, P.J., 2001. The amygdala: vigilance and emotion. Mol. Psychiatry 6, 13–34.
Decosterd, I., Woolf, C.J., 2000. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain 87, 149–158.
Decosterd, I., Buchser, E., Gilliard, N., Saydoff, J., Zurn, A.D., Aebischer, P., 1998. Intrathecal implants of bovine chromaffin cells alleviate mechanical allodynia in a rat model of neuropathic pain. Pain 76, 159–166.
Devor, M., 2006. Sodium channels and mechanisms of neuropathic pain. J. Pain 7 (Suppl 1), S3–S12 Review.
Doetsch, F., Garcia-Verdugo, J.M., Alvarez-Buylla, A., 1997. Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. J. Neurosci. 17, 5046–5061.
Drevets, W.C., 2000. Neuroimaging studies of mood disorders. Biol. Psychiatry 48, 813–829.
Frodl, T., Meisenzahl, E., Zetzsche, T., Bottlender, R., Born, C., Groll, C., Jäger, M., Leinsinger, G., Hahn, K., Möller, H.-J., 2002. Enlargement of the amygdala in patients with a first episode of major depression. Biol. Psychiatry 51, 708–714.
Gao, X., Kim, H.K., Chung, J.M., Chung, K., 2005. Enhancement of NMDA receptor phosphorylation of the spinal dorsal horn and nucleus gracilis neurons in neuropathic rats. Pain 116, 62–72.
Gibb, R., Kolb, B., 1998. A method for vibratome sectioning of Golgi-Cox stained whole rat brain. J. Neurosci. Methods 79, 1–4.
Gonçalves, L., Silva, R., Pinto-Ribeiro, F., Pego, J.M., Bessa, J.M., Pertovaara, A., Sousa, N., Almeida, A., 2006. Chronic neuropathic pain induces neurogenesis in the rat amygdala and is associated with altered emotional behavior. Society Neurosci Abstr, No 443.17, Abstract Viewer/Itinerary Planner.
Gould, E., Gross, C.G., 2002. Neurogenesis in adult mammals: some progress and problems. J. Neurosci. 22, 619–623.
Gould, E., Reeves, A.J., Graziano, M.S., Gross, C.G., 1999a. Neurogenesis in the neocortex of adult primates. Science 286, 548–552.

Gould, E., Reeves, A.J., Fallah, M., Tanapat, P., Gross, C.G., 1999b. Hippocampal neurogenesis in adult Old World primates. Proc. Natl. Acad. Sci. U. S. A. 96, 5263–5267.
Han, J.S., Neugebauer, V., 2004. Synaptic plasticity in the amygdala in a visceral pain model in rats. Neurosci. Lett. 361, 254–257.
Ikeda, R., Takahashi, Y., Inoue, K., Kato, F., 2007. NMDA receptor-independent synaptic plasticity in the central amygdala in the rat model of neuropathic pain. Pain 127, 161–172.
Iwai, M., Sato, K., Kamada, H., Omori, N., Nagano, I., Shoji, M., Abe, K., 2003. Temporal profile of stem cell division, migration, and differentiation from subventricular zone to the olfactory bulb after transient forebrain ischemia in gerbils. J. Cereb. Blood Flow Metab. 23, 331–341.
Jin, K., Minami, M., Lan, J.Q., Mao, X.O., Batteur, S., Simon, R.P., Greenberg, D.A., 2001. Neurogenesis in the dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. Proc. Natl. Acad. Sci. U. S. A. 98, 4710–4715.
Kauppila, T., Xu, X.J., Yu, W., Wiesenfeld-Hallin, Z., 1998. Dextromethorphan potentiates the effect of morphine in rats with peripheral neuropathy. Neuroreport 9, 1071–1074.
Kempermann, G., Gage, F.H., 2000. Neurogenesis in the adult hippocampus. Novartis Found. Symp. 231, 220–235 discussion 235–241, 302–306.
Kempermann, G., Jessberger, S., Steiner, B., Kronenberger, G., 2004. Milestones of neuronal development in the adult hippocampus. Trends Neurosci. 27, 447–552.
Lima, D., Almeida, A., 2002. The medullary dorsal reticular nucleus as a pronociceptive centre of the pain control system. Prog. Neurobiol. 66, 81–108.
Manning, B.H., 1998. A lateralized deficit in morphine antinociception after unilateral inactivation of the central amygdala. J. Neurosci. 18, 9453–9470.
Manning, B.H., Mayer, D.J., 1995. The central nucleus of the amygdala contributes to the production of morphine antinociception in the rat tail-flick test. J. Neurosci. 15, 8199–8213.
Manning, B.H., Merin, N.M., Meng, I.D., Amaral, D.G., 2001. Reduction in opioid- and cannabinoid-induced antinociception in rhesus monkeys after bilateral lesions of the amygdaloid complex. J. Neurosci. 21, 8238–8246.
Meguro, R., Lu, J., Gavrilovici, C., Poulter, M.O., 2004. Static, transient and permanent organization of GABA receptor expression in calbindin-positive interneurons in response to amygdala kindled seizures. J. Neurochem. 91, 144–154.
Merskey, H., 1965. Psychiatric patients with persistent pain. J. Psychosom. Res. 9, 299–309.
Merskey, H., Bogduk, N. (Eds.), 1994. Classification of Chronic Pain: Descriptions of Chronic Pain Syndromes and Definitions of Pain Terms, 2nd ed. IASP Press, Seattle.
Mesquita, A.R., Tavares, H.B., Silva, R., Sousa, N., 2006. Febrile convulsions in developing rats induce a hyperanxious phenotype later in life. Epilepsy Behav. 9, 401–406.
Miller, M.W., Nowakowski, R.S., 1988. Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. Brain Res. 457, 44–52.
Mullen, R.J., Buck, C.R., Smith, A.M., 1992. NeuN, a neuronal specific nuclear protein in vertebrates. Development 116, 201–211.
Neugebauer, V., Li, W., 1992. Processing of nociceptive mechanical and thermal information in central amygdala neurons with knee-joint input. J. Neurophysiol. 87, 103–112.
Neugebauer, V., Li, W., Bird, G.C., Bhave, G., Gereau IV, R.W., 2003. Synaptic plasticity in the amygdala in a model of arthritic pain: differential roles of metabotropic glutamate receptors 1 and 5. J. Neurosci. 23, 52–63.
Neugebauer, V., Li, W., Bird, G.C., Han, J.S., 2004. The amygdala and persistent pain. Neuroscientist 10, 221–234.
Parent, J.M., Vexler, Z.S., Gong, C., Derugin, N., Ferriero, D.M., 2002. Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. Ann. Neurol. 52, 802–813.
Paxinos, G., Watson, C., 1998. The Rat Brain in Stereotaxic Coordinates, Fourth Ed. Academic Press, New York.
Pertovaara, A., 2000. Plasticity in descending pain modulatory systems. Prog Brain Res. 129, 231–242.
Porsolt, R.D., Bertin, A., Jalfre, M., 1977. Behavioural despair in mice: a primary screening test for antidepressants. Arch. Int. Pharmacodyn. Ther. 229, 327–336.
Porsolt, R.D., Anton, G., Blavet, N., Jalfre, M., 1978. Behavioural despair in rats: a new model sensitive to antidepressant treatments. Eur. J. Pharmacol. 47, 379–391.
Porreca, F., Ossipov, M.H., Gebhart, G.F., 2002. Chronic pain and medullary descending facilitation. Trends Neurosci. 25, 319–325.
Rasmussen, K.G., Rummans, T.A., 2002. Electroconvulsive therapy in the management of chronic pain. Curr. Pain Headache Rep. 6, 17–22.
Rasmussen, P.V., Sindrup, S.H., Jensen, T.S., Bach, F.W., 2004. Symptoms and signs in patients with suspected neuropathic pain. Pain 110, 461–469.
Reeves, S., Helman, L., Allison, A., Israel, M., 1989. Molecular cloning and primary structure of human glial fibrillary acidic protein. Proc. Natl. Acad. Sci. 86, 5178–5182.
Rietze, R., Poulin, P., Weiss, S., 2000. Mitotically active cells that generate neurons and astrocytes are present in multiple regions of the adult mouse hippocampus. J. Comp. Neurol. 424, 397–408.
Ren, K., Dubner, R., 1996. Enhanced descending modulation of nociception in rats with persistent hindpaw inflammation. J. Neurophysiol. 76, 3025–3037.
Rhudy, J.L., Meagher, M.W., 2000. Fear and anxiety: divergent effects on human pain thresholds. Pain 84, 65–75.
Schaible, H.G., Neugebauer, V., Cervero, F., Schmidt, R.F., 1991. Changes in tonic descending inhibition of spinal neurons with articular input during the development of acute arthritis in the cat. J. Neurophysiol. 66, 1021–1032.
Sousa, N., Almeida, O.F.X., Wotjak, C.T., 2006. A hitchhiker's guide to behavioral analysis in laboratory. Rodents Genes. Brain Behav. 5 (Suppl. 2), 5–24.
Steiner, B., Kronenberg, G., Jessberger, S., Brandt, M.D., Reuter, K., Kempermann, G., 2004. Differential regulation of gliogenesis in the context of adult hippocampal neurogenesis in mice. Glia 46, 41–52.
Strakowski, S.M., DelBello, M.P., Sax, K.W., Zimmerman, M.E., Shear, P.K., Hawkins, J.M., Larson, E.R., 1999. Brain magnetic resonance imaging of structural abnormalities in bipolar disorder. Arch. Gen. Psychiatry 56, 254–260.
Takemura, N.U., 2005. Evidence for neurogenesis within the white matter beneath the temporal neocortex of the adult rat brain. Neuroscience 134, 121–132.
Tal, M., Bennett, G.J., 1994. Extra-territorial pain in rats with a peripheral mononeuropathy: mechano-hyperalgesia and mechano-allodynia in the territory of an uninjured nerve. Pain 57, 375–382.
Tebartz van Elst, L., Woermann, F., Lemieux, L., Trimble, M.R., 2000. Increased amygdala volumes in female and depressed humans. A quantitative magnetic resonance imaging study. Neurosci. Lett. 281, 103–106.
Tershner, S.A., Helmstetter, F.J., 2000. Antinociception produced by mu opioid receptor activation in the amygdala is partly dependent on activation of mu opioid and neurotensin receptors in the ventral periaqueductal gray. Brain Res. 865, 17–26.
Van Kampen, J.M., Hagg, T., Robertson, H.A., 2004. Induction of neurogenesis in the adult rat subventricular zone and neostriatum following dopamine D receptor stimulation. Eur. J. Neurosci. 19, 2377–2387.
Willoughby, S.G., Hailey, B.J., Mulkana, S., Rowe, J., 2002. The effect of laboratory-induced depressed mood state on responses to pain. Behav. Med. 28, 23–31.
Yoshimi, K., Ren, Y.R., Seki, T., Yamada, M., Ooizumi, H., Onodera, M., Saito, Y., Murayama, S., Okano, H., Mizuno, Y., Mochizuki, H., 2005. Possibility for neurogenesis in substantia nigra of Parkinsonian brain. Ann. Neurol. 58, 31–40.
Zelman, D.C., Howland, E.W., Nichols, S.N., Cleeland, C.S., 1991. The effects of induced mood on laboratory pain. Pain 46, 105–111.
Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109–110.

Chapter 2.3

Gonçalves L, Coutada R, Pertovaara A, Almeida A

Newborn neurons are present in the amygdala after prolonged neuropathic pain: evidence for local neurogenesis.

(Manuscript in preparation)

Newborn neurons are present in the amygdala after prolonged neuropathic pain: evidence for local neurogenesis

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ABSTRACT

Neuropathic pain is strongly associated with the development of affective disorders like depression. The amygdala (AMY) has a significant role in the processing of emotions and is an important area in pain modulation and in emotional response to pain. Affective disorders (i.e. depression) resulting from chronic pain conditions are associated with structural plasticity in the AMY. We have previously described that after eight weeks of neuropathic pain induced by the spared nerve injury model (SNI) in rats, newborn neurons are present in the AMY, in association with a depressive-like behaviour. The aim of this study was to replicate the behaviour results and clarify what is the actual origin of these newborn neurons observed in the AMY. The SNI model was induced in a group of animals and prolonged during eight weeks. Behavioural tests were performed to assess mechanical allodynia (von Frey filaments), hyperalgesia (pin-prick test), depressive-like behaviour (forced swimming test) and anxiety-like behaviour (elevated plus-maze). Immunohistochemical analysis was performed in order to detect, in the rat brain, the location of different markers: doublecortin (DCX; protein expressed in migrating and differentiating neurons) + Ki-67 (nuclear protein expressed in proliferating cells in all phases of the active cell cycle); PSA-NCAM (specifically expressed in committed neuronal precursors present in regions that are undergoing some kind of structural plasticity) + GFAP (glial fibrillary acidic protein); and nestin (protein marker for immature neural cells) + GFAP. Depressive-like behaviour was confirmed, as well as the absence of anxious-like behaviour, after eight weeks of induced neuropathy. Additionally, we observed in basal and central AMY nuclei DCX+Ki-67-positive cells (co-localizing), PSA-NCAM-positive and GFAP-positive cells (not co-localizing), and nestin-positive and GFAP-positive cells (not co-localizing). These data strongly indicate that the cell division inducing newborn neurons in the central nucleus of the AMY after 8 weeks SNI neuropathy took place in

the amygdalar region and did not result from a long distance migration from other regions of the brain.

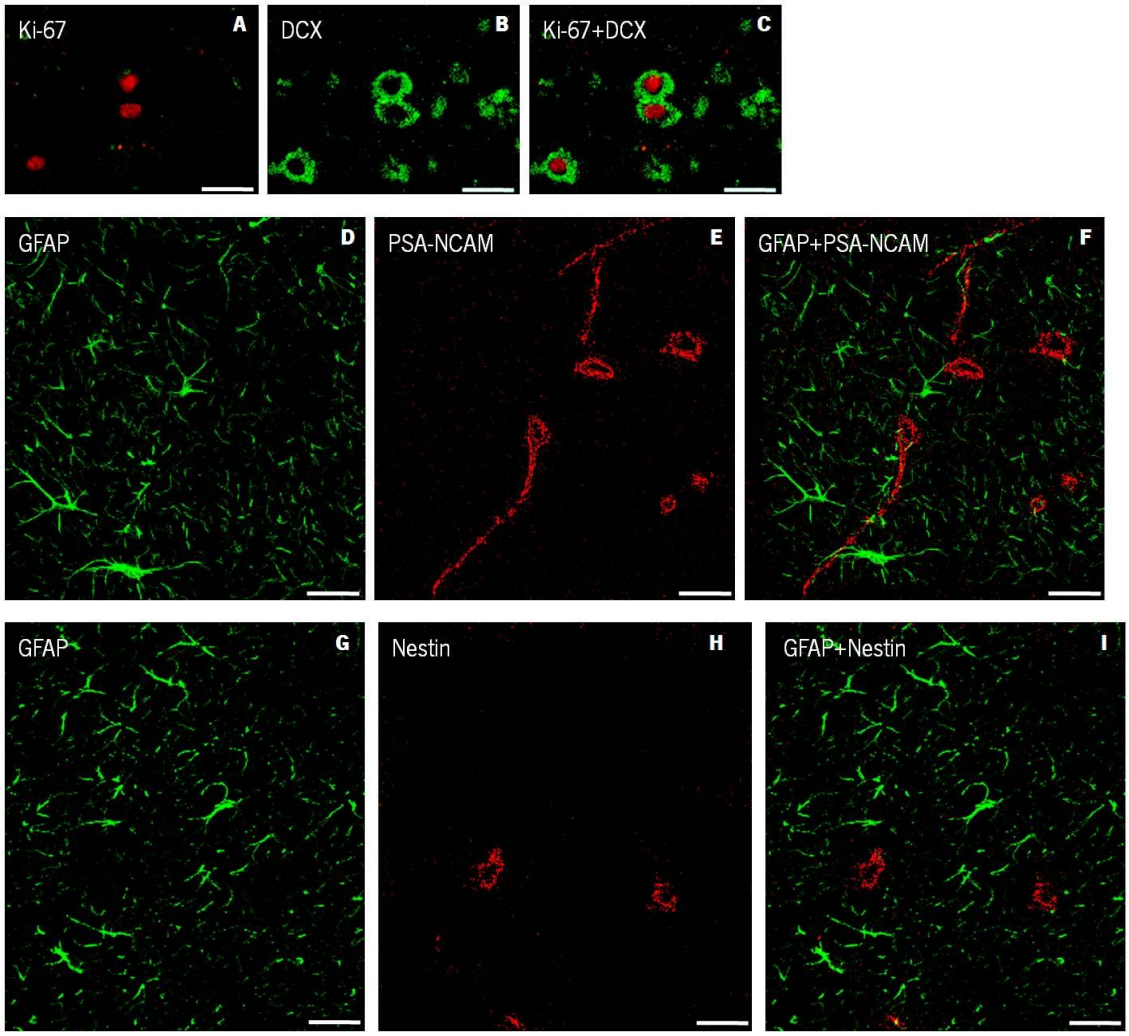

Figure 1 – Some examples of the immunohistochemical reacted sections observed through confocal microscope. A) Representative images of Ki-67-positive cells in the central nucleus of the AMY (CeA); B) Representative images of DCX-positive cells in the CeA; C) Representative images of Ki-67+DCX-double positive cells in the CeA; D) Representative images of GFAP-positive cells in the CeA; E) Representative images of PSA-NCAM-positive cells in the CeA; F) Representative images of GFAP+PSA-NCAM-double positive cells in the CeA; G) Representative images of GFAP-positive cells in CeA; H) Representative images of nestin-positive cells in the CeA; I) Representative images of GFAP+nestin-double positive cells in the CeA. Magnification bar: 20 μm.

Chapter 2.4

Ansah O, Gonçalves L, Almeida A, Pertovaara A

Enhanced pronociception by amygdaloid group I metabotropic glutamate receptors in 2 nerve-injured animals

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Enhanced pronociception by amygdaloid group I metabotropic glutamate receptors in nerve-injured animals

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ABSTRACT

Peripheral neuropathy has been associated with structural and functional changes of the amygdala, a key player in emotions. Here we study whether peripheral neuropathy influences pain regulation by the amygdala. For this purpose, we determined discharge rates of presumably pro- and antinociceptive pain-regulatory neurons in the rostral ventromedial medulla (RVM) following microinjection of various glutamatergic compounds into the central nucleus of the amygdala. RVM neurons were recorded in pentobarbitone-anesthetized rats with a peripheral nerve injury or sham-operation. In a separate behavioral experiment, we determined whether the influence of amygdaloid administration of a glutamatergic compound on affective pain-related behavior, as assessed by an aversive place-conditioning test, is changed by neuropathy. While glutamate or an NMDA receptor antagonist in the amygdala failed to induce marked changes in discharge rates of RVM cells, amygdaloid administration of DHPG, a group I metabotropic glutamate receptor (mGluR) agonist acting on $mGluR_1$ and $mGluR_5$, increased discharge rates of presumably pronociceptive RVM ON-cells in nerve-injured but not sham-operated animals. This pronociceptive effect of DHPG was reversed by MPEP ($mGluR_5$ antagonist) and CPCCOEt ($mGluR_1$ antagonist). CHPG, an $mGluR_5$ agonist, failed to influence ON-cell activity and DHPG failed to influence activity of presumably antinociceptive RVM OFF-cells. Amygdaloid administration of DHPG increased and that of CPCCOEt decreased affective pain-related behavior in nerve-injured animals. The results suggest that following nerve injury, the amygdaloid group I mGluR, particularly subtype $mGluR_1$, has an enhanced pronociceptive effect providing a potential mechanism for emotional enhancement of pain in peripheral neuropathy.

Introduction

The amygdala is a major player in emotions (Phelps and LeDoux, 2005). It also receives ascending nociceptive signals (Bernard et al., 1996) and it has efferent projections to structures that are involved in pain modulation (e.g., Rizvi et al., 1991; Van Bockstaele et al., 1996). These findings, together with chemical or electrical stimulation and lesion studies (see below), indicate that the amygdala has a role in pain modulation. Interestingly, the role is a dual one varying from antinociception (Helmstetter and Bellgowan, 1993; Helmstetter et al., 1998; Manning and Mayer, 1995; McGaraughty and Heinricher, 2002; Mena et al., 1995; Nandigama and Borszcz, 2003) to pronociception (Greenwood-Van Meerveld et al., 2001; Manning, 1998; Quin et al., 2003).

Sustained nociception produces synaptic plasticity in the amygdala. This has been shown in electrophysiological recordings performed in animals with inflammatory pain (Neugebauer et al., 2004; Neugebauer, 2006) and in control animals following tetanic stimulation of the parabrachial nucleus that relays nociceptive inputs to the amygdala (Lopez de Armentia and Sah, 2007). Peripheral nerve injuries may cause chronic neuropathic pain that is associated with plastic changes in pain-mediating (Woolf and Salter, 2006) and -regulating (Almeida et al., 2006; Pertovaara, 2000; Porreca et al., 2002) pathways. Recent studies indicate that peripheral nerve injury induces neural plasticity in the amygdala, as shown by increased postsynaptic currents evoked by ascending inputs (Ikeda et al., 2007) and generation of new amygdala neurons (Gonçalves et al., 2008). These findings still leave open whether the pain regulatory role of the amygdala is changed by peripheral nerve injury.

In the present study we test a hypothesis that peripheral nerve injury influences pain regulation by the amygdala. Partial support for this hypothesis is provided by a recent finding showing that amygdaloid activation by glutamate suppressed presumably antinociceptive neurons in the noradrenergic locus coeruleus of nerve-injured animals (Viisanen and Pertovaara, 2007). To test further this hypothesis, we determined whether administration of glutamatergic compounds into the amygdala has a differential influence on discharge rates of putative pain-regulatory neurons in the rostroventromedial medulla (RVM) of nerve-injured versus sham-operated

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animals. For this purpose, we recorded discharge rates of presumably pronociceptive ON-cells and antinociceptive OFF-cells in the RVM (Fields et al., 2006). Moreover, we assessed whether the effect by amygdaloid administration of a glutamatergic compound on affective pain-related behavior in an aversive place-conditioning test is changed following peripheral nerve injury.

Materials and methods

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

Techniques for producing neuropathy

The unilateral axotomy and ligation of the tibial and common peroneal nerves was performed under pentobarbitone anesthesia (50 mg/kg i.p.) as described in detail earlier (Decosterd and Woolf, 2000; Gonçalves et al., 2007). Briefly, the skin of the lateral surface of the thigh was incised and a section made directly through the biceps femoris muscle exposing the sciatic nerve and its three terminal branches. Following ligation and removing 2–4 mm of the distal nerve stumps of the tibial and common peroneal nerves, muscle and skin were closed in two layers. In sham-operated animals, the surgical procedure was identical, except that the tibial and common peroneal nerves were not ligated or sectioned. After the surgery, the animals were allowed to recover before the actual testing that was performed either 1 or 8 weeks after the operation.

Behavioral verification of neuropathy

Development of hypersensitivity was verified behaviorally in animals habituated to the experimental conditions 1–2 h daily for 2 to 3 days. For assessment of tactile allodynia, the hind limb withdrawal threshold was determined stimulating the sural nerve area in the hind paw of the operated limb with monofilaments. The calibrated series of monofilaments used in this study produced forces ranging from 0.16 to 15 g (North Coast Medical, Inc. Morgan Hill, CA, USA). The monofilaments were applied to the foot pad with increasing force until the rat withdrew its hind limb. The lowest force producing a withdrawal response was considered the threshold. The threshold for each hind paw of each rat was based on three separate measurements and the median of these values was considered to represent the threshold. Threshold values <1 g were considered to represent hypersensitivity. It should be noted that the currently used strain of rats delivered by Harlan (Horst, the Netherlands) has an exceptionally low withdrawal threshold to monofilament stimulation in baseline (unoperated) condition: in ten unoperated control animals the lowest withdrawal threshold was only 4 g and therefore, the criterion for hypersensitivity was set to as low as <1 g in this study as we did earlier with the same strain of animals (Gonçalves et al., 2007).

Electrophysiological recordings

For electrophysiological recordings the anesthesia was induced by pentobarbitone at a dose of 50 mg/kg i.p. and the animal was placed in a standard stereotaxic frame according to the atlas of Paxinos and Watson (1998). Anesthesia was maintained by infusing pentobarbitone (15–20 mg/kg/h). The level of anesthesia was frequently monitored by observing the size of the pupils and by assessing withdrawal responses to noxious stimulation. When necessary, the infusion rate of pentobarbitone was adjusted to keep the level of anesthesia steady. Although a change in the level of anesthesia may significantly influence neuronal responses, anesthesia is not likely to explain differences among different experimental groups and drug treatments in the present study. This is because anesthesia was induced and maintained in an identical manner in all experimental conditions. The rats were spontaneously breathing. A warming blanket was used to maintain body temperature within physiological range. Peripheral perfusion was checked by evaluating the color of ears and extremities. The skull was exposed and a hole drilled for placement of recording electrode in the RVM. The desired recording site in the RVM was 1.8–2.3 mm posterior from the ear bar, 0.4–0.9 mm lateral from the midline, and 8.9–10.7 mm ventral from the dura mater (Fig. 1).

Single neuron activity was recorded extracellularly with lacquer-coated tungsten electrodes (tip impedance 3–10 MΩ at 1 kHz) and then 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, U.K.).

Actual recordings did not start until the animal was under light anesthesia; i.e., the animals gave a brief withdrawal response to noxious pinch, but the pinch did not produce any longer lasting motor activity, nor did the animals have spontaneous limb movements. Neurons were classified based on their response to noxious pinch of the tail with a hemostatic clamp (Fig. 2). This stimulus was painful when applied to the finger of the experimenters. Neurons giving excitatory responses to pinch were considered ON-cells, those giving inhibitory responses were considered OFF-cells and neurons showing no or only a negligible (<10%) change in their discharge rates as a response to pinch were considered NEUTRAL-cells. This classification scheme of medullary neurons was modified from that described by Fields et al., (2006). A noteworthy difference is that we did not verify whether pinch-evoked responses of RVM neurons were associated with spinal reflex responses as in the original classification scheme (Fields et al., 2006). Therefore, the populations of ON- and OFF-cells in this study may not be identical with those in a study in which cells are classified strictly according to the classification scheme of Fields et al.,

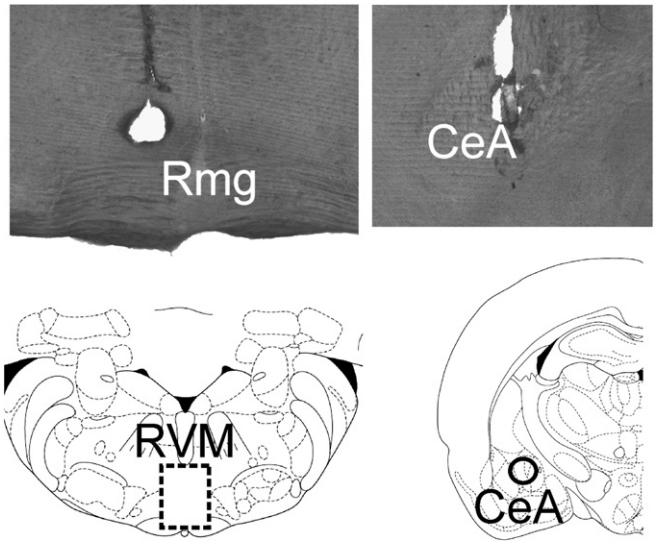

Fig. 1. Microelectrode recording sites in the RVM (left column) and microinjection sites in the amygdala (right column). The upper row shows an example of an electrolytic lesion made by the recording electrode in the RVM (left) and the track of the injection cannula in the amygdala (right). The rectangle and the circle in the schematic graphs of the lower row indicate the dorsolateral extent of areas, across several anteroposterior sections, in which the tips of medullary recording electrodes and amygdaloid microinjection cannulae were located, respectively. RVM = rostroventromedial medulla, CeA = central nucleus of the amygdala, Rmg = raphe magnus.

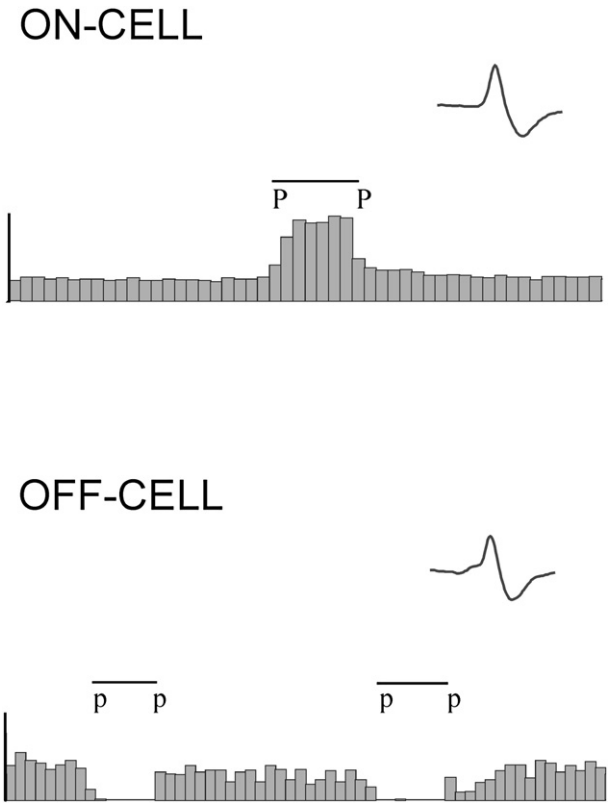

Fig. 2. Examples of an RVM ON-cell (upper graph) and OFF-cell (lower graph) response to noxious pinch of the tail in a neuropathic animal. $\overline{PP}$ indicates the duration of noxious tail pinch. The horizontal bars indicating tail pinch represent 5 s and the vertical calibration bars for the peristimulus time histograms represent 50 impulses/s. The insets show the shapes of the action potential.

(2006). Our previous results suggest, however, that there is only a little difference in the classification of RVM neurons whether or not spinal reflex responses are concurrently measured in lightly anesthetized animals (Pertovaara et al., 2001).

Intracerebral drug injections

The animals had a guide cannula for drug administrations into the amygdala ipsilateral to the spared nerve injury or sham-operated limb (left side), except for one group that had the cannula contralateral to the nerve injury (right side), and a control group that had the cannula in the hippocampus. Additionally, a group of animals tested in behavioral experiments only had a bilateral guide cannula for drug injections into the central nucleus of the amygdala. For placement of the guide cannula (26 gauge), the skull was exposed and a hole drilled for its placement. The desired injection site was in the central nucleus of the amygdala: 7.12 mm anterior from the ear bar, 3.40 mm lateral from the midline, and 8.00 mm ventral from the dura mater. A control injection site in a group of neuropathic animals was in the hippocampus, ipsilateral to nerve injury: 6.20 mm anterior from the ear bar, 1.00 mm lateral from the midline, and 3.20 mm ventral from the dura mater. The tip of the guide cannula was positioned 2 mm above the desired injection site. The cannula was fixed into the skull using a dental screw and dental cement. Drug administration to the brain and experimental protocols were performed 1 week after fixation of the guide cannula to the skull. When testing animals at the one-week postoperative time point, the guide cannula for amygdala injections was installed in the same operating session as sham or nerve surgery. When testing animals at the eight-week postoperative time point, the guide cannula was installed in a separate session at least 1 week prior to electrophysiological recordings.

Drugs or saline control were microinjected into the amygdala through a 33-gauge stainless steel injection cannula inserted through and protruding 2 mm beyond the tip of the guide cannula. The microinjection was made using a 10 µl Hamilton syringe that was connected to the injection cannula by a length of polyethylene (PE-10) tubing. The volume of injection was 0.5 µl. At this volume, the spread of the injected drugs within the brain was at least 1 mm (Myers, 1966). The efficacy of injection was monitored by watching the movement of a small air bubble through the tubing. The injection lasted 30 s and the injection cannula was left in place for an additional 30 s to minimize flow of the drug solution back up the injector track. At the completion of the experiment, the microinjection sites were histologically verified and plotted on a standardized section derived from the stereotaxic atlas of Paxinos and Watson (1998).

Course of the electrophysiological study

There were four groups of animals that were included in the electrophysiological study and had a guide cannula for amygdala injections ipsilateral to nerve injury or sham operation: i) sham group tested 1 week after operation, ii) sham group tested 8 weeks after operation, iii) SNI group tested 1 week after operation, and iv) SNI group tested 8 weeks after operation. Additionally, there was a fifth group of SNI animals tested 1 week after operation that had the guide cannula for amygdala injections contralateral to nerve injury, and a sixth group of animals with neuropathy of 1 week duration that had the guide cannula for drug injections into a control site in the hippocampus ipsilateral to nerve injury. In each of these groups, neuropathic hypersensitivity was verified with the monofilament test (see above) before the start of the electrophysiological experiment.

After induction of anesthesia, the microelectrode was lowered to the RVM. After finding a single cell, it was first classified based on its response to noxious tail pinch (see above) and then its spontaneous activity was recorded for 2 to 3 min. Next, one of the studied drugs or saline control was administered in a varied order to the amygdala and the spontaneous activity was recorded for up to 30 min (except with glutamate and MK-801 for 6 min). One to two drug conditions were tested in one cell, and one to four cells were tested in each animal. The minimum interval to the next drug testing condition was 30 min following saline or glutamate, while it was 60 min following other drugs, except for MK-801 that was always the last drug tested in each animal. When attempting to reverse the effect induced by the glutamatergic agonist DHPG, the antagonist was injected into the amygdala immediately (MPEP) or 15 min (CPCCOEt) before the agonist. In the data analysis, the discharge rate before injection was compared with the discharge rate determined after the injection. This was done by subtracting the mean post-injection discharge frequency during 1 min at various time points following microinjection from the mean discharge frequency before microinjection; i.e., a positive value represents increase of activity in the RVM by amygdala injection, and vice versa.

Assessment of aversive avoidance behavior and its modulation by glutamatergic receptors of the amygdala

Place avoidance test was performed, as described earlier (LaBuda and Fuchs, 2000), to obtain a measure of affective pain induced by mechanical stimulation of the neuropathic hind paw. Before testing, the animals were habituated to the test conditions by spending 1 to 2 h daily for 2 days in the test box. In the actual testing, the rat was placed within a Plexiglas chamber (60×30×30 cm; one half of which was painted black on the external surface) placed upon an elevated metal grid. The rats were placed over the midline of the chamber and stimulation of the plantar surface of the hind paw initiated with a 60 g monofilament once every 15 s for 15 min. When residing within the dark side of the chamber the injured or sham-operated hind paw was

stimulated. Conversely, the non-operated hind paw was stimulated when residing within the light side of the chamber. Throughout the 30 min test period rats were allowed unrestricted movement throughout the chamber. The percent time spent in the light side of the chamber during the 30 min observation period was determined in each condition for each animal. It is assumed that the more aversive the mechanical stimulation of the hind paw, the more the animal spends time in the light side of the chamber; i.e., the place avoidance test is considered to assess affective-emotional pain behavior (LaBuda and Fuchs, 2000).

Three experimental groups of rats were tested in the place avoidance test: i) SNI animals with amygdaloid injections ipsilateral to the nerve injury ii) SNI animals with bilateral amygdaloid injections, iii) sham-operated animals with amygdaloid injections ipsilateral to the sham operation. In the bilateral treatment group, the drug conditions were saline, DHPG at the dose of 5 nmol or 10 nmol/amygdala (10 nmol or 20 nmol/animal, respectively), and CPCCOEt at the dose of 20 nmol or 40 nmol/amygdala (40 nmol or 80 nmol/animal/respectively). In the ipsilateral treatment groups, drug conditions were saline, 10 nmol of DHPG, or 40 nmol of CPCCOEt. In all experimental conditions, drugs were administered into the amygdala immediately before the start of the place avoidance test. In each experimental group, each drug condition was assessed in a separate day, 1 to 2 weeks following nerve or sham injury. Each animal participated in three to four drug testing sessions, the interval in testing different drug conditions in one animal was at least 2 days. The order of testing different drug conditions was varied within the groups to avoid serial effects.

Drugs

(S)-3,5-Dihydroxyphenylglycine (DHPG; an $mGluR_1$ and $mGluR_5$ agonist), (RS)-2-Chloro-5-hydroxy (CHPG; an $mGluR_5$ agonist), 6-Methyl-2-(phenylethynyl)pyridine (MPEP; an $mGluR_5$ antagonist), (+)-MK-801 hydrogen maleate (MK-801; an NMDA-R antagonist) and glutamate were purchased from Sigma (St. Louis, MO) and 7-Hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt; an $mGluR_1$ antagonist) was purchased from Tocris (Bristol, U.K.). Physiological saline (OrionPharma, Espoo, Finland) was used for control injections. Drugs were dissolved in saline, except for CPCCOEt that was dissolved in DMSO.

Previous studies indicate that DHPG and CPCCOEt at the currently used dose of 10 nmol have proved effective in activating group I mGluRs within the currently used observation period of 30 min following intracerebral administration in the rat (e.g., Kim et al., 2007; Renoldi et al., 2007). Previous studies indicate that the currently used doses of glutamate (50 nmol) and MK-801 (3 nmol) induce a significant antinociception (Zhuo and Gebhart, 1997) or antiallodynia (Pertovaara and Wei, 2003), respectively, following supraspinal microinjection. The maximum antinociceptive effect induced by central injection of glutamate has been obtained within 2 min (Zhuo and Gebhart, 1997), whereas the maximum antiallodynic effect induced by central injection of MK-801 was reached within 15 min (Pertovaara and Wei, 2003). Thus, the currently used observation period of 6 min following the injection of glutamate in the amygdala was appropriate for detecting the maximum effect induced by glutamate but only a submaximal effect induced by MK-801.

At the completion of the study, an electrolytic lesion was made in the recording site, the animals were given a lethal dose of pentobarbitone and the brains removed for verification of recording and microinjection sites.

Statistics

Data are presented as mean±S.E.M. One- or two-way ANOVA followed by Student-Newman-Keuls test or *t*-test (differences between two groups) were used for assessing differences between the experimental conditions. Grubb's test was used to exclude potential outliers (www.graphpad.com/quickcalcs/). $P<0.05$ was considered to represent a significant difference.

Results

Response characteristics of RVM neurons

Spontaneous activity of RVM ON-cells was significantly influenced by SNI ($F_{1,131}=4.79$, $P=0.030$; 2-w-ANOVA) and postoperative time point of testing ($F_{1,131}=4.19$, $P=0.043$; 2-w-ANOVA). *Post hoc* tests indicated that the spontaneous discharge rate of ON-cells was increased in the SNI group 1 week after nerve injury, while 8 weeks following injury it was reduced to the same level as in sham controls (Fig. 3a). Spontaneous activity of RVM OFF-cells was influenced by postoperative time point of testing ($F_{1,100}=8.0$, $P=0.0057$; 2-w-ANOVA), and this time-dependent effect varied with the experimental group (SNI versus sham; $F_{1,100}=4.50$, $P=0.036$; 2-w-ANOVA). *Post hoc* tests indicated that the discharge rate of OFF-cells was significantly decreased 1 week following nerve injury, while 8 weeks following injury it was at the same level as in sham controls (Fig. 3b). In the present sample of neurons, postoperative time point of testing (one versus 8 weeks) had no significant influence on spontaneous discharge rates of ON- or OFF-cells in the sham control group (Fig. 3).

Discharge rates of RVM cells following amygdaloid administration of glutamatergic compounds

When studying the influence of glutamatergic compounds on discharge rates of RVM cells, the studied compounds were microinjected at a volume of 0.5 µl into the amygdala ipsilateral to nerve injury or sham operation, except for one group in which it was injected

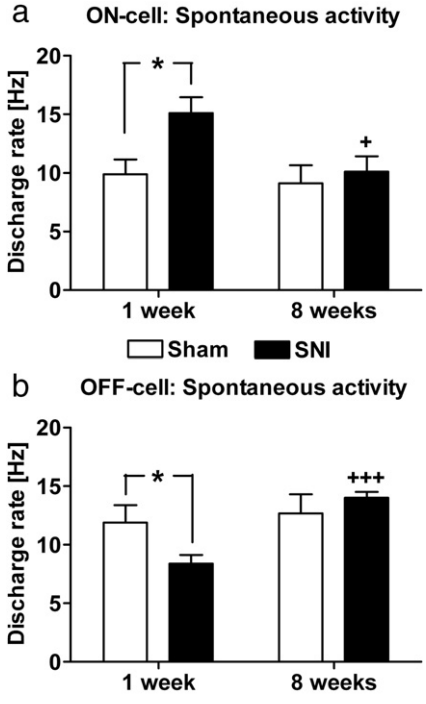

Fig. 3. Spontaneous discharge rates of RVM ON-cells (a) and OFF-cells (b) in nerve-injured (SNI) and sham-operated animals. 1 and 8 weeks refer to the postoperative time point of testing. *$P<0.05$ (reference: the corresponding value in the sham-operated group at the same postoperative time point). +$P<0.05$, +++$P<0.005$ (Student-Newman-Keuls test; reference: the corresponding value at an earlier postoperative time point). Error bars represent S.E.M. ($n=19$–29).

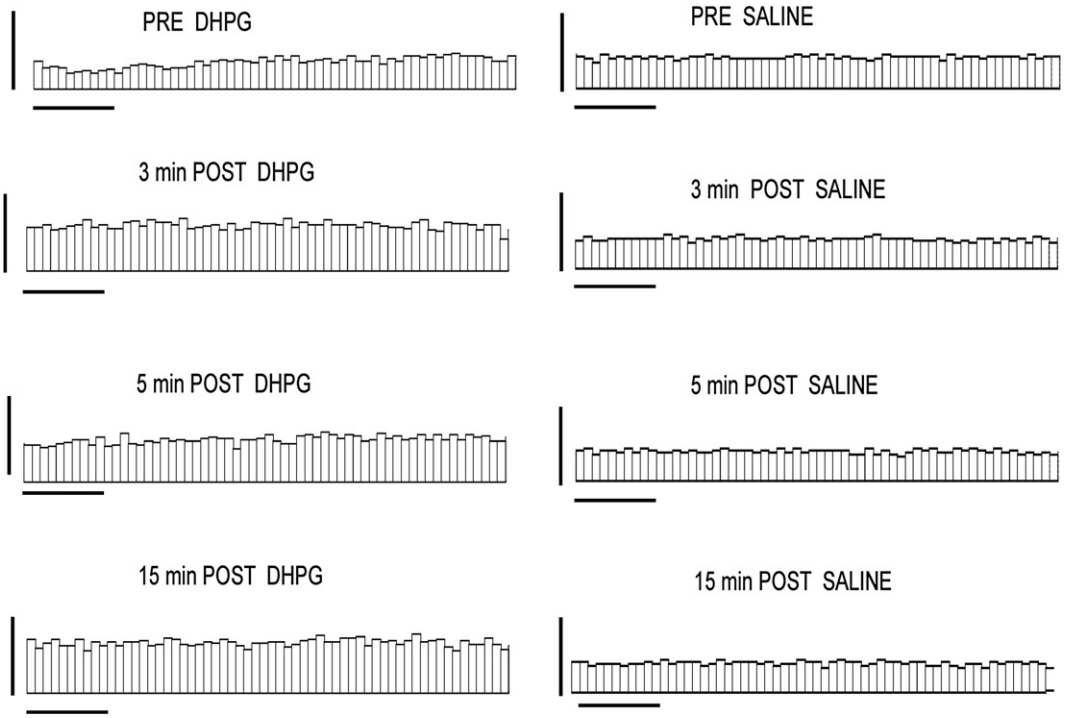

Fig. 4. Influence of DHPG (10 nmol; left column) or saline (right column) injection into the amygdala on the discharge rate of an RVM ON-cell in a neuropathic animal 1 week following the nerve injury. Time point of testing before and after the injection is shown above each row. The horizontal calibration bar represents 10 s and the vertical one 50 impulses/s.

into the amygdala contralateral to nerve injury. Microinjection of DHPG, an agonist of group I metabotropic glutamate receptor (mGluR) subtypes $mGluR_1$ and $mGluR_5$, at a dose of 10 nmol produced a significant increase in the discharge rate of RVM ON-cells in the SNI group (Fig. 4). DHPG induced the maximum increase in the discharge rate of ON-cells within 5 min, and the increase was of equal magnitude 1 and 8 weeks following nerve injury (Figs. 5a, b). The increase in the discharge rate of RVM ON-cells by 10 nmol of

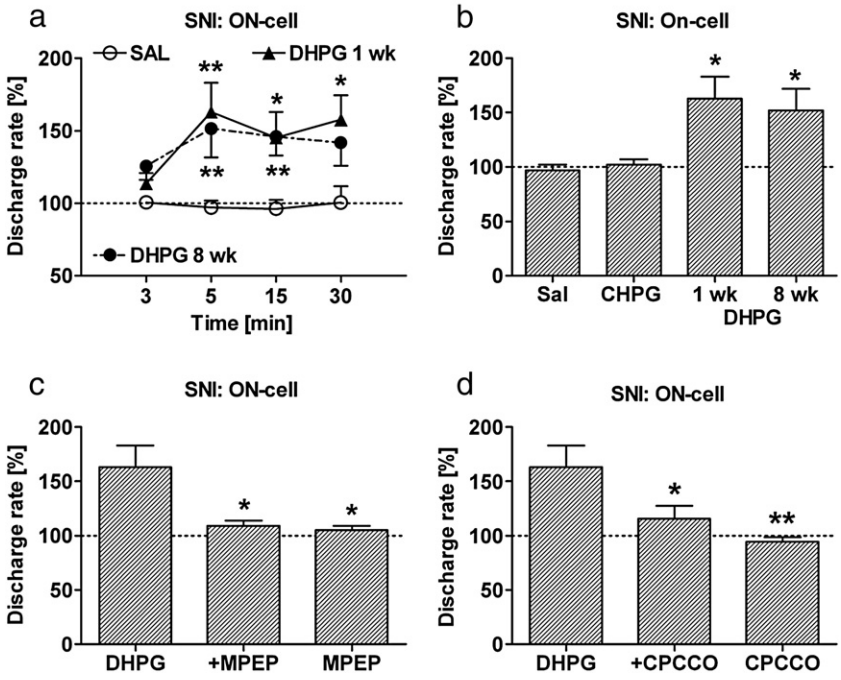

Fig. 5. Discharge rates of RVM ON-cells in nerve-injured (SNI) animals following administration of group I metabotropic glutamatergic compounds into the amygdala ipsilateral to the nerve injury. (a) Time course of the effect by DHPG (10 nmol), an $mGluR_1$ and $mGluR_5$ agonist. (b) The effect by DHPG versus CHPG (10 nmol), an $mGluR_5$ agonist. (c) Reversal of the DHPG-induced increase in the discharge rate by MPEP (50 nmol), an $mGluR_5$ antagonist. (d) Reversal of the DHPG-induced increase in the discharge rate by CPCCOEt (40 nmol), an $mGluR_1$ antagonist. 1 and 8 weeks refer to the postoperative time point of testing. Sal = saline. MPEP = MPEP alone, CPCCO = CPCCOEt alone, +MPEP or +CPCCO = MPEP or CPCCOEt co-administered with DHPG. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$ (Student-Newman-Keuls test; in a, reference is the corresponding pre-injection value. In b, reference is the saline group. In c and d, reference is the DHPG group). Error bars represent S.E.M. ($n_{sal}=16$, $n_{DHPG\_1wk}=8$, $n_{DHPG\_8wk}=14$, $n_{CHPG}=4$, $n_{MPEP}=5$, $n_{+MPEP}=6$, $n_{CPCCO}=10$, $n_{+CPCCO}=8$). 100% (dotted horizontal line) represents the pre-injection discharge rate.

DHPG was of equal magnitude following its microinjection into the amygdala ipsi- (n=14) as contralateral (n=4) to the nerve injury in animals that were operated 8 weeks before time point of testing (F=0.05; 2-w-ANOVA; not shown). CHPG, an $mGluR_5$ agonist (10 nmol), failed to influence the discharge rate of RVM ON-cells in nerve-injured animals (Fig. 5b). The DHPG-induced increase of ON-cell activity in nerve-injured animals was completely reversed by pretreatment of the amygdala with MPEP, an $mGluR_5$ antagonist, at a dose of 50 nmol that produced no significant effect when administered alone (Fig. 5c). The DHPG-induced increase of ON-cell activity in nerve-injured animals was also completely reversed by pretreatment of the amygdala with CPCCOEt, an $mGluR_1$ antagonist, at a dose (40 nmol) that failed to produce a change in ON-cell discharge rate when administered alone (Fig. 5d). Administration of DHPG at the dose of 10 nmol into a control site, the hippocampus, failed to produce any significant change on the discharge rate of five ON-cells (not shown) in neuropathic animals, while in one ON-cell DHPG administration into the hippocampus was followed at a 15 min post-injection time point by a sudden increase of the discharge rate from the pre-injection baseline rate by 384% (not shown). According to Grubb's test, the RVM ON-cell with a sudden increase in the discharge rate 15 min following hippocampal injection of DHPG was a significant outlier (P<0.05) and therefore, it was not included in the statistical assessment of the over-all effect.

DHPG (10 nmol) in the amygdala failed to influence OFF-cell activity in the SNI group, independent of postoperative time point (Fig. 6a). Moreover, DHPG (10 nmol) in the amygdala had no significant effect on discharge rates of ON- or OFF-cells in the sham group (Fig. 6b).

Microinjections of glutamate at a dose of 50 nmol or MK-801, an NMDA receptor (NMDA-R) antagonist, at a dose of 3 nmol failed to produce significant changes in the discharge rates of ON- or OFF-cells in the SNI or sham group (Fig. 7).

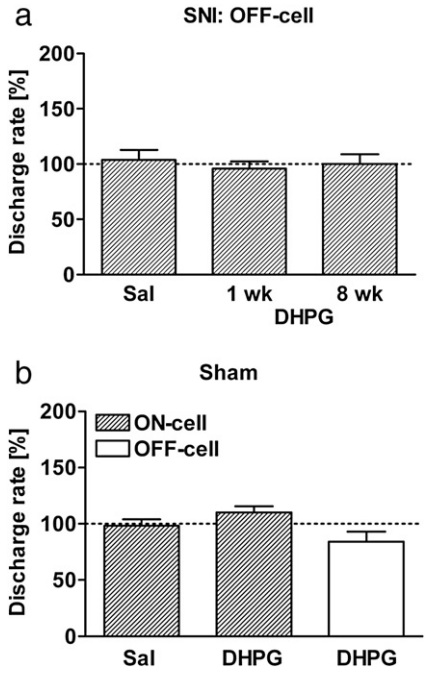

Fig. 6. (a) Discharge rates of RVM OFF-cells in nerve-injured (SNI) animals following administration of saline (Sal) or DHPG (10 nmol), an $mGluR_1$ and $mGluR_5$ agonist, into the amygdala. (b) Discharge rates of RVM ON- and OFF-cells in sham-operated animals following amygdaloid administration of saline or DHPG. 1 and 8 weeks refer to the postoperative time point of testing. Error bars represent S.E.M. (In a, n_{Sal}=6, n_{DHPG_1wk}=9, n_{DHPG_8wk}=6. In b, n_{Sal}=8, $n_{DHPG\_ON\text{-}cell}$=8, $n_{DHPG\_OFF\text{-}cell}$=5). 100% represents the pre-injection discharge rate.

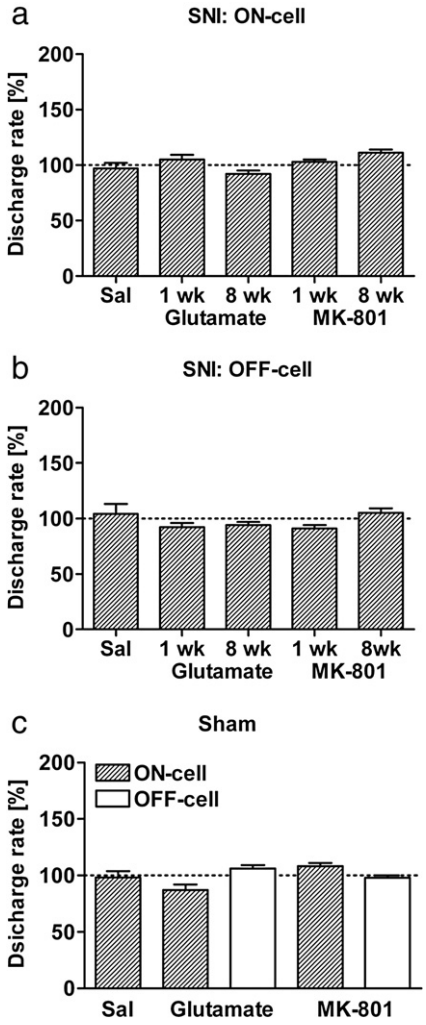

Fig. 7. Discharge rates of RVM cells following administration of glutamate (50 nmol) or MK-801 (3 nmol), an NMDA-R antagonist, into the amygdala. (a) ON-cells in nerve-injured (SNI) animals. (b) OFF-cells in nerve-injured animals. (c) ON- and OFF-cells in sham-operated animals. 1 and 8 weeks refer to the postoperative time point of testing. Sal=saline Error bars represent S.E.M. (n=6–10). 100% represents the pre-injection discharge rate.

Affective pain-related behavior following amygdaloid administration of DHPG or CPCCOEt

A behavioral place avoidance paradigm was used to assess whether amygdaloid administration of DHPG or CPCCOEt influences aversive quality of mechanical stimulation of the neuropathic hind paw. In sham-operated animals, mechanical stimulation of the operated hind paw induced no or negligible avoidance behavior, independent whether saline or DHPG (10 nmol) was injected into the ipsilateral amygdala (Fig. 8). In nerve-injured animals, mechanical stimulation of the neuropathic hind paw induced a marked avoidance behavior (as revealed by increased time spent in light) that was increased by administration of DHPG into the amygdala: while the increase of avoidance behavior induced by ipsilateral injection of 10 nmol of DHPG was short of significance, bilateral administration of DHPG produced a dose-related increase in place avoidance ($F_{2,14}$=8.7, P<0.01; 1-w-ANOVA) that was significant at a dose of 10 nmol of DHPG/amygdala, corresponding to 20 nmol of DHPG/animal (Fig. 8a). In contrast, avoidance behavior was reduced by administration of CPCCOEt in the amygdala: while the decrease of avoidance behavior was short of significance following ipsilateral administration of 40 nmol of CPCCOEt, bilateral administration of CPCCOEt produced a

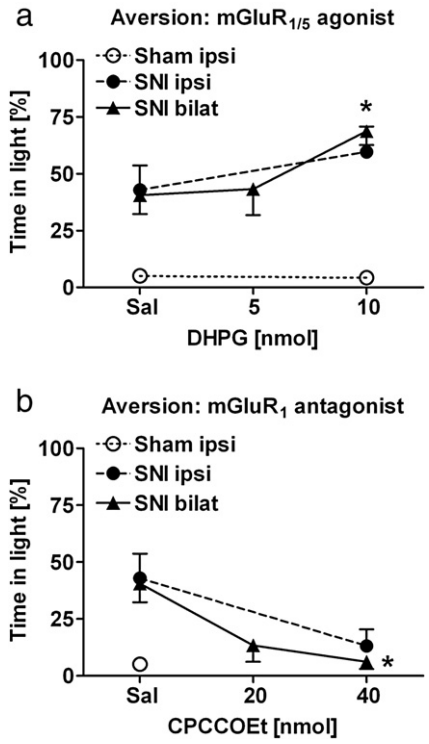

Fig. 8. Behavior in aversive place-conditioning test following administration DHPG, an $mGluR_1$ and $mGluR_5$ agonist (a), or CPCCOEt, an $mGluR_1$ antagonist (b), into the amygdala in nerve-injured (SNI) or sham-operated animals. An increase in time spent in light is considered to reflect an increase in affective pain induced by monofilament stimulation of the hind paw. ipsi = amygdaloid injection was performed only ipsilateral to the nerve injury/sham operation, bilat = amygdaloid injection was performed bilaterally. In the *Y*-axis, doses represent the dose/side; e.g., the dose 10 nmol represents 10 nmol/animal with ipsilateral injections and 20 nmol/animal with bilateral injections. $*P<0.05$ (Student-Newman-Keuls test; reference: the corresponding saline condition). Error bars represent S.E.M. (In a, $n_{SNI\text{-}ipsi}=9$, $n_{SNI\text{-}bilat}=5$, $n_{Sham\text{-}ipsi}=5$. In b, $n_{SNI\text{-}ipsi}=4$, $n_{SNI\text{-}bilat}=4$, $n_{Sham\text{-}ipsi}=5$).

dose-related decrease in place avoidance ($F_{2,10}=4.86$, $P<0.04$; 1-w-ANOVA) that was significant at a dose of 40 nmol of CPCCOEt/amygdala (Fig. 8b).

Discussion

In the present study, amygdaloid administration of DHPG, an $mGluR_{1/5}$ agonist, increased the discharge rate of presumably pronociceptive ON-cells in the RVM of nerve-injured but not sham-operated animals. This enhanced pronociceptive effect was at least due to action on the amygdaloid $mGluR_1$, since DHPG, an agonist of the $mGluR_1$ and $mGluR_5$, but not CHPG, an $mGluR_5$ agonist, increased discharge rates of RVM ON-cells and this DHPG-induced effect was reversed by CPCCOEt, an $mGluR_1$ antagonist. However, since MPEP, an $mGluR_5$ antagonist, applied at a high dose also reversed the DHPG-induced increase of the ON-cell discharge rate, we cannot exclude contribution of the $mGluR_5$ to the DHPG-induced pronociception. Administration of DHPG into a control site, the hippocampus, failed to produce a change in the discharge rate of RVM ON-cells in neuropathic animals. Amygdaloid administration of DHPG also failed to influence discharge rates of presumably antinociceptive OFF-cells of the RVM indicating that the nerve injury-induced change was selective for the pronociceptive cell type. Since amygdaloid administration of NMDA-R or group I mGluR antagonists alone failed to influence discharge rates of pro- or antinociceptive RVM cells, the amygdaloid NMDA-R or group I mGluRs may not contribute to tonic maintenance of neuropathic pain and hypersensitivity.

In behavioral experiments of the present study, affective pain of nerve-injured animals was increased by amygdaloid administration of DHPG and decreased by CPCCOEt. Since the aversive place-conditioning test used in assessing affective pain-related behavior (LaBuda and Fuchs, 2000) provides an emotional challenge putatively activating the amygdala, the decrease of affective pain by amygdaloid administration of an $mGluR_1$ antagonist is in line with the hypothesis that emotions processed by the amygdala may enhance pain in nerve-injured animals, due to action on the amygdaloid $mGluR_1$.

Interaction between the amygdala and pain

Psychophysical studies suggest that emotions presumably processed by the amygdala produce significant changes in human pain reactivity (Craig, 2006). The direction of the change has varied from pain facilitation in anxious subjects to suppression of pain sensitivity in subjects with intense fear (Rhudy and Meagher, 2000 & 2003). In line with this, previous studies in non-neuropathic animals have shown that the amygdala has a dual role in the regulation of nociception varying from pronociception to antinociception (see the Introduction). There is accumulating evidence indicating that sustained pain induces plastic changes in the amygdala (Neugebauer, 2006). Pain-induced neural plasticity of the amygdala may influence its pain regulatory action as indicated by a recent study showing that activation of the extracellular signal-regulated kinase in the amygdala contributes to inflammatory hypersensitivity (Carrasquillo and Gereau, 2007). In addition to inflammation, peripheral neuropathy induces neural plasticity in the amygdala as shown by the findings that postsynaptic currents evoked by ascending inputs in the central amygdala were enhanced (Ikeda et al., 2007) and new amygdala neurons were generated (Gonçalves et al., 2008) following peripheral nerve injury. While the enhanced synaptic responses to ascending signals indicate that the relay of pain-related signals to the amygdala is facilitated in neuropathy (Ikeda et al., 2007), these previous results still leave open whether the amygdala-induced pain regulation is changed by peripheral nerve injury.

In neuropathic animals, not only pain processing within the amygdala (Ikeda et al., 2007) but also pain regulatory influence of the amygdala may be changed as suggested by the following findings. Amygdaloid administration of a $GABA_A$ receptor agonist suppressed aversive pain-related behavior and hypersensitive spinal reflex responses in nerve-injured animals (Pedersen et al., 2007). This behavioral finding suggests that the amygdala may contribute to regulation of neuropathic hypersensitivity, possibly through action on descending pathways relaying in the RVM. Also, amygdaloid administration of glutamate suppressed presumably antinociceptive neurons in the noradrenergic locus coeruleus of nerve-injured but not sham-operated animals suggesting that activation of the amygdala may have a pronociceptive effect in peripheral neuropathy (Viisanen and Pertovaara, 2007). In line with this, the present results suggest that activation of the group I mGluR in the amygdala of nerve-injured animals promotes activity of presumably pronociceptive neurons in the RVM. Together these results suggest that the amygdala-induced pain modulation is changed in neuropathy. In neuropathic animals, activation of the amygdala may promote hypersensitivity by, at least, two different types of actions on brain regulatory nuclei of the brainstem: by facilitating pronociceptive neurons in the RVM and by inhibiting antinociceptive neurons in the locus coeruleus. While the pronociceptive influence by the amygdala may be partly tonic in peripheral neuropathy (Pedersen et al., 2007), the present results with amygdaloid administrations of specific receptor antagonists alone suggest that the amygdaloid NMDA-R or group I mGluR do not explain the tonic pronociceptive action of the amygdala.

Interestingly, while the enhancement of synaptic currents evoked by afferent inputs to the amygdala occurred predominantly in the central nucleus of the amygdala contralateral to peripheral nerve

injury (Ikeda et al., 2007), the pronociceptive action of DHPG on the RVM was obtained following its amygdaloid administration ipsi- as well as contralateral to peripheral nerve injury. This result is in line with the earlier finding that activation of the amygdala ipsilateral to nerve injury attenuated presumably antinociceptive locus coeruleus neurons (Viisanen and Pertovaara, 2007) and it also fits the finding that the amygdala receives ascending nociceptive inputs from the ipsi- as well as contralateral body half (Bernard et al., 1996). It may be proposed that the enhancement of contralateral afferent inputs in the amygdala (Ikeda et al., 2007) and the pronociceptive change in the amygdala-induced pain regulatory effect that was observed also ipsilateral to nerve injury (Viisanen and Pertovaara, 2007; the present results) have, at least partly, different underlying mechanisms.

Glutamatergic receptor types sensitized in the amygdala

Earlier results indicate that in arthritis, the responses of multireceptive neurons in the central nucleus of the amygdala are sensitized to administration of DHPG, an $mGluR_1$ and $mGluR_5$ agonist but not to CHPG, an $mGluR_5$ agonist (Li and Neugebauer, 2004b; Neugebauer et al., 2003). This finding suggests that the amygdaloid $mGluR_1$ plays a role in arthritic pain-related sensitization (Li and Neugebauer, 2004b; Neugebauer et al., 2003). The present results on the amygdaloid influence on the RVM and affective pain-related behavior extend this earlier finding by showing that a change in the function of the amygdaloid group I mGluR, and particularly the subtype $mGluR_1$, may contribute to pain-related sensitization in peripheral neuropathy as well as in arthritis. In contrast, while the NMDA-R contributes to pain-related sensitization of amygdala neurons in arthritis (Li and Neugebauer, 2004a), a recent study indicated that sensitization of amygdala neurons in peripheral neuropathy is not dependent on the NMDA-R (Ikeda et al., 2007). In line with this, amygdaloid administration of an NMDA-R antagonist failed to induce a change in the discharge rate of RVM neurons in nerve-injured animals of the present study. While studies performed in non-neuropathic animals have provided evidence suggesting that the central nucleus of the amygdala plays a significant role in promoting affective pain behavior induced by noxious visceral stimulation, its lesion has attenuated place aversion induced by noxious cutaneous stimulation, too (Tanimoto et al., 2003), a finding which is in line with the present results. It should be noted, however, that the currently administered injection volume of 0.5 μl into the central nucleus of the amygdala is likely to spread also to immediately adjacent areas (Myers, 1966), particularly other amygdaloid subnuclei. Therefore, we cannot exclude the possibility that the group I mGluRs e.g. in the basolateral nucleus of the amygdala contribute to the DHPG-induced pronociceptive effect in the present study. In a distant control site, the hippocampus, DHPG, however, had no pronociceptive effect indicating that the pronociceptive effect was region-specific.

Influence of the amygdala on discharge rates of RVM cells

An earlier study showed that microinjection of morphine into the basolateral nucleus of the amygdala produced antinociception that was accompanied by a decrease in the discharge rate of RVM ON-cells and an increase in the discharge rate of RVM OFF-cells, while morphine in the central or lateral nuclei of the amygdala failed to influence pain-related behavior or discharge rates of RVM ON- or OFF-cells (McGaraughty and Heinricher, 2002). Since the main amygdaloid output nucleus is the central nucleus receiving convergent information from various amygdaloid nuclei (Pitkänen et al., 1997), the antinociceptive effect by morphine in the basolateral nucleus was likely to be relayed to the RVM via the amygdaloid central nucleus. In the present study, DHPG was microinjected into the central nucleus of the amygdala, although the results do not allow excluding that group I mGluRs in adjacent amygdaloid subnuclei contributed to its pronociceptive effect. Together, earlier (McGaraughty and Heinricher, 2002) and the present results indicate that the amygdala has an influence on neuronal discharge rates in the RVM, a major pain regulatory region (Gebhart, 2004). The pain-regulatory effect originating in or relaying through the amygdala may have been mediated to the RVM directly (Hermann et al., 1997) or indirectly through the periaqueductal gray (Rizvi et al., 1991). Moreover, efferent pathways other than those projecting directly or indirectly to the RVM may contribute to pain regulation by the amygdala. It should also be noted that the increased affective pain response to mechanical stimulation of the neuropathic limb in the aversive place-conditioning task by the amygdaloid $mGluR_1$ may reflect increased afferent barrage evoked by the mechanical test stimulus due to increased RVM ON-cell activity, a distinct mechanism facilitating affective pain, or both.

Spontaneous discharge rate of RVM cells following nerve injury

Increase in the ongoing discharge rate of RVM ON-cells and a decrease in the ongoing discharge rate of RVM OFF-cells have been associated with sustained hyperalgesia induced by inflammation (Kincaid et al., 2006). The cumulative results of the present and our preceding study (Gonçalves et al., 2007) support the hypothesis that an increased spontaneous discharge rate of RVM ON-cells and a decreased discharge rate of RVM OFF-cells contribute to maintenance of hypersensitivity also during the early phase (first week) of peripheral neuropathy in animals with SNI. During the late phase (eighth week) of neuropathy, the discharge rates of RVM ON- and OFF-cells were returned to control levels indicating that an abnormality in the ongoing discharge rate of RVM ON- or OFF-cells may not explain hypersensitivity induced by SNI. The return of the ongoing discharge rate to control levels during the late phase of SNI-induced neuropathy, however, does not exclude the possibility that the RVM ON- or OFF-cells contributed to maintenance of hypersensitivity through a change in the synaptic efficacy of their efferent connections. A change in the ongoing discharge rates of RVM ON- and OFF-cells is not pathognomic for the early phase of peripheral neuropathy in all experimental models as indicated by observations made in animals with ligation of the spinal nerves (Carlson et al., 2007; Pertovaara et al., 2001) or chronic constriction of the sciatic nerve (Luukko and Pertovaara, 1993). Additionally, peripheral nerve injury may induce enhanced responses of RVM cells to peripheral stimulation that potentially contributes to abnormal feedback regulation of ascending nociceptive signals (Carlson et al., 2007; Gonçalves et al., 2007).

Conclusions

Present results indicate that group I mGluRs in the amygdala may promote nociception in nerve-injured animals. This was shown by increased excitation of pronociceptive RVM ON-cells following amygdaloid administration of a group I mGluR agonist in neuropathic animals. Since the amygdala has a key role in processing emotions and since the administration of glutamatergic compounds into the amygdala potentially mimics emotional activation of the amygdala, it may be proposed that activation of the amygdaloid group I mGluR, provides a possible mechanism for emotional enhancement of nerve injury-related hypersensitivity and pain. This proposal is supported by the behavioral finding indicating that amygdaloid administration of an $mGluR_{1/5}$ agonist increased and that of an $mGluR_1$ antagonist decreased affective pain-related behavior when the nerve-injured animal was exposed to an emotional challenge provided by the aversive place-conditioning test.

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References

Almeida, A., Leite-Almeida, H., Tavares, I., 2006. Medullary control of nociceptive transmission: reciprocal dual communication with the spinal cord. Drug Discov. Today Dis. Mech. 3, 305–312.

Bernard, J.F., Bester, H., Besson, J.M., 1996. Involvement of the spino-parabrachio-amygdaloid and -hypothalamic pathways in the autonomic and affective emotional aspects of pain. Prog. Brain Res. 107, 243–255.

Carlson, J.D., Maire, J.J., Martenson, M.E., Heinricher, M.M., 2007. Sensitization of pain-modulating neurons in the rostral ventromedial medulla after peripheral nerve injury. J. Neurosci. 27, 13222–13231.

Carrasquillo, Y., Gereau IV, R.W., 2007. Activation of the extracellular signal-related kinase in the amygdala modulates pain perception. J. Neurosci. 27, 1543–1551.

Craig, K.D., 2006. Emotions and psychobiology, In: McMahon, S.B., Koltzenburg, M. (Eds.), Wall and Melzack's Textbook of Pain, fifth ed. Elsevier, China, pp. 231–239.

Decosterd, I., Woolf, C.J., 2000. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain 87, 149–158.

Fields, H.L., Basbaum, A.I., Heinricher, M.M., 2006. Central nervous system mechanisms of pain modulation, In: McMahon, S.B., Koltzenburg, M. (Eds.), fifth ed. Elsevier, China, pp. 125–142.

Gebhart, G.F., 2004. Descending modulation of pain. Neurosci. Biobehav. Rev. 27, 729–737.

Gonçalves, L., Almeida, A., Pertovaara, A., 2007. Pronociceptive changes in response properties of rostroventromedial medullary neurons in a rat model of peripheral neuropathy. Eur. J. Neurosci. 26, 2188–2195.

Gonçalves, L., Silva, R., Pinto-Ribeiro, F., Pêgo, J.M., Bessa, J.M., Pertovaara, A., Sousa, N., Almeida, A., 2008. Neuropathic pain is associated with depressive behaviour and induces neuroplasticity in the amygdala of the rat. Exp. Neurol. 213, 48–56.

Greenwood-Van Meerveld, B., Gibson, M., Gunder, W., Shepard, J., Foreman, R., Myers, D., 2001. Stereotaxic delivery of corticosterone to the amygdala modulates colonic sensitivity in rats. Brain Res. 893, 135–142.

Helmstetter, F.J., Bellgowan, P.S., 1993. Lesions of the amygdala block conditional hypoalgesia on the tail flick test. Brain Res. 612, 253–257.

Helmstetter, F.J., Tershner, S.A., Poore, L.H., Bellgowan, P.S., 1998. Antinociception following opioid stimulation of the basolateral amygdala is expressed through the periaqueductal gray and rostral ventromedial medulla. Brain Res. 779, 104–118.

Hermann, D., Luppi, P., Peyron, C., Hinckel, P., Jouvet, M., 1997. Afferent projections to the rat nuclei raphe magnus, raphe pallidus, and reticularis gigantocellularis pars alpha demonstrated by iontophoretic application of choleratoxin. J. Chem. Neuroanat. 13, 1–21.

Ikeda, R., Takahashi, Y., Inoue, K., Kato, F., 2007. NMDA receptor-independent synaptic plasticity in the central amygdala in the rat model of neuropathic pain. Pain 127, 161–172.

Kim, J., Lee, S., Park, H., Song, B., Hong, I., Geum, D., Shin, K., Choi, S., 2007. Blockade of amygdala metabotropic glutamate receptor subtype 1 impairs fear extinction. Biochem. Biophys. Res. Comm. 355, 188–193.

Kincaid, W., Neubert, M.J., Xu, M., Kim, C.J., Heinricher, M.M., 2006. Role of medullary pain facilitating neurons in secondary thermal hyperalgesia. J. Neurophysiol. 95, 33–41.

LaBuda, C.J., Fuchs, P.N., 2000. A behavioral test paradigm to measure the aversive quality of inflammatory and neuropathic pain in rats. Exp. Neurol. 163, 490–494.

Li, W., Neugebauer, V., 2004a. Block of NMDA and non-NMDA receptor activation results in reduced background and evoked activity of central amygdala neurons in a model of arthritic pain. Pain 110, 112–122.

Li, W., Neugebauer, V., 2004b. Differential roles of mGluR1 and mGluR5 in brief and prolonged nociceptive processing in central amygdala neurons. J. Neurophysiol. 91, 13–24.

Lopez de Armentia, M., Sah, P., 2007. Bidirectional synaptic plasticity at nociceptive afferents in the rat central amygdala. J. Physiol. (Lond.) 581, 961–970.

Luukko, M., Pertovaara, A., 1993. Influence of an experimental peripheral mononeuropathy on the responses of medial bulboreticular neurons to noxious skin stimulation and the modulation of the responses by an α_2-adrenoceptor agonist in the rat. Exp. Neurol. 124, 390–394.

Manning, B.H., 1998. A lateralized deficit in morphine antinociception after unilateral inactivation of the central amygdala. J. Neurosci. 18, 9453–9470.

Manning, B.H., Mayer, D.J., 1995. The central nucleus of the amygdala contributes to the production of morphine antinociception in the rat tail-flick test. J. Neurosci. 15, 8199–8213.

McGaraughty, S., Heinricher, M.M., 2002. Microinjection of morphine into various amygdaloid nuclei differentially affects nociceptive responsiveness and RVM neuronal activity. Pain 96, 153–162.

Mena, N.B., Mathur, R., Nayar, U., 1995. Amygdaloid involvement in pain. Indian J. Physiol. Pharmacol. 39, 339–346.

Myers, R.D., 1966. Injection of solutions into cerebral tissue: relation between volume and diffusion. Physiol. Behav. 1, 171–174.

Nandigama, P., Borszcz, G.S., 2003. Affective analgesia following the administration of morphine into the amygdala of rats. Brain Res. 959, 343–354.

Neugebauer, V., 2006. Subcortical processing of nociceptive information: basal ganglia and amygdala. In: Cervero, F., Jensen, T.S. (Eds.), Handbook of Clinical Neurology, vol. 81. Elsevier, Amsterdam, pp. 141–158.

Neugebauer, V., Li, W., Bird, G.C., Bhave, G., Gereau IV, R.W., 2003. Synaptic plasticity in the amygdala in a model of arthritic pain: differential roles of metabotropic glutamate receptors 1 and 5. J. Neurosci. 23, 52–63.

Neugebauer, V., Li, W., Bird, G.C., Han, J.S., 2004. The amygdala and persistent pain. Neuroscientist 10, 221–234.

Paxinos, G., Watson, C., 1998. The Rat Brain in Stereotaxic Coordinates. Academic Press, New York.

Pedersen, L.H., Scheel-Kruger, J., Blackburn-Munro, G., 2007. Amygdala GABA-A receptor involvement in mediating sensory-discriminative and affective-motivational pain responses in a rat model of peripheral nerve injury. Pain 127, 17–26.

Pertovaara, A., 2000. Plasticity in descending pain modulatory systems. Prog. Brain Res. 129, 231–242.

Pertovaara, A., Wei, H., 2003. A dissociative change in the efficacy of supraspinal versus spinal morphine in the neuropathic rat. Pain 101, 237–250.

Pertovaara, A., Keski-Vakkuri, U., Kalmari, J., Wei, H., Panula, P., 2001. Response properties of neurons in the rostroventromedial medulla of neuropathic rats: attempted modulation of responses by [1DMe]NPYF, a neuropeptide FF analogue. Neuroscience 105, 457–468.

Phelps, E.A., LeDoux, J.E., 2005. Contributions of the amygdala to emotion processing: from animal models to human behavior. Neuron 48, 175–187.

Pitkänen, A., Savander, V., LeDoux, J.E., 1997. Organization of intra-amygdaloid circuitries in the rat: an emerging framework for understanding functions of the amygdala. Trends Neurosci. 20, 517–523.

Porreca, F., Ossipov, M.H., Gebhart, G.H., 2002. Chronic pain and medullary descending facilitation. Trends Neurosci. 25, 319–325.

Quin, C., Greenwood-Van Meerveld, B., Myers, D.A., Foreman, R.D., 2003. Corticosterone acts directly at the amygdala to alter spinal neuronal activity in response to colorectal distension. J. Neurophysiol. 89, 1343–1352.

Renoldi, G., Calcagno, E., Borsini, F., Invernizzi, R.W., 2007. Stimulation of group I mGlu receptors in the ventrotegmental area enhances extracellular dopamine in the rat medial prefrontal cortex. J. Neurochem. 100, 1658–1666.

Rhudy, J.L., Meagher, M.W., 2000. Fear and anxiety: divergent effects on human pain thresholds. Pain 84, 65–75.

Rizvi, T.A., Ennis, M., Behbehani, M.M., Shipley, M.T., 1991. Connections between the central nucleus of the amygdala and the midbrain periaqueductal gray: topography and reciprocity. J. Comp. Neurol. 303, 121–131.

Tanimoto, S., Nakagawa, T., Yamauchi, Y., Minami, M., Satoh, M., 2003. Differential contributions of the basolateral and central nuclei of the amygdala in the negative affective component of chemical somatic and visceral pains in rats. Eur. J. Neurosci. 18, 2343–2350.

Van Bockstaele, E.J., Chan, J., Pickel, V.M., 1996. Input from central nucleus of the amygdala efferents to pericoerulear dendrites, some of which contain tyrosine hydroxylase immunoreactivity. J. Neurosci. Res. 45, 289–302.

Viisanen, H., Pertovaara, A., 2007. Influence of peripheral nerve injury on response properties of locus coeruleus neurons and coeruleospinal antinociception in the rat. Neuroscience 146, 1785–1794.

Woolf, C.J., Salter, M.W., 2006. Plasticity and pain: role of the dorsal horn, In: McMahon, S.B., Koltzenburg, M. (Eds.), Wall and Melzack's Textbook of Pain, fifth ed. Elsevier, China, pp. 91–106.

Zhuo, M., Gebhart, G.F., 1997. Biphasic modulation of spinal nociceptive transmission from the medullary raphe nuclei in the rat. J. Neurophysiol. 78, 746–758.

Chapter 2.5

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Response Properties of Amygdala Nociceptive Neurons to Peripherally-Evoked Stimulation and Cortical Influence in the Neuropathic Rat

(Manuscript in preparation)

Response Properties of Amygdala Nociceptive Neurons to Peripherally-Evoked Stimulation and Cortical Influence in the Neuropathic Rat

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ABSTRACT (271 words)

The amygdala (AMY) receives nociceptive inputs from the periphery and central modulatory influence from various limbic sites, including the anterior cingulate cortex (ACC). However, the influence of neuropathic pain on AMY neurons is poorly known. We determined the response characteristics of AMY nociceptive neurons to peripheral stimulation and their modulation by ACC manipulation following induction (1 week - 1W) or sustained (8 weeks – 8W) spared nerve injury (SNI) neuropathy. Additionally, in order to determine behavioural correlates for neuronal findings, the aversive quality of noxious stimulation was evaluated.

After 1W, but not 8W SNI, ACC inhibition with the NMDA-receptor antagonist MK-801 decreased the aversive behaviour resulting from noxious stimulation of the lesioned hindpaw. Concerning the activity of nociceptive neurons from basolateral (BLA) and central (CeA) AMY nuclei: (I) their spontaneous activity generally increased in both nuclei following 1W and 8W neuropathy; (II) after 1W SNI, the peripherally-evoked activity of ipsilateral BLA neurons decreased more strongly and that of CeA neurons less strongly following all types of noxious stimuli, when compared with Sham group, whereas in contralateral SNI neurons the decrease of the activity was the opposite in BLA and CeA neurons; (III) ACC glutamate stimulation increased neuronal activity of ipsilateral BLA neurons after 8W SNI, an effect that was reverted by MK-801 block of the ACC..

Results indicate that SNI results in (i) alterations of BLA and CeA neuronal activity from a short to a sustained neuropathic period and in (ii) opposite neuronal changes in the activity of BLA / CeA and ipsilateral / contralateral AMY neurons. These alterations are discussed integrating the different modulatory roles of BLA and in pain processing.

INTRODUCTION (591 words)

Pain is a multidimensional experience with sensitive-discriminative and motivational-affective components. While encoding of the sensory quality of pain is conveyed by the "lateral pain system" through specific thalamocortical connections, pathways implicated in the emotional processing of pain are far from being firmly established (Treede et al, 1999; Craig, 2003). This "medial pain system" relays diffusely in intralaminar and midline thalamic nuclei (Cliffer et al, 1991) and projects massively to areas like the amygdala (AMY; Su & Bentivoglio, 1990) and anterior cingulate cortex (ACC; Hoover & Vertes, 2007), major telencephalic areas implicated in pain processing (Rainville, 2002; Lowe et al, 2007).

The rostral ACC has been implicated in the encoding of the affective component of pain or unpleasantness of noxious stimulation, as its integrity is necessary for the "aversiveness" reaction to nociception (Johansen et al, 2001) and the negative affect associated with neuropathy-induced hypersensitivity (LaGraize et al, 2004, LaBuda and Fuchs, 2005) without inducing changes on sensory processing. Since the ACC receives and integrates nociceptive information to regulate behaviour, it is a potential source of descending nociceptive modulation. Accordingly, the ACC has a pain facilitating effect mediated by projections to the medullary dorsal reticular nucleus (Zhang et al, 2005), an area reciprocally connected with the spinal cord and implicated in nociceptive facilitation (Lima & Almeida, 2002). The ACC role in pain modulation seems to be mediated, at least in part, by NMDA-receptors (Lei et al, 2004; Tang et al, 2005; Zhang et al, 2005).

There is considerable evidence that the amygdala is involved in fear conditioning (LeDoux, 1995; Maren et al., 1996), consolidation of inhibitory avoidance memory (Malin et al, 2007), pain processing (Bernard and Besson, 1990; Neugebauer and Li, 2002) and pain modulation (Manning & Mayer, 1995). The BLA is a storage site for affective information, including negative pain affect (Tanimoto et al, 2003), and fear-related memories (LeDoux, 2000), whereas the CeA projects to the brainstem and controls arousal and response systems (Cardinal et al, 2002). The AMY modulatory role upon the supraspinal control system is mediated by projections to the brainstem, which contains centres of descending circuitries that facilitate or inhibit nociception (Xu et al, 2003; Han & Neugebauer, 2005; Han et al, 2005). The AMY is also associated with the

synaptic plasticity underlying painful memories as nociceptive inputs innervating the lateral and capsular regions of the CeA (Bernard et al. 1993) undergo long-term changes following persistent peripheral pain (Neugebauer & Li, 2003; Neugebauer et al, 2003; Ikeda et al, 2007). Additionally, structural neuroplasticity of the BLA and CeA associated to depressive-like behaviour was shown to develop following a peripheral neuropathy (Gonçalves et al, 2008).

The ACC and AMY are integrated in the limbic corticostriatal loop regulating emotions (Cardinal et al, 2002). The ACC terminate on AMY interneurons that are largely inhibitory and regulates AMY emotional responses through conscious evaluation, whereas AMY projections back to ACC influence directly cortical output (Hariri et al, 2003). The anatomical (Gabbott et al, 2005; Hoover & Vertes, 2007) and functional (Malin et al, 2007) interactions between the ACC and AMY should contribute to the regulation of the endogenous pain modulatory systems (Rainville, 2002). However, little is known about the close interaction between these higher centres for controlling the emotional component of pain.

The objective of the present study was to analyse (I) alterations in the activity of BLA and CeA amygdalar neurons resulting from a one-week (induction) or 8-weeks (sustained) peripheral neuropathy and (II) the influence that the rostral ACC may have in these alterations, by local cortical glutamate stimulation or NMDA-antagonist inhibition.

MATERIAL AND METHODS

Animals

All procedures were performed in adult, male Hannover-Wistar rats weighing 180–190 g at the beginning of the experiment (Harlan, Horst, The Netherlands). The experimental protocol was accepted by the Institutional Ethics Committee and the experiments were performed according to the guidelines of European Communities Council Directive of 24 November 1986 (86 / 609 / EEC) and IASP ethical guidelines for pain experimentation in awake animals (Zimmerman, 1983).

Neuropathy induction

Neuropathy was induced through the Spared Nerve Injury (SNI) model, as described earlier by Decosterd & Woolf (2000). Briefly, the sciatic nerve and its three terminal branches were

exposed through a small surgery, followed by the ligation and removal of 2–4 mm of the distal nerve stumps of the tibial and common peroneal nerves and leaving the sural nerve intact. The surgery was performed only after the animals were anaesthetised with pentobarbitone (50 mg / kg i.p.). In Sham-operated animals, the surgical procedure was identical, except that the tibial and common peroneal nerves were not ligated or sectioned.

Placement of cannulas in the rACC

A cannula was placed in the rostral anterior cingulate cortex (rACC) of all animals, under light anaesthesia, according to the atlas of Paxinos & Watson (1998; coordinates 1.56 mm rostrocaudally from Bregma, 0.0 mm laterally and 2.0–3.0 mm dorsoventrally). In the group submitted to one week neuropathy, surgical implantation of a cannula was performed in the same operation as ligation of nerves. In those animals subjected to 8 weeks experiment, surgical implantation of cannulas was performed 5 weeks after SNI induction.

Nociceptive tests

Nociceptive tests were performed a day before (baseline) and every two days after the surgery procedure, in order to verify the development of hypersensitivity. For assessment of tactile mechanical allodynia, the hind limb withdrawal threshold was determined by stimulating the hind paw of the operated limb with von Frey monofilaments in the area innervated by the sural nerve. The calibrated series of monofilaments used produced forces ranging from 0.16 to 15 g (North Coast Medical, Inc. Morgan Hill, CA, USA) and were applied to the foot pad with increasing force until the rat withdrew its hind limb. The lowest force producing a withdrawal response was considered the mechanical nociceptive threshold. For assessment of tactile mechanical hyperalgesia, the sural nerve area in the hind paw of the operated limb was briefly stimulated with a safety pin with an intensity sufficient to touch but not penetrate the skin (Pin Prick test). The duration of paw withdrawal was measured, with an arbitrary minimal time of 0.5 seconds (sec) (for the brief normal response) and maximal cut-off of 20 sec (for tissue protection) (Tal and Bennett, 1994).

Place escape/avoidance test

The place escape/avoidance testing (LaBuda and Fuchs, 2000a,b, 2001, 2005; LaGraize et al., 2003a,b, 2004a,b, 2006) was performed 1 week after surgical implantation of a cannula in the rACC. No training is necessary for this behavioural test. Animals were placed within a 16×40.5×30.5 cm Plexiglas chamber that was positioned on top of a grid. One half of the chamber is transparent (light area), and the other half of the chamber is painted black (dark area). During behavioural testing, animals were allowed unrestricted movement throughout the test chamber for the duration of a 30min test period. The animal is placed inside the chamber, between the two areas and the test begins following pre-microinjection withdrawal threshold testing and drug administration (saline, glutamate and MK-801), starting afterwards the suprathreshold mechanical stimulation (0.07g von Frey monofilament) applied to the plantar surface of the hindpaws at 15seconds intervals. The mechanical stimulus was applied to the right paw (ipsilateral to ligation) when the animal was within the preferred dark area of the test chamber and the left paw (contralateral to injury) when the animal was within the non-preferred light area of the test chamber. Sham-ligated animals were mechanically stimulated in an identical manner as the experimental group. The location of the animal at each 15-s interval when it is stimulated is recorded and converted to the percentage of time spent in the light side of the chamber.

Electrophysiological recordings

For electrophysiological recordings, the animals were lightly anaesthetized with pentobarbitone at a dose of 50mg/kg i.p., and maintained anaesthetized by infusing pentobarbitone (15–20 mg/kg/h). The level of anaesthesia was frequently monitored by observing the size of the pupils and by assessing withdrawal responses to noxious stimulation. When necessary, the infusion rate of pentobarbitone was increased. The rats were spontaneously breathing. A warming blanket was used to maintain body temperature within the physiological range. Peripheral perfusion was checked by evaluating the colour of ears and extremities. The animals were placed in a standard stereotaxic frame, the skull was exposed and holes drilled for: placement of recording electrodes in the basolateral (BLA; coordinates -2.28 mm rostrocaudally from Bregma, 5 or -5 mm laterally and 8.6 mm dorsoventrally, according to the atlas of Paxinos & Watson, 1998) and central amygdala (CeA; coordinates -2.16 mm rostrocaudally from Bregma,

4.2 or -4.2 mm laterally and 8.2 mm dorsoventrally) nuclei, and a guide cannula placed at the rACC (coordinates 2.7 mm rostrocaudally from Bregma, 0.5 or -0.5 mm laterally and -1.5 mm dorsoventrally), but kept 2 mm above the target. Single neuron activity was recorded extracellularly with lacquer-coated tungsten electrodes (tip impedance 3–10 MW at 1 kHz) and then 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). Actual recordings did not start until the animal was under light anaesthesia; that is, the animals gave a brief withdrawal response to a noxious pinch, but the pinch did not produce any longer-lasting motor activity, and nor did the animals have spontaneous limb movements.

Peripheral stimulation and drug administration

In electrophysiological experiments, heat stimuli (peak stimulus temperature, 40ºC; baseline temperature, 35ºC; rate of stimulus temperature increase, 4ºC/s; duration of the peak temperature of 40ºC, 10 s) and cold stimuli (peak stimulus temperature, 4ºC; baseline temperature, 35ºC; rate of stimulus temperature decrease, 4ºC/s; duration of the peak temperature of 4ºC, 10 s) were applied with a feedback-controlled Peltier device (LTS-3 Stimulator; Thermal Devices Inc., Golden Valley, MN, USA) to the lateral side of the lesioned hind paw innervated by the sural nerve. Whereas some models of neuropathy may be associated with significant skin temperature changes that provide a confounding factor to assessment of thermal responses, particularly when using radiant heat for stimulation (Luukko et al., 1994), applying thermal stimuli with a contact thermo-stimulator and starting from a standard adapting temperature reduces the possibility that a neuropathy associated change in skin temperature will influence the results (Gonçalves et al., 2007). Mechanical stimulation of the skin consisted of applying a haemostatic clamp to the tail for 5 seconds. When applied to the experimenter's hand, this stimulus produced a painful pinch sensation. Noxious visceral stimulation consisted of colorectal distension (CRD) at a noxious intensity (80 mmHg) (Ness et al., 1991). CRD was applied for 10 seconds by inflating with air a 7–8-cm flexible latex balloon inserted transanally into the descending colon and rectum. The pressure in the balloon was controlled by an electronic device (Anderson et al., 1987). Administration of glutamate (5 nmol; Sigma, St.Louis,

MO) and the NMDA-receptor antagonist MK-801 (3 nmol; Sigma, St.Louis, MO) into rACC was performed by an injection syringe inserted through the guide cannula, but 2 mm longer than the latter in order to reach the target. Neuronal recording started immediately after glutamate injection and during 5 minutes, and 5 min after MK-801 injection, during 10 minutes. When the stimulus-evoked responses were analysed, the baseline discharge frequency recorded during a corresponding period just before the stimulation was subtracted from the discharge frequencies determined during stimulation; that is, positive values represent excitatory responses evoked by peripheral stimulation and negative ones inhibitory responses.

Course of the study

Four groups of animals were included in the electrophysiological study: (i) Sham group tested 1 week after surgery (1W Sham); (ii) sham group tested 8 weeks after surgery (8W Sham); (iii) SNI group tested 1 week after surgery (1W SNI); and (iv) SNI group tested 8 weeks after surgery (8W SNI). In each of these groups, behavioural assessment of sensitivity to monofilament and pin-prick stimulation was performed before the surgery, every two days then forward and before the start of the electrophysiological experiment. After induction of anaesthesia, the microelectrode was lowered to the ipsilateral or contralateral BLA. After a single cell had been found, its spontaneous activity was recorded for 2–3 min. Then, cold stimulation was applied to the sural nerve area in the operated limb followed by pinch of the tail and CRD at 1-min intervals. The testing procedure, including the order of testing different sub-modalities of nociception, was the same in all experimental groups.

To minimize serial effects, every other animal tested in this series belonged to the Sham group and every other to the SNI group. At the completion of the study, an electrolytic lesion was made in the recording site, the animals were given a lethal dose of pentobarbitone and the brains were removed for verification of recording and microinjection sites.

Statistic analysis

Data are presented as mean ± standard deviation (S.D.). For evaluation of differences between Sham, 1W SNI and 8W SNI groups, in the avoidance paradigm test, ANOVA and Tukey post-hoc test were performed. In the evaluation of the differences of the activity of various types

of neurons in different experimental conditions, the t-test was used. $p< 0.05$ was considered to represent a significant difference.

RESULTS

1. Avoidance paradigm test

The emotional component of pain-like behaviour was evaluated by the time spent by the animals in the light chamber (**Fig. 1**). Although it is clear that Sham animals preferred the dark chamber in all three experimental situations, neuropathic animals showed different results when evaluated after 1 or 8 weeks of SNI. After 1 week neuropathy, SNI animals spent most of the time in the light chamber after saline and glutamate injection in the rACC. However, after MK-801 injection in the rACC, the behaviour changed drastically and the animals spent most of the time in the dark chamber (decreased avoidance behaviour to pain). The group tested 8 weeks after the SNI surgery still preferred the light side of the chamber and was not affected byMK-801 administered to the ACC (**Fig. 1**).

2. Changes in BLA and CeA neuronal activity induced by SNI

The activity of nociceptive BLA and CeA AMY neurons was recorded in a baseline state (spontaneous activity), after different somatic and visceral peripheral stimulation and after pharmacological stimulation of the rACC.

2.1.1. Spontaneous neuronal activity of AMY neurons

Spontaneous activity in BLA neurons recorded in SNI animals is changed, when compared with Sham animals. In the ipsilateral side, neuronal activity in SNI animals with 1 week neuropathy increased significantly, while in the 8 week SNI group the spontaneous activity was significantly decreased. In the contralateral side, the activity of the neurons of SNI animals was significantly increased only after a 8 week neuropathy (**Fig. 2A**). Concerning the spontaneous

activity of CeA neurons recorded in SNI groups, it was always significantly increased, when compared with Sham groups (**Fig. 2B**).

2.1.2. Peripherally-evoked neuronal activity

AMY neurons decreased their activity after peripheral noxious stimulation. After 1W SNI, contralateral BLA neurons showed a clear higher response than corresponding neurons in the sham group to any type of peripheral stimulation (**Fig. 3A**), whereas the opposite occurred in neurons recorded in the contralateral CeA (**Fig. 3B**). Peripheral stimulation induced a stronger decrease in the activity of ipsilateral BLA neurons of SNI animals than in Sham to most of the noxa (cold, tail-pinch, CRD and von Frey) (**Fig 3C**), whereas the opposite occurred after cold noxious stimulation (**Fig. 3D**) in the CeA.

After a prolonged neuropathy (8W), almost no differences were present between SNI and Sham neuronal responses to peripheral stimuli in the 8 week group of contralateral BLA neurons (**Fig. 4A**), whereas concerning contralateral CeA neurons, SNI neuronal responses were variable, showing a significantly higher response after cold and tail-pinch stimuli and a weaker one after CRD stimulation (**Fig. 4B**). On the other side of the AMY, after 8W SNI, the BLA neuronal responses were variable, being weaker than Sham group after heat and tail-pinch stimuli, and weaker than Sham group after cold and CRD stimuli (**Fig. 4C**)

In what concerns CeA neurons, 1 week group ipsilateral neuropathic neurons responded less strongly, specially to cold stimuli, than Sham neurons (**Fig. 3D**), whereas 8 week group neuropathic neurons responded with higher activity to peripheral stimuli than Sham neurons, with differences being significant after thermal (heat and cold) stimulation (**Fig. 4D**). Contralaterally, CeA neuronal responses to peripheral stimulation 1 week after the surgery were significantly stronger in the neuropathic than in Sham animals (**Fig. 4C**).

3. Changes in BLA neuronal activity induced by rACC manipulation

3.1. Effect on neuropathic BLA neurons after Glutamate injection in the rACC

In 8W SNI neuropathic animals, BLA ipsilateral neurons responded to glutamate activation of the rACC with a progressive greater increase than correspondent Sham neurons (**Fig. 5A**),

reaching its maximum 2-5 minutes after the beginning of the injection and returning to control levels after 6 minutes.

3.2. Effect on neuropathic BLA neurons after MK-801 injection in the rACC

The injection of the glutamate NMDA-receptor antagonist MK-801 in the rACC in 8W SNI animals resulted in a constant decreased activity of BLA neurons during the entire recording period, when compared with BLA neurons of Sham-operated animals (**Fig. 5B**).

DISCUSSION (1912 words)

In this study we analysed the changes induced by neuropathic pain upon spontaneous and peripherally-evoked neuronal activity of BLA and CeA amygdalar nuclei, and also the influence that the rACC has in these alterations following a short (1W) or prolonged (8W) neuropathic condition. Neuropathic pain increased the emotional aversive behaviour after both 1W and 8W SNI, with a NMDA-receptor pathway centred in the ACC mediatingthis behaviour (LaGraize et al, 2004; Lei et al, 2004) only at the initial (1W SNI) neuropathic period. At the cellular level, there was an increased spontaneous activity of ipsilateral BLA neurons from 1W SNI rats and ipsi- and contralateral CeA neurons from 1W and 8W SNI, when compared with neurons recorded from Sham-operated animals. Additionally, the response activity of these AMY neurons to peripheral noxious stimulation was also significantly changed in the passage from 1W to 8W SNI, not only when SNI and Sham animals were compared but also between SNI ipsilateral and contralateral BLA/CeA neurons of SNI. These alterations may reflect the morphological and functional plastic changes occurring in the AMY following chronic pain (Neugebauer at al, 2003; Ikeda et al, 2007; Gonçalves et al, 2008). Finally,manipulation of NMDA receptors in the ACC revealed an influence of these receptors in modulating the activity of BLA neurons after 8W SNI, which was not relevant, however, to drive the aversive pain behaviour at this SNI period. This suggests that after 8W SNI, other brain areas or other neurotransmitter systems in the ACC should mediate the aversive behaviour induced by the place escape/avoidance test (LaBuda and Fuchs, 2000).

Aversive behaviour induced by SNI is partly modulated by the rACC

In order to measure the aversive nature of neuropathic pain, the avoidance of a preferred location was analyzed using the place avoidance paradigm test (LaBuda and Fuchs, 2000). This test evaluates the affective-motivational component of pain, rather than the sensory/discriminative dimension. This is a relevant approach for the present study, as the amygdala is mainly associated with behavioural responses to emotional stimuli (Davis and Whalen, 2001; Han and Neugebauer, 2001; Neugebauer and Li, 1992), and is also deeply involved in nociceptive modulation (Manning and Mayer, 1995; Manning, 1998; Manning et al., 2001). There is evidence that the rACC is involved in modulating the storage of emotional events and aversive learning and direct connections between the rACC and BLA suggest that these areas may interact in consolidating aversive memories In this test, both 1W and 8W SNI animals remained more time in the light area after both saline and glutamate injection in the rACC. These results are in accordance with literature, since SNI animals seem to prefer the light chamber to the dark one, with the intention of avoiding the noxious stimulus (LaBuda and Fuchs, 2000). However, after MK-801 injection into the rACC, the response of 1W and 8W SNI animals was different. While the 8W group still remained more time in the light chamber, blocking rACC NMDA-r in 1W SNI animals inverted the aversive effect of pain to sham levels, as they stayed now more time in the dark chamber. It has been previously described that NMDArs in the ACC play an important role in the induction and expression of neuropathic pain (Zhuo M, 2004). These receptors are probably activated in an activity-dependent manner or via abnormal neuronal activities triggered under pathological conditions (Zhuo, 2006) that result in permanent alterations in the CNS, including structural changes in the ACC (Zhao et al, 2006) and AMY (Gonçalves et al, 2008). Accordingly, we hypothesize that the lack of effect of rACC NMDA-r manipulation after 8Wdeveloping neuropathic pain, central mechanisms may have changed in such a way that: (i) rACC influence in the "neuropathic pain system" is diminished, (ii) NMDArs number in the rACC is reduced, (iii) NMDArs functional role in the rACC is diminished, or (iv) other brain areas have now a major role in the control of the aversive pain behaviour.

Spontaneous activity of AMY nociceptive neurons increases with SNI

Electrophysiological recordings revealed that the spontaneous activity of BLA and CeA nociceptive neurons was higher in SNI group after 1 and 8W neuropathies, with the only

exception of 8W ipsilateral BLA recordings. In the BLA nucleus, the major changes observed were in the ipsilateral side. Interestingly, there was a shift from great increase to great decrease in this ipsilateral BLA, from 1 week to 8 weeks after surgery. After 1 week of SNI surgery, neuropathy signs were already present, as mechanical allodynia and hyperalgesia are clearly perceptible. BLA is responsible for the facilitation of memory consolidation produced by moderate emotional arousal (McGaugh et al., 2002; Wingard and Packard, 2008). BLA heavily projects to the hippocampus, main responsible for memory formation and preservation (Petrovich et al., 2001). It was described that after an emotionally arousing event, the firing rate of BLA neurons increases for several hours (Pelletier et al., 2005; Popescu et al., 2007), which may be seen in our ipsilateral neurons recordings, 1 week after surgery. On the contrary, the neuronal activity of ipsilateral BLA neurons recorded 8 weeks after the surgery is decreased. We think this may turn out because after a long-term time neuropathy, pain memory is already consolidated. The increased activity of contralateral BLA neurons may reflect ongoing nociceptive input arriving polyssynaptically to the BLA (Barnett et al, 1995). Concerning the increase of CeA spontaneous activity obtained in SNI animals, it is in accordance with literature, as it has been previously described (i) an increase in the input and output of neurotransmission at the pontine parabrachial area (PB)-CeA synapse (indicative of plasticity and enhanced processing of incoming signals to the CeA) and (ii) an increase in neuronal excitability (increased action potential frequency) of CeA neurons in neuropathic models (Han and Neugebauer, 2004).

Peripherally-induced activity of AMY neurons changes differently after 1 or 8W SNI

After 1W sham or SNI, the peripheral-induced activity of virtually all recorded neurons was diminished, comparatively to baseline activity. At this time point, in SNI animals, contralateral BLA neurons showed an increase of activation and contralateral CeA neurons were deeply inhibited following most types of noxious stimuli when compared with Sham group neurons, whereas in ipsilateral BLA and ipsilateral CeA SNI group neurons the opposite occurred. Nociceptive information reaching the AMY involves (i) the polymodal information arriving from the thalamus and cortex that reaches the lateral-BLA nuclei (McDonald, 1998; Neugebauer et al, 2004) and the spino-parabrachio-AMY pathway and the ascending input from the spinal dorsal horn, both targeting the contralateral CeA (Cliffer et al, 1991; Garieu and Bernard, 2002, 2004).

The CeA is also the major output source of AMY projections to brainstem nuclei linked to the supraspinal pain control system, such as the periaqueductal gray matter (PAG), parabrachial nuclei (PBN), locus coeruleus (Pitkänen, 2000) and dorsal reticular nucleus (DRt; Almeida et al, 2002), which make it a major regulator of endogenous nociception. Since the BLA projects to CeA (Swanson and Petrovich, 1998), it would be expected that, after peripheral stimulation, the response of neurons in both nuclei was similar. However, not only the CeA receives input from other brain areas (Swanson and Petrovich, 1998) that could had a overcome input, but also it has been described that the projections BLA→CeA can be inhibitory (Rosenkranz et al., 2006; Royer et al 1999) by the presence of intercalated cell masses (ICM; Royer et al 1999; Sun et al 1994), a band of -aminobutyric acid (GABA)-containing inhibitory neurons present between the BLA and the CeA. Furthermore, CeA neurons targeting autonomic regions are GABAergic- (Batten et al 2002; Jia et al, 2005) and more likely to inhibit downstream targets that mediate affective responses. Thus, it is possible that the BLA output suppresses CeA neuronal activity and that this suppression of activity allows disinhibition of downstream targets. This would allow the production of an affective response and justify the opposite activity shown by BLA and CeA neurons following peripheral stimulation, mainly after 1W SNI. Interestingly, neuronal recordings in the AMY showed that approximately the same percentage of CeA neurons were excited and inhibited (46/34%) after peripheral noxious stimulation (Bernard et al., 1992). On the other hand, after 8W SNI the response of both BLA and CeA nociceptive neurons was clearly changed, with the disappearance of the opposite interaction between BLA and CeA neuronal activity. These alterations may represent the consequence of plastic changes occurring in the AMY following the same period of neuropathic pain induced by the SNI model (Gonçalves et al., 2008).

rACC-induced activity of BLA neurons is NMDA-r mediated after 8W SNI

Glutamate stimulation of the rACC showed an increase in neuronal activity of ipsilateral BLA neurons in SNI animals 8 weeks after the surgery, an effect that was reduced by MK-801 activity in the ACC. Since the ACC has a major projection to the BLA (McDonald, 1998), we can conclude that a rAAC→BLA pathway mediated by NMDA-r is still present after 8W SNI. However, an MK-801 injection into the rACC did not affect aversive behaviour after this neuropathic time period (8W; see above); this suggests that, although NMDA receptors in the anterior cingulate

cortex mediate pain-related aversion (Lei et al, 2004) probably through the BLA (Deyama et al, 2007), alterations induced by prolonged chronic pain may have altered modulatory pain pathways. In accordance with this hypothesis, an enhanced synaptic transmission of nociceptive specific inputs (PB→CeA synapse) and polymodal sensory inputs (BLA→CeA synapse) has been demonstrated already in a persistent pain model of arthritis (Neugebauer et al, 2003). CeA neurons from arthritic rats developed also increased excitability compared with control CeA neurons, as synaptic plasticity in the CeA was accompanied by increased presynaptic mGluR1 function and upregulation of mGluR1 metabotropic glutamate receptors (Neugebauer et al, 2003). These plastic changes probably explain the lack of effect of rACC administration of MK-801, a NMDA ionotropic glutamate receptor, in emotional pain-like behaviour after 8W SNI (see above). However, we cannot exclude that other neurotransmitters acting in the BLA same area, or other brain areas can influence the results observed in the avoidance paradigm test. In fact, the ACC is known to project to the AMY (McDonald, 1998), but also to exert descending pain modulatory actions through the DRt (Zhang et al., 2005) and areas that have been consistently implicated in the pain facilitation that is associated with neuropathic pain, namely the RVM (Calajesan et al, 2000 Burgess et al, 2002). Further studies are needed to elucidate this essential point of neuroplasticity in the higher processing of nociceptive information observed between 1 and 8 weeks of neuropathic pain.

Conclusion

BLA and CeA neuronal basal activity is increased in animals with peripheral neuropathy, 1 and 8 weeks after the SNI model induction. This result was somehow expected, due to the known neuronal hyperexcitabilty that is present in neuropathic pain (Bleakman et al., 2006). However, as far as we know, the neuronal basal activity at these amygdalar nuclei in animal models of neuropathy has not been described before. The peripheral induced activity revealed that, not only the BLA influence over the CeA is inhibitory, but also that the BLA is not the strongest influence in the CeA in this neuropathy model. These effects are not so linear in the longest period of time here analysed. This observation, together with the behaviour results and previous experimental work published by our and other groups (Gonçalves et al., 2008) lead us to believe that, after a

long period of time developing neuropathy, there are central changes in the pain modulatory system, which is subsequently expressed in other systems.

REFERENCES

Bernard JF, Alden M, Besson JM. The organization of the efferent projections from the pontine parabrachial area to the amygdaloid complex: a *Phaseolus vulgaris* leucoagglutinin (PHA-L) study in the rat. J Comp Neurol. 1993; 329(2):201-29.

Bernard JF, Besson JM. The spino(trigemino)pontoamygdaloid pathway: electrophysiological evidence for an involvement in pain processes. J Neurophysiol. 1990; 63(3):473-90.

Bleakman D, Alt A, Nisenbaum ES. Glutamate receptors and pain Seminars in Cell & Developmental Biology. 2006; 17:592–604. Review

Cardinal RN, Parkinson JA, Hall J, Everitt BJ. Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. Neurosci Biobehav Rev. 2002; 26(3):321-52. Review.

Craig AD. Distribution of trigeminothalamic and spinothalamic lamina I terminations in the cat. Somatosens Mot Res. 2003; 20(3-4):209-22.

Cliffer KD, Burstein R, Giesler GJ Jr. Distributions of spinothalamic, spinohypothalamic, and spinotelencephalic fibers revealed by anterograde transport of PHA-L in rats. J Neurosci. 1991; 11(3):852-68.

Davis M, Whalen PJ. The amygdala: vigilance and emotion. Mol Psychiatry. 2001; 6:13-34.

Decosterd, I., Woolf, C.J., 2000. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain. 87:149-158.

Gabbott PL, Warner TA, Jays PR, Salway P, Busby SJ. Prefrontal cortex in the rat: projections to subcortical autonomic, motor, and limbic centers. J Comp Neurol. 2005; 492(2):145-77.

Gonçalves L, Almeida A, Pertovaara A. Pronociceptive changes in response properties of rostroventromedial medullary neurons in a rat model of peripheral neuropathy. Eur J Neurosci. 2007; 26:2188–2195.

Gonçalves L, Silva R, Pinto-Ribeiro F, Pêgo JM, Bessa JM, Pertovaara A, Sousa N, Almeida A. Neuropathic pain is associated with depressive behaviour and induces neuroplasticity in the amygdala of the rat. Exp Neurol. 2008; 213(1):48-56.

Han JS, Neugebauer V. Synaptic plasticity in the amygdala in a visceral pain model in rats. Neurosci Lett. 2004; 361:254-257.

Han JS, Neugebauer V. mGluR1 and mGluR5 antagonists in the amygdala inhibit different components of audible and ultrasonic vocalizations in a model of arthritic pain. Pain. 2005; 113(1-2):211-22.

Han JS, Li W, Neugebauer V. Critical role of calcitonin gene-related peptide 1 receptors in the amygdala in synaptic plasticity and pain behavior. J Neurosci. 2005; 25(46):10717-28.

Hariri AR, Mattay VS, Tessitore A, Fera F, Weinberger DR. Neocortical modulation of the amygdala response to fearful stimuli. Biol Psychiatry. 2003; 15;53(6):494-501.

Hoover WB, Vertes RP. Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat. Brain Struct Funct. 2007; 212(2):149-79. Epub 2007; 27.

Johansen JP, Fields HL, Manning BH. The affective component of pain in rodents: direct evidence for a contribution of the anterior cingulate cortex. Proc Natl Acad Sci U S A. 2001; 98(14):8077-82.

Koo JW, Han JS, Kim JJ. Selective neurotoxic lesions of basolateral and central nuclei of the amygdala produce differential effects on fear conditioning. J Neurosci. 2004; 24(35):7654-62.

LaBuda CJ, Fuchs P. Behavioral test paradigm to measure the aversive quality of inflammatory and neuropathic pain in rats. Exp Neurol. 2000; 163, 490–494.

LaBuda CJ, Fuchs PN. Catecholamine depletion by reserpine blocks the anxiolytic actions of ethanol in the rat. Alcohol. 2002; 26(1):55-9.

LaBuda CJ, Fuchs PN. Attenuation of negative pain affect produced by unilateral spinal nerve injury in the rat following anterior cingulate cortex activation. Neuroscience. 2005; 136(1):311-22.

LaGraize SC, Borzan J, Rinker MM, Kopp JL, Fuchs PN. Behavioral evidence for competing motivational drives of nociception and hunger. Neurosci Lett. 2004; 372(1-2):30-4.

LeDoux JE. Emotion circuits in the brain. Annu Rev Neurosci. 2000; 23:155-84. Review.

LeDoux JE. Emotion: clues from the brain. Annu Rev Psychol. 1995; 46:209-35. Review.

Lei LG, Sun S, Gao YJ, Zhao ZQ, Zhang YQ. NMDA receptors in the anterior cingulate cortex mediate pain-related aversion. Exp Neurol. 2004; 189(2):413-21.

Lima, D., Almeida, A., 2002. The medullary dorsal reticular nucleus as a pronociceptive centre of the pain control system. Prog Neurobiol. 66:81-108.Lowe et al, 2007

Malin EL, Ibrahim DY, Tu JW, McGaugh JL. Involvement of the rostral anterior cingulate cortex in consolidation of inhibitory avoidance memory: interaction with the basolateral amygdala. Neurobiol Learn Mem. 2007; 87(2):295-302.

Manning BH. A lateralized deficit in morphine antinociception after unilateral inactivation of the central amygdala. J Neurosci 1998;18:9453-70.

Manning BH, Mayer DJ. The central nucleus of the amygdala contributes to the production of morphine antinociception in the rat tail-flick test. J Neurosci. 1995, 15:8199-8213.

Manning BH, Merin NM, Meng ID, Amaral DG. Reduction in opioid- and cannabinoid-induced antinociception in rhesus monkeys after bilateral lesions of the amygdaloidcomplex. J Neurosci. 2001, 21:8238-46.

Maren S, Aharonov G, Fanselow MS. Retrograde abolition of conditional fear after excitotoxic lesions in the basolateral amygdala of rats: absence of a temporal gradient. Behav Neurosci. 1996; 110(4):718-26.

McGaugh JL, McIntyre CK, Power AE. Amygdala modulation of memory consolidation: interaction with other brain systems. Neurobiol Learn Mem. 2002; 78(3):539-52.

Nakagawa T, Katsuya A, Tanimoto S, Yamamoto J, Yamauchi Y, Minami M, Satoh M. Differential patterns of c-fos mRNA expression in the amygdaloid nuclei induced by chemical somatic and visceral noxious stimuli in rats. Neurosci. Lett. 2003; 344:197–200.

Neugebauer V, Li W. Processing of nociceptive mechanical and thermal information in central amygdala neurons with knee-joint input. J Neurophysiol. 1992; 87:103-12.

Neugebauer V, Li W. Processing of nociceptive mechanical and thermal information in central amygdala neurons with knee-joint input. J Neurophysiol. 2002; 87(1):103-12.

Neugebauer V, Li W. Differential sensitization of amygdala neurons to afferent inputs in a model of arthritic pain. J Neurophysiol. 2003; 89(2):716-27.

Neugebauer V, Li W, Bird GC, Bhave G, Gereau RW 4th. Synaptic plasticity in the amygdala in a model of arthritic pain: differential roles of metabotropic glutamate receptors 1 and 5. J Neurosci. 2003; 23(1):52-63.

Pelletier JG, Likhtik E, Filali M, Paré D. Lasting increases in basolateral amygdala activity after emotional arousal: implications for facilitated consolidation of emotional memories. Learn Mem. 2005 Mar-Apr;12(2):96-102.

Petrovich GD, Canteras NS, Swanson LW. Combinatorial amygdalar inputs to hippocampal domains and hypothalamic behavior systems. Brain Res. Rev. 2001; 38: 247– 289 (21).

Pikkarainen M, Ronkko S, Savander V, Insausti R, Pitkänen A. Projections from the lateral, basal, and accessory basal nuclei of the amygdala to the hippocampal formation in rat. J. Comp. Neurol. 1999; 403: 229– 260.

Pitkänen A. Connectivity of the rat amygdaloid complex. In. "The Amygdala: a functional analysis", Aggleton JP (Ed.), 2nd Ed. Oxford, Oxford University Press, pp 31-115

Pitkänen A, Pikkarainen M, Nurminen N, Ylinen A. Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat, Ann. N.Y. Acad. Sci. 2000; 911: 369– 391.

Popescu AT, Saghyan AA, Paré D. NMDA-dependent facilitation of corticostriatal plasticity by the amygdala. Proc Natl Acad Sci U S A. 2007 Jan 2;104(1):341-6. Epub 2006 Dec 20.

Rainville P. Brain mechanisms of pain affect and pain modulation. Curr Opin Neurobiol. 2002; 12(2):195-204. Review.

Rosenkranz JA, Buffalari DM, Grace AA. Opposing influence of basolateral amygdala and footshock stimulation on neurons of the central amygdala. Biol Psychiatry. 2006 May 1;59(9):801-11. Epub 2005 Dec 20.

Royer S, Martina M, Pare D (1999): An inhibitory interface gates impulse traffic between the input and output stations of the amygdala. *J Neurosci* 19:10575–10583.

Sah P, Nicoll RA. Mechanisms underlying potentiation of synaptic transmission in rat anterior cingulate cortex in vitro. J Physiol. 1991; 433:615– 630.

Su HS, Bentivoglio M. Thalamic midline cell populations projecting to the nucleus accumbens, amygdala, and hippocampus in the rat. J Comp Neurol. 1990; 297(4):582-93.

Sun N, Yi H, Cassell MD. Evidence for a GABAergic interface between cortical afferents and brainstem projection neurons in the rat central extended amygdala. J Comp Neurol. 1994; 340:43– 64.

Swanson LW and Petrovich GD. What is the amygdala? Trends Neurosci. 1998; 21, 323–331.

Xu W, Lundeberg T, Wang YT, Li Y, Yu LC. Antinociceptive effect of calcitonin gene-related peptide in the central nucleus of amygdala: activating opioid receptors through amygdala-periaqueductal gray pathway. Neuroscience. 2003; 118(4):1015-22.

Tanimoto S, Nakagawa T, Yamauchi Y, Minami M, Satoh M. Differential contributions of the basolateral and central nuclei of the amygdala in the negative affective component of chemical somatic and visceral pains in rats. Eur J Neurosci. 2003 Oct;18(8):2343-50.

Tang J, Ko S, Ding HK, Qiu CS, Calejesan AA, Zhuo M. Pavlovian fear memory induced by activation in the anterior cingulate cortex. Mol Pain. 2005; Feb 9;1:6.

Traub RJ, Silva E, Gebhart GF, Solodkin A. Noxious colorectal distention induced c-Fos protein in limbic brain structures in the rat. Neurosci. Lett. 1996; 215:165–168.

Treede RD, Vogel H, Rios M, Krauss G, Lesser RP, Lenz FA. Pain-related evoked potentials from parasylvian cortex in humans. Electroencephalogr Clin Neurophysiol Suppl. 1999; 49:250-4. Review.

Wei F, Li P, Zhuo M. Loss of synaptic depression in mammalian anterior cingulate cortex after amputation. J Neurosci. 1999; 19:9346–9354.

Wingard JC, Packard MG. The amygdala and emotional modulation of competition between cognitive and habit memory. Behav Brain Res. 2008 May 8. [Epub ahead of print]

Tal M, Bennett GJ. Extra-territorial pain in rats with a peripheral mononeuropathy: mechano-hyperalgesia and mechano-allodynia in the territory of an uninjured nerve. Pain. 1994; 57, 375–382.

Zhang L, Zhang Y, Zhao ZQ. Anterior cingulate cortex contributes to the descending facilitatory modulation of pain via dorsal reticular nucleus. Eur J Neurosci. 2005; 22(5):1141-8.

Zhao MG, Ko SW, Wu LJ, Toyoda H, Xu H, Quan J, Li J, Jia Y, Ren M, Xu ZC, Zhuo M. Enhanced presynaptic neurotransmitter release in the anterior cingulate cortex of mice with chronic pain. J Neurosci. 2006; 26(35):8923-30.

Zhuo M. Central plasticity in pathological pain. Novartis Found Symp. 2004;261:132-45; discussion 145-54.

Zhuo M. Molecular Mechanisms of Pain in the Anterior Cingulate Cortex. Journal of Neuroscience Research 84:927–933 2006.

FIGURES

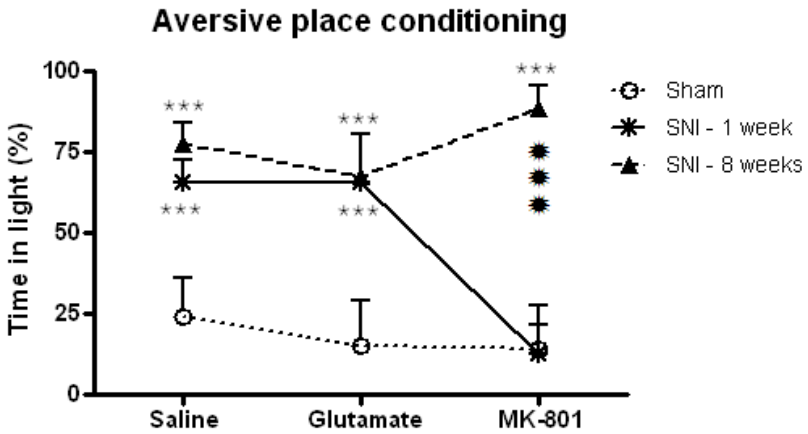


Figure 1 – The aversive nature of neuropathic pain was assessed in SNI and Sham groups, 1 and 8 weeks after surgery, by the place escape/avoidance test following the injection of saline, glutamate or MK-801 in the rACC. Note that the time spent by both 1W and 8W SNI groups in the light chamber was similar, but significantly higher when compared with Sham group after saline or glutamate injection in the rACC (ANOVA<0.05; 1W SNI x Sham, $p<0.05$; 8W SNI x Sham, $p<0.05$, Tukey tests); on the other hand, after MK-801 injection in the rACC, a significantly higher avoidance behaviour was still observed in 8W SNI animals when compared with Sham group, but this aversive behaviour was reverted to Sham levels in 1W SNI animals (ANOVA<0.05; 1W SNI x 1W SNI, $p<0.05$; 1W SNI x Sham, $p>0.05$; 8W SNI x Sham, $p<0.05$, Tukey tests). The symbols and error bars represent mean ± S.D. in this and subsequent figures.

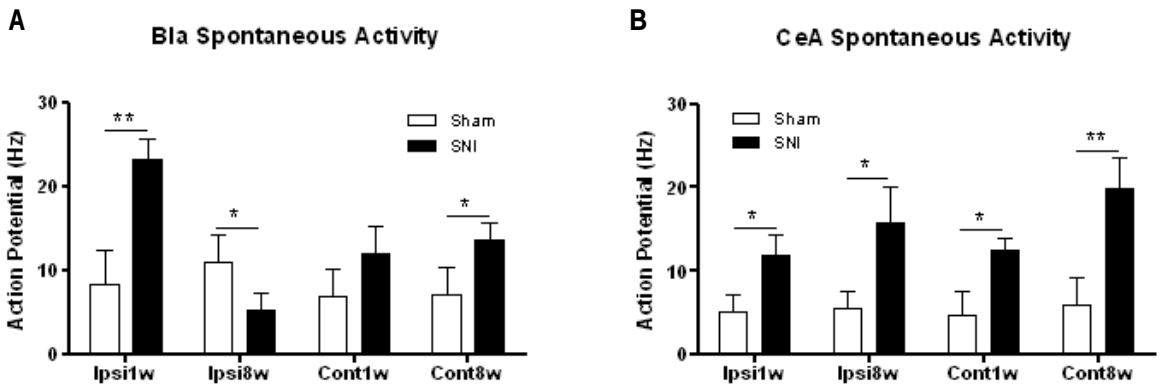


Figure 2 – Spontaneous activity recordings of amygdalar neurons. (**A**) Basal activity of ipsilateral BLA neurons is significantly increased 1 week after the SNI surgery (1W SNI BLA_{ipsi} x Sham, $p<0.01$, t-test) and significantly decreased 8 week after the surgery (8W SNI BLA_{ipsi} x Sham, $p<0.05$, t-test). In contralateral BLA, basal activity is increased after both 1 and 8 weeks SNI, with differences from BLA Sham neurons being statistically different only after a prolonged neuropathy (1W SNI BLA_{cont} x Sham, $p=0.08$; 8W SNI BLA_{cont} x Sham, $p<0.05$,, t-tests) . (**B**) In the CeA nucleus, spontaneous activity was increased in both sides, at both time neuropathic periods (1W SNI CeA_{ipsi} x Sham, $p<0.05$; 8W SNI CeA_{ipsi} x Sham, $p<0.05$; 1W SNI CeA_{cont} x Sham, $p<0.05$; 8W SNI CeA_{cont} x Sham, $p<0.01$, t-tests).

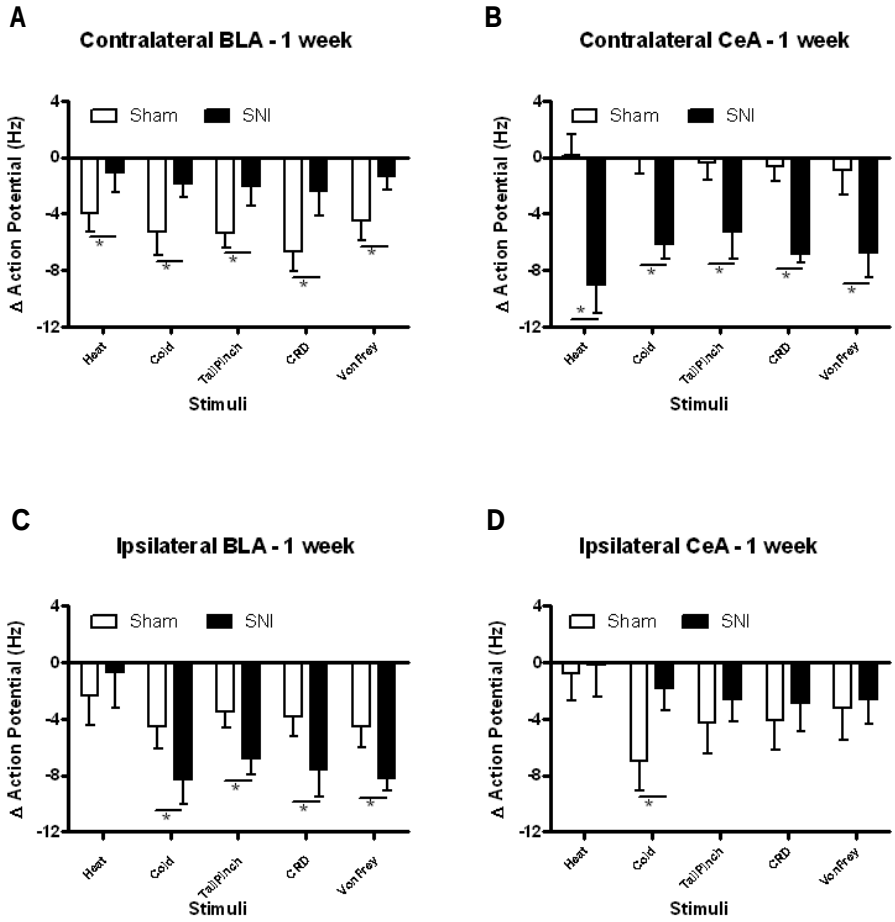


Figure 3 – Peripherally-evoked changes in basal activity of BLA and CeA neurons induced by somatic and visceral stimulation, 1 week after SNI or Sham surgery. Neuronal activity graphic representation is always compared with its relative basal (spontaneous) activity, shown in the graphs has a 0 (zero) Hz line. Note that the activity of most AMY neurons recorded, both from SNI or Sham animals, from any side of the brain or after 1W or 8W surgery, decreased after peripheral noxious stimulation when compared with their original basal activity. (A) Although the activity of contralateral BLA neurons decreased in both Sham and SNI groups, the SNI group changed the least after all the stimuli and were always more active than Sham neurons. The differences between Sham and SNI groups are statistically significant for all the stimuli ($p<0.05$, t-tests). (B) The activity of contralateral CeA neurons was almost unchanged in Sham animals following peripheral stimulation, whereas that of SNI neurons was significantly higher inhibited after any type of noxa applied ($p<0.05$, t-tests). (C) The activity of ipsilateral BLA neurons decreased in both Sham and SNI groups, with the strongest inhibition being that of SNI animals after all stimuli ($p<0.05$, t-test), with the exception of heat stimulation. (D) The activity of ipsilateral CeA activity decreased in both Sham and SNI groups, with no relevant differences between Sham and SNI groups, with exception of the cold stimulus response, where SNI neurons were higher activated than Sham cells ($p<0.05$, t-test)

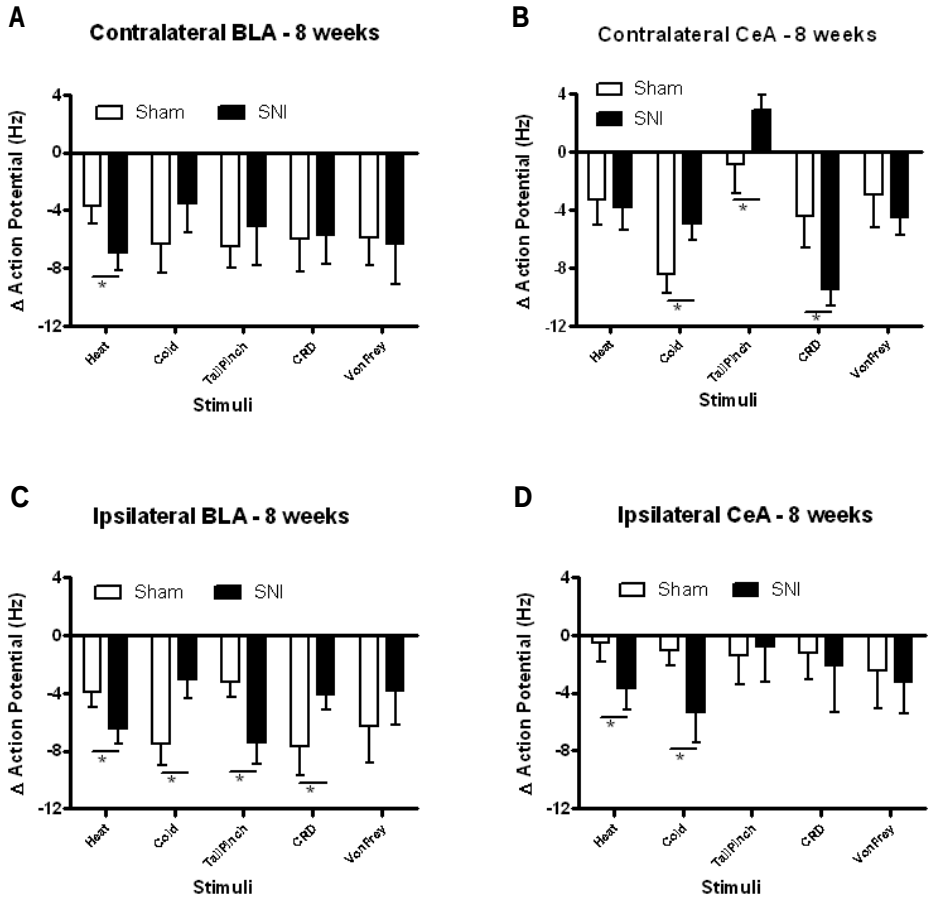


Figure 4 – Peripherally-evoked changes in basal activity of BLA and CeA neurons induced by somatic and visceral stimulation, 8 weeks after SNI or Sham surgery. Neuronal activity graphic representation is always compared with its relative basal (spontaneous) activity, shown in the graphs has a 0 (zero) Hz line. Note that again, with very few exceptions, the activity of AMY neurons recorded, both from SNI or Sham animals, from any side of the brain or after 1W or 8W surgery, decreased after peripheral noxious stimulation when compared with their original basal activity. Importantly, the response pattern of BLA and CeA neurons in 8W SNI versus Sham groups, did not remain as clearly similar along the different noxa as after 1W SNI (Fig. 3). (A) Contralateral BLA activity decreased in both Sham and SNI groups, with no relevant differences between Sham and SNI groups, with exception of the heat stimulus response, where SNI neurons showed a higher decrease ($p<0.05$, t-test). (B) Contralateral CeA activity decreased in both Sham and SNI groups to all the stimuli, except for neuropathic nerons responding to tail-pinch, where the evoked-activity was higher than the basal (spontaneous) one; the response of CeA SNI neurons was significantly higher than Sham after cold and tail-pinch stimuli ($p<0.05$, t-tests) and significantly decreased after CRD stimulation. (C) Ipsilateral BLA activity decreased in both Sham and SNI groups, with a significant stronger decrease of SNI neurons after heat and tail pinch stimuli ($p<0.05$, t-tests), whereas after cold and CRD stimulation SNI neurons were more activated than Sham ($p<0.05$, t-tests). (D) Ipsilateral CeA activity decreased in both Sham and SNI groups, with significantly stronger decreases in the activity of SNI cells after heat and cold stimuli responses ($p<0.05$, t-tests).

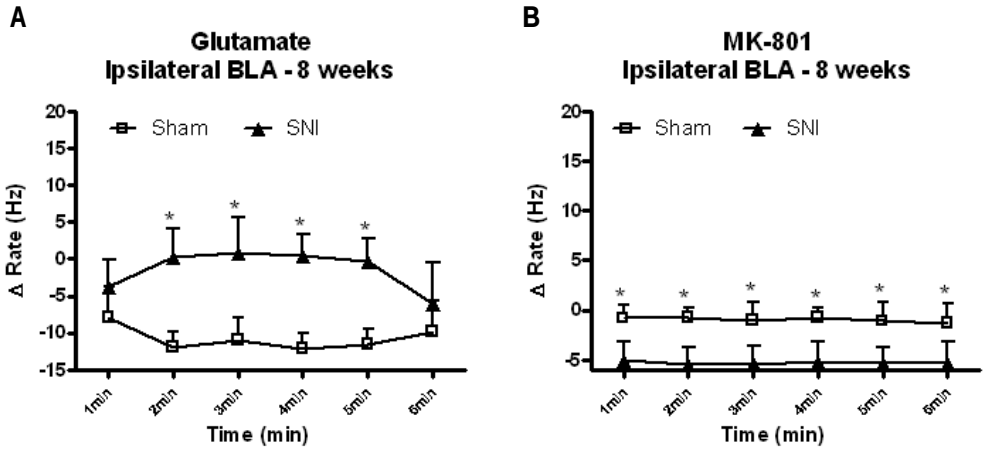


Figure 5 – Microinjection of Glutamate and MK-801 into the rACC produces changes in BLA neuronal spontaneous activity, 8 weeks after SNI induction. (A) Glutamate produced a significant variation in SNI neuronal activity relatively to baseline (0 Hz), and, more importantly, a statistically different response when compared with Sham neuronal activity ($p<0.05$, t-test), which has a strongest response to the NMDArs agonist. (B) MK-801 also induced a significant variation in SNI neuronal activity relatively to baseline (0 Hz), and a statistically different response when compared with Sham neuronal activity ($p<0.05$, t-test).

Chapter 3

DISCUSSION

The mechanisms underlying the development of neuropathic pain are far from being entirely revealed. Nonetheless, the state of the art provides a considerable amount of knowledge that contributes to the comprehension of this painful condition. In this thesis, the intention was to analyze changes in the interaction between the limbic and supraspinal pain control systems following a persistent neuropathic pain condition. The discussion focuses on evaluating the most significant anatomical, electrophysiological and behavioural results and how they may contribute to understand changes in supraspinal processing following the development of neuropathic pain.

3.1 Neuropathy induces pain- and emotional-like behavioural alterations

3.1.1 Sensory and emotional changes

In this work we compared different behavioural parameters in neuropathic- and sham-operated animals, using several different tests. These tests showed that neuropathic animals have both sensory and emotional components of pain-like behaviour altered: they consistently presented mechanical allodynia and hyperalgesia, depressive-like behaviour and an increase in emotional pain-like behaviour. However, no anxiety-like behaviour was detected.

Clinical studies have shown that neuropathic pain has hallmark characteristics: spontaneous and evoked pain (such as allodynia, hyperalgesia; Jensen et al., 2001; Herrero et al., 2000). In rats, the spared nerve injury (SNI) model of neuropathy that we used (Decosterd and Woolf, 2000) is characterized by a marked hypersensitivity to normally innocuous mechanical stimuli (allodynia), as well as a marked hyper-responsiveness to a suprathreshold pin-prick (hyperalgesia). Consequently, Von Frey and pin-prick tests were performed in every experiment to verify the presence of allodynia and hyperalgesia respectively, and they confirmed the success of the SNI surgery (**Chapter 2.2**).

Although not all chronic pain patients develop affective disorders (McBeth et al., 2002), there is a high incidence of these emotional conditions, most notably depression and anxiety disorders, in chronic pain patients (Bennett et al., 1996). Clinical studies have shown clearly the importance of chronic painful physical conditions in subjects with major depressive disorder: chronic pain is strongly associated with major depressive disorder, whereas occurrence of chronic painful physical conditions increased the likelihood of having a major depressive disorder (Barsky et al., 1986; Macfarlane et al., 1999; Ohayon and Schatzberg, 2003). We have shown here that a two month neuropathy resulted also in a depressive-like behaviour in the rat, measured by the forced-swimming test (FST). The open field (OF) test confirmed that differences found in FST were not

due to neuropathy-induced motor impairments, as there were no changes in locomotor activity and exploratory behavior. As mentioned before, although depressive-like behaviour in rats has been extensively studied in association with several different factors (chronic mild stress, acute stress, drug addiction, maternal separation), there is a lack of information in what concerns depressive-like behaviour associated with pain in animals. Wistar-Kyoto (WKY) rats, used as preclinical model of depression, show mechanical allodynia (Zeng et al., 2008). In this study, intra-ACC administration of melatonin (used as antidepressant) decreased mechanical allodynia and depression-like behavior in WKY rats without changing the nociceptive response in normal Wistar rats. In this thesis, we observed depressive-like behaviour in rats, 8 weeks after neuropathy induction. Differently from Zeng and colleagues study, we observed neuropathic pain signs 2-6 days after surgery and depressive-like behaviour only 8 weeks after the surgery. Although it can be argued that we did not assessed depressive-like behaviour in animals at an earlier stage, a previous study by Kontinen and colleagues reported no depressive-like behaviour in animals subjected to the SNL model, 14 days after the nerve lesion (1999). Additionally, a more recent study showed, also with the SNL model, that changes in affective behaviour were detectable at 15 days and, more obviously, at 30 days after surgery (Suzuki et al., 2007). Consequently, we can conclude there is definitely a connection in the processing of neuropathic pain and depressive-like behaviour (**Chapter 2.2**). After our experiments and existent literature, we hypothesize that neuropathic pain leads to depressive-like behaviour in the rat.

Regarding anxiety assessment, however, no alterations were detected in the elevated plus-maze or in the OF tests (**Chapter 2.2**). Although anxiety symptoms in neuropathic pain patients have been reported (Bair et al., 2008; Keller et al; 2008), studies that assess anxiety-like behaviour in different models of experimental neuropathy pain have shown different results. Hasnie and colleagues (2007) described a positive correlation between mechanical hypersensitivity and anxiety-like behaviour in a rat model of varicella zoster virus (VZV)-associated pain. However, in the same study, it is shown that while animals subjected to VZV and spinal nerve transection (SNT) models presented anxiety-like behaviour, the same was not true to those subjected to partial sciatic nerve injury (PSNI) model (Hasnie et al., 2007). Furthermore, anxiety-like behaviour was observed in a rodent model for human immunodeficiency virus (HIV)-related neuropathic pain (Wallace et al., 2007) and in animals subjected to chronic constriction injury

(CCI), whereas in partial nerve ligation (PNL) animals no anxiety-like behavior was observed. As far as we know, herein we present the first results concerning anxiety behaviour assessment in animals subjected to SNI model. As previously described before (see Introduction, section 1.1.1.6), the existent animal models for neuropathic pain develop and express differently and do not superimpose totally with humans states.

3.1.2 Pharmacological basis for increased emotional pain

In order to measure the aversive nature of neuropathic pain, the avoidance of a preferred location was analyzed using the place avoidance paradigm (PAP) test (LaBuda and Fuchs, 2000). This test evaluates the affective-motivational component of pain, rather than the sensory/discriminative dimension. This is a relevant approach for the present study, as the amygdala (AMY) is mainly associated with behavioural responses to emotional stimuli (Davis and Whalen, 2001; Han and Neugebauer, 2005), and is also deeply involved in nociceptive modulation (Manning and Mayer, 1995; Manning, 1998; Manning et al., 2001). In the herein presented work, affective pain in nerve-injured animals was increased by amygdaloid administration of an $mGluR_{1/5}$ agonist and decreased by an $mGluR_1$ antagonist, whereas no effect was observed in Sham-operated animals (**Chapter 2.4**). These results point to a role of amygdaloid $mGluR_1$ in mediating the increased emotional pain component observed in nerve-injured animals and are in accordance with previous studies involving these receptors in amygdalar plasticity following neuropathic pain. In fact, metabotropic glutamate receptors have also been shown to modulate the induction and/or maintenance of central sensitization, through pre- or postsynaptic action (Armstrong and Gouaux, 2000). Group I metabotropic glutamate receptors (mGluRs), which comprise $mGluR_1$ and $mGluR_5$ subtypes, are involved in neuroplasticity associated with normal brain functions and with neurological and psychiatric disorders (Fundytus, 2001; Neugebauer, 2001, 2002). Additionally, Neugebauer and colleagues (2003) reported that, in a model of arthritic pain, a group $mGluR_{1/5}$ agonist potentiated synaptic transmission at the PB→CeA synapse (a connection "par excellence" in nociceptive processing at the AMY), while a selective $mGluR_1$ antagonist reduced transmission in CeA neurons.

It has been reported that the rostral anterior cingulate cortex (rACC) is involved in the processing of the affective component of pain, as its integrity is necessary for the "aversive" reaction to nociception (Johansen et al, 2001) and to the negative affect associated with

neuropathy-induced hypersensitivity (LaGraize et al, 2004; LaBuda and Fuchs, 2005), but not to sensory processing. Also part of the limbic system, the ACC is strongly reciprocally connected with the basolateral amygdala (BLA; Gabbott et al, 2005; Hoover & Vertes, 2007). It has been shown that NMDA receptors play important roles in various forms of plasticity in the nervous system (Dingledine et al., 1999; Hollmann and Heinemann, 1994; Malenka and Nicoll, 1999), and in disorders affecting and in peripheral and spinal pain mechanisms (Carlton, 2001; Fundytus, 2001; Schaible et al., 2002; Varney and Gereau, 2002). The rACC is involved in pain-related fear memory and in pain-related aversive behaviours through NMDA-receptors (Lei et al, 2004; Tang et al, 2005; Zhang et al, 2005).

There is evidence that the rACC is involved in the storage of emotional events and aversive learning (Farr et al., 2000; Johansen and Fields, 2004) and direct connections between the rACC and BLA suggest that these areas may interact for the consolidation of aversive memories (Malin et al., 2007). In the place avoidance paradigm (PAP), SNI animals remained more time in the light area after both saline and glutamate injection in the rACC, when compared with sham operated rats, both after 1W or 8W neuropathy (**Chapter 2.5**). These results are in accordance with literature, since SNI animals seem to prefer the light chamber to the dark one, with the intention of avoiding noxious stimulation (LaBuda and Fuchs, 2000). These data suggest an association between depressive-like behaviour of SNI animals (see above) and an increase in the emotional component of the pain response. This is in accordance with increasing evidence that the emotional state of a patient with chronic pain increases the possibility of an associated depressive condition, as deeply depressed patients have pain as an important associated symptom (Peveler et al., 2006). Contrary to glutamate/saline rACC injection, the administration of an NMDAr antagonist to the rACC resulted in a different behavioural response of SNI animals one (1W) versus eight (8W) weeks after injury: while the 8W group still remained more time in the light chamber, the 1W SNI animals inverted the aversive effect of pain in comparison with sham levels, by preferring the dark chamber. It has been reported that NMDA receptors (NMDArs) play important roles in nervous system plasticity and various forms of disorders (Dingledine et al., 1999; Hollmann and Heinemann, 1994; Malenka and Nicoll, 1999), and in peripheral and spinal pain mechanisms (Carlton, 2001; Fundytus, 2001; Schaible et al., 2002; Varney and Gereau, 2002). NMDAr activation in the ACC mediates excitatory synaptic transmission (Sah and Nicoll, 1991; Wei et al., 1999, 2001) and mediates pain-related aversion (Lei et al, 2004). Importantly, while MK-801 is a specific antagonist for NMDArs, glutamate activates not only NMDAr, but also

AMPA and kainate receptors (Gong et al., 2005). AMPA receptor-mediated synaptic inputs are involved in descending facilitatory pathways from AMY and ACC to the periaqueductal grey (PAG) and the RVM (Huang et al., 2006), while kainate participate in pain signaling in the BLA (Li et al., 2001). Curiously, the occurrence of central sensitization in chronic pain seems to occur mostly within the glutamatergic pathways in supraspinal nociceptive regions (Huang et al., 2006) and to be primarily expressed as alterations in the biophysical properties of ionotropic glutamate receptors (NMDA, AMPA and kainate receptors) and/or promotion of AMPA receptor trafficking to the postsynaptic membrane (Bleakman et al., 2006). Furthermore, as the metabotropic glutamate receptors (mGluRs) present in the in the ACC also play an important role in pain regulation (Tang et al., 2005; Calejesan et al., 2000), and analyzing in our results, we cannot exclude the contribution of the mGluRs in pain regulation originating in the ACC. Hence, we propose that our data showing differences of NMDAr-antagonist effect on pain behaviour after 1W but not 8W is based on the effect of preferential activation of non-NMDA ionotropic glutamate receptors in the ACC after prolonged neuropathy (8W), when major plastic changes have also occurred in other brain areas (**Chapter 2.5**); on the contrary, after a shorter period neuropathy (1W), ionotropic glutamate receptors in the ACC probably activate preferentially the NMDArs.

3.2 Neuropathy induces brain neuroplasticity towards pronociception

3.2.1 Alterations in the supraspinal pain control system - the RVM

The evidence of endogenous pain-modulating mechanisms is well established, but during decades research has been concentrated in the descending antinociceptive system (Basbaum and Fields, 1984). Although the presence of descending nociceptive facilitating pathways is now perfectly accepted (Almeida et al., 2002; Porreca et al., 2002; Vanegas and Schaible, 2004), the exact mechanisms by which descending projections facilitate pain transmission are not yet clarified (Watkins and Mayer, 1982; Mason et al., 1999; Vanegas and Schaible, 2004; Suzuki et al., 2002). Nonetheless, the presence of pain facilitation mechanisms emphasizes the fact that their abnormal sustained activity should contribute to the development of chronic pain (Vanderah et al., 2001).

One of the main observations of this thesis is that hypersensitivity to peripheral stimulation is predominantly observed in ON-cells of the RVM, considered to have a pronociceptive role, by increasing pain perception through facilitation of spinal nociceptive transmission (**Chapter 2.1**).

This is in accordance with the hypothesis that ON-cells are involved in promoting neuropathic hypersensitivity to peripheral stimulation. Previous studies have shown that lidocaine microinjections in RVM blocked persistent nerve injury-induced pain (Pertovaara et al., 1996; Kovelowski et al., 2000). These and other studies have provided evidence for the hypothesis that it is the tonic activity of descending facilitatory cells that mediates nociceptive descending facilitation (Fields et al., 1991; Fields, 1992). ON-cells have been previously accountable for the descending facilitation of nociception, having their pericarya within the RVM and descending axons projecting to the spinal dorsal horn (Fields et al., 1991; Heinricher et al., 1992; Fields et al, 1995).

As mentioned before, after nerve injury there are changes in central processing, with an increase of nociceptive barrage to the dorsal horn (Lu et al., 2007). This, in turn, leads to alterations in brainstem processing, which eventually contributes to the increased activity of RVM ON-cells (Burgess et al., 2002; Carlson et al., 2007), and the resulting selective descending action upon the spinal cord that is dependent on the type of peripheral input (Bee and Dickenson, 2008). As our results showed that SNI produces a different hypersensitivity to cold and mechanical stimuli, we conclude that they are in accordance with the hypothesis that descending facilitatory neurons discriminate between stimulus modality (Bee and Dickenson, 2008).

This is also supported by earlier behavioural studies showing that nerve injury-induced hypersensitivity to mechanical stimulation (Pertovaara, 2000; Porreca et al., 2002) and cold (Urban et al., 2003) is dependent on descending facilitatory influence from the RVM. Additionally, clinical studies have shown that hypersensitivity to mechanical stimulation and cold are frequent and prominent symptoms after nerve injuries, whereas hyperalgesia to heat occurs only occasionally after neuropathic conditions (Scadding & Koltzenburg, 2006). Hypersensitivity to cold and mechanical stimulation is also a prominent finding in rats with the SNI model of neuropathy (Decosterd & Woolf, 2000). Therefore, we conclude that our data are in accordance with the existence literature and reinforce the fact that, after prolonged neuropathy, sensitivity and its enhancement varies with the submodality of stimulation (Chapter 2.1).

3.2.2 Alterations in the limbic system - the AMY

3.2.2.1 Physiological plasticity

CeA receives highly processed polymodal sensory information from other AMY nuclei and from the thalamus and cortex via the BLA (Paré et al., 2004), as well as nociceptive-specific

information from the spino-PB pathway via the PB tract (Bernard and Besson, 1990; Jasmin et al., 1997; Gauriau and Bernard, 2002; Neugebauer et al., 2004). Importantly, the CeA is the output nucleus for major amygdala functions and regulates behavior through widespread projections to forebrain and brainstem areas (Bernard et al., 1996; Davis, 1998; LeDoux, 2000). The latero-capsular part of CeA is defined as the 'nociceptive amygdala' due to its high content in neurons implicated in nociceptive processing (Bernard et al., 1996; Neugebauer et al., 2004). Previous studies have shown increased neurotransmission activity in the PB-CeA synapse, which is indicative of plasticity and enhanced processing of incoming signals to the CeA and leads to an increased activity of CeA neurons and an enhancement of the nucleus outputs (Han and Neugebauer, 2004). Synaptic transmission at the BLA-CeA synapse was shown not to be significantly altered in the visceral pain state (Han and Neugebauer, 2004). In the arthritis pain model, however, enhanced synaptic transmission was measured both at the PB-CeA synapse and the BLA-CeA synapse (Neugebauer et al., 2003). Finally, Ikeda and colleagues found that unilateral neuropathic pain potentiated contralateral PB–CeA transmission and, to a lesser extent, bilateral BLA–CeA transmission (2007). Taking together, these results indicated that pain-related synaptic plasticity in the CeA, although depending both on the type of pain and on pathway, always converges to an augmented output activity of the central nucleus. In the present work, CeA spontaneous (basal) activity was increased bilaterally in animals with peripheral neuropathy in both time points (1W and 8W), while BLA spontaneous activity was increased only at the ipsilateral side after 1W and on the contralateral side after 8W SNI. These observations are partially in accordance with Ikeda and colleagues results (2007), which stated that there is a consolidation of the PB-CeA synapse in persistent neuropathic pain; the increase of CeA activity is most probably due to PB–CeA synapses and less to the BLA–CeA transmission, since BLA and CeA activities changed with different patterns. Additionally, our observations showed an increase in ipsilateral CeA activity at 8W that might be due to an increase in PB–CeA transmission, as the ipsilateral BLA activity, at that time point, was decreased. Finally, our results show not only that the highest significant increase in CeA neuronal basal activity after 8W was on the contralateral side, but also that, after peripheral stimulation, contralateral CeA neurons showed all significant changes recorded at both time points (**Chapter 2.5**).

We found that all peripherally evoked stimuli did not activate the BLA, but resulted in a decrease in the activity of BLA and CeA neurons of SNI neuropathic animals. Previous studies revealed the involvement of several different brain regions in the processing of mechanical and

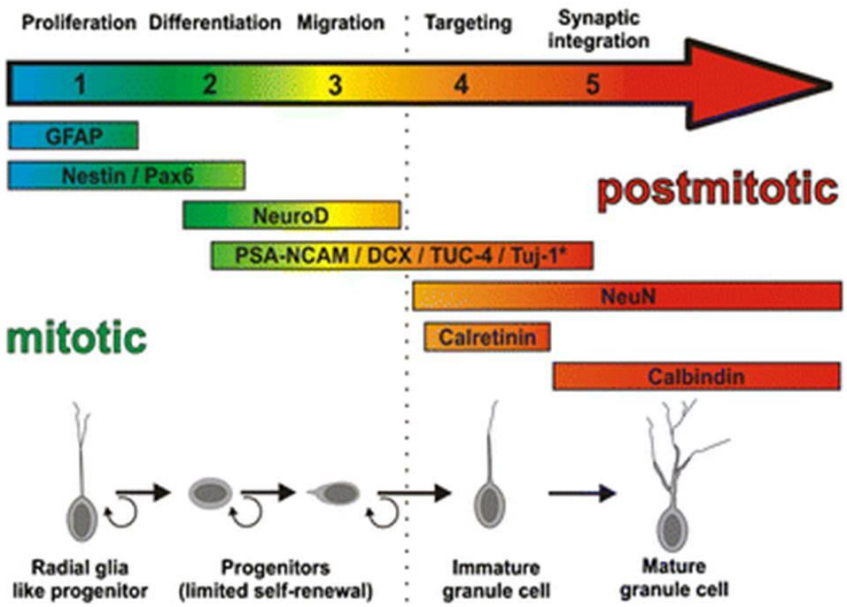
cold allodynia, such as insular cortex, ACC, S1 and S2, thalamus, cerebellum, parietal association areas and the midbrain, but not the AMY (Bushnell et al., 1993; Craig et al., 2000; Davis et al., 1998; Tracey et al., 2000; Seifert and Maihöfner, 2007). Curiously, in an fMRI study of imagined allodynia, Krämer and colleagues have shown activation of AMY and other limbic structures, only if subjects had a previous knowledge of the allodynic sensation (2008). In summary, there seems to be no specific AMY activation during cold or mechanical allodynia stimulation in neuropathic pain (**Chapter 2.5**).

3.2.2.1 Structural plasticity

In addition to physiological alterations of amygdalar nociceptive neurons, a profound structural plasticity in the AMY was also found, mainly in BLA and CeA. Human studies have previously shown changes in different brain areas, usually related with different conditions: stress-related increase of hippocampus volume (Czéh et al., 2001), bilateral volume reduction of insular cortex gray matter in first-episode patients with schizophrenia (Lee et al., 2002), volume reduction in left temporal pole gray matter and absence of normal left-greater-than-right asymmetry gray matter in first-episode psychosis groups (Hirayasu, et al., 1999), and increased AMY volumes measured by structural magnetic resonance in patients with depression and anxiety (Frodl et al., 2002; Tebartz van Elst et al., 2000). Clinical data also reveal that prolonged pain conditions are associated with a high incidence of emotional disorders, including anxiety and depression (Rasmussen et al., 2004; Campbell et al., 2003; Baliki et al., 2003). As our results showed depressive-like behaviour in rats after prolonged neuropathic pain, we decided to evaluate AMY structural changes. We found a significant increase in the volume of BLA and CeA nuclei after 8 weeks of neuropathy. The 3D morphological analysis showed that this increased volume in SNI animals was not due to differences in pericarya size, dendritic length or spine number, but to the presence of newly proliferating cells in these AMY nuclei. Furthermore, double-immunoreactions (BrdU+NeuN) confirmed that the increased number of newborn cells in SNI animals were neurons, demonstrating that these neurons were responsible, at least in part, for the higher cell number and the increase of AMY volume of SNI- in comparison with Sham-operated animals. This was the first time that newborn neurons were observed in association with the development of a neuropathic pain condition (**Chapter 2.2**). Importantly, these new neurons reach synaptic integrity within the neuronal network, as shown by the present of BrdU+Calb double-labeled cells

in the AMY of neuropathic animals (Figure 3). Although we cannot establish a clear link between persistent neuropathic pain and depressive-like behaviour associated to neuroplasticity of the AMY, it is also the first time that these three characteristics are observed simultaneously in animals where plasticity in the AMY is described.

An additional novelty presented in this study is given by our most recent results, which indicate that cell divisions originating new neurons in the AMY of neuropathic rats occurred inside the limits of the AMY (**Chapter 2.3**). It is known that doublecortin (DCX), present in migrating neuroblasts and young neurons, promotes microtubule polymerization (Gleeson et al. 1999; Francis et al. 1999) and can serve as a marker of adult neurogenesis in the hippocampus (von Bohlen und Halbach, 2007). Hence, the observation of DCX+Ki-67-positive cells in the BLA and CeA only in SNI animals, show that the cell division had occurred recently. Supporting our observations, Shapiro and colleagues observed DCX-positive cells not only in the AMY, but also in other "non-neurogenic" areas, in naive rats (2009). Also in the adult brain, the polysialylated form of the neural cell adhesion molecule (PSA-NCAM) is only present in regions that are undergoing some kind of structural plasticity, such as the hypothalamo-neurohypophyseal system (Theodosis et al., 1994), the olfactory bulb (Miragall et al., 1988) or the piriform and entorhinal cortices or the hippocampus (Seki and Arai, 1991a,b). PSA-NCAM has also been previously observed in the adult AMY, confirming structural plasticity associated to memory consolidation (Nacher et al., 2002). In our results, the presence of PSA-NCAM only in SNI animals reflects both structural plasticity, and the formation of new neurons. Finally, nestin-positive newborn cells are known to identify cells involved in neurogenesis (Doyle et al. 2001; Yue et al. 2006), and nestin-positive cell spheres are thought to differentiate into neurons and glial cells (Figure 3; Itoh et al. 2006). Additionally, it is also known that only nestin-positive but not the astrocyte (GFAP)-positive precursors are involved in neurogenesis (Cao et al. 2006), since GFAP expression is restricted to astrocyte (glial cells). On the other hand, there are reports of nestin expression in many cell types in the CNS, including non-neural cells (Nakagawa et al., 2004). All together, we propose that our observations, point to the fact that cell proliferation giving rise to new neurons in the AMY of neuropathic animals occurred inside AMY borders (**Chapter 2.3**).



Adapted from Bohlen and Halbach, Cell Tissue Res (2007) 329:409–420

Figure 3 - Stages of adult neurogenesis in the dentate gyrus (DG) and expression pattern of specific markers. Generation of new neurons within the granular layer of the DG can be subdivided into five developmental stages. A stem cell might be located outside the SVZ (*1, blue*). The generated precursors start to proliferate and give rise to transient amplifying cells (*2, green*), which differentiate into immature neurons. At stage 3 (*3*, *yellow*), the immature neurons migrate over a short distance to reach the granular layer of the DG. During stage 4 (*4*, *orange*), the immature and postmitotic neurons extend their axons toward the pyramidal layer of the hippocampal area CA3 and send their dendrites in the direction of the molecular layer of the DG. By stage 5 (*5*, *red*), the new granule cells are synaptically integrated into the network of the hippocampal formation, receiving inputs from the entorhinal cortex and sending outputs to the hippocampal area CA3 and the hilus.

3.2.3 Alterations in the interaction between limbic and pain control systems

As mentioned before (see section 3.1.2), $mGluR_{1/5}$ are involved in the main processes of neuropathic pain development. In the present work, after $mGluR_{1/5}$ agonist administration in the CeA, the discharge rate of pronociceptive ON-cells in the RVM increased in neuropathic animals only, an effect reversed by an $mGluR_1$ and by an $mGluR_5$ antagonist. It has been reported that the systemic administration of selective $mGluR_1$ or $mGluR_5$ blockers reduces mechanical allodynia in the CCI and SNL models of persistent pain (Varty et al., 2005; Zhu et a., 2004), and that an $mGluR_5$ antagonist completely reverses thermal hyperalgesia in the SNL (Dogrul et al., 2000).

These data add evidence to the already present notion that the amygdala contributes to the modulation of neuropathic hypersensitivity and to the pronociceptive effect in peripheral neuropathy, namely through action on descending pathways relaying in the RVM: Pedersen and colleagues showed suppression of aversive pain-related behavior and hypersensitive spinal reflex responses in nerve-injured animals after injecting a GABA-A receptor agonist in the AMY (2007); also, glutamate administered in the AMY suppressed antinociceptive neurons in the noradrenergic locus coeruleus of nerve-injured but not sham-operated animals (Viisanen and Pertovaara, 2007). Accordingly, our results propose that activation of the group I mGluR in the amygdala of SNI animals enhances ON-cells activity in the RVM, indicating the occurrence of neuropathic changes in the interplay between the limbic and pain control systems following neuropathic pain (**Chapter 2.4**).

References

Almeida A, Cobos A, Tavares I, Lima D. Brain afferents to the medullary dorsal reticular nucleus: a retrograde and anterograde tracing study in the rat. Eur J Neurosci. 2002;16:81-95.

Armstrong N, Gouaux E. Mechanisms for activation and antagonism of an AMPA-sensitive glutamate receptor: crystal structures of the GluR2 ligand binding core. Neuron. 2000;28:165-81.

Bair MJ, Wu J, Damush TM, Sutherland JM, Kroenke K. Association of depression and anxiety alone and in combination with chronic musculoskeletal pain in primary care patients. Psychosom Med. 2008;70:890-7.

Baliki M, Al-Amin HA, Atweh SF, Jaber M, Hawwa N, Jabbur SJ, Apkarian AV, Saadé NE. Attenuation of neuropathic manifestations by local block of the activities of the ventrolateral orbito-frontal area in the rat. Neuroscience. 2003;120:1093-104.

Basbaum AI, Fields HL. Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. Annu Rev Neurosci. 1984;7:309-38.

Barsky AJ, Wyshak G, Klerman GL. Medical and psychiatric determinants of outpatient utilization. Med Care 1986;24:548–560.

Bee LA, Dickenson AH. Descending facilitation from the brainstem determines behavioural and neuronal hypersensitivity following nerve injury and efficacy of pregabalin. Pain. 2008;140:209-23.

Bennett RM, Burckhardt CS, Clark SR, O'Reilly CA, Wiens AN, Campbell SM. Group treatment of fibromyalgia: a 6 month outpatient program. J Rheumatol. 1996;23:521-8.

Bernard JF, Besson JM. The spino(trigemino)pontoamygdaloid pathway: electrophysiological evidence for an involvement in pain processes. J Neurophysiol. 1990;63:473-90.

Bernard JF, Bester H, Besson JM. Involvement of the spinoparabrachio-amygdaloid and -hypothalamic pathways in the autonomic and affective emotional aspects of pain. Prog. Brain Res. 1996;107:243–255.

Bleakman D, Alt A, Nisenbaum ES. Glutamate receptors and pain. Seminars in Cell & Developmental Biology 2006;17:592–604. Review.

von Bohlen Und Halbach O. Immunohistological markers for staging neurogenesis in adult hippocampus. Cell Tissue Res. 2007;329:409–420.

Bowsher D, Haggett C. Paradoxical burning sensation produced by cold stimulation in patients with neuropathic pain. Pain 2005;117:230.

Burgess SE, Gardell LR, Ossipov MH, Malan TP Jr, Vanderah TW, Lai J, Porreca F. Time-dependent facilitation from the rostral ventromedial medulla maintains, but does not initiate, neuropathic pain. J Neurosci. 2002;22:5129–38.

Bushnell MC, Duncan GH, Tremblay N. Thalamic VPM nucleus in the behaving monkey. I. Multimodal and discriminative properties of thermosensitive neurons. J. Neurophysiol. 1993;69:739–752.

Calejesan AA, Kim SJ, Zhuo M. Descending facilitatory modulation of a behavioral nociceptive response by stimulation in the adult rat anterior cingulate cortex. Eur J Pain. 2000;4:83-96.

Campbell LC, Clauw DJ, Keefe FJ. Persistent pain and depression: a biopsychosocial perspective. Biol Psychiatry. 2003;54:399-409. Review.

Campero M, Serra J, Ochoa JL. C-polymodal nociceptors activated by noxious low temperature in human skin. J. Physiol. 1996;497:565–572.

Campero M, Serra J, Bostock H, Ochoa JL. Slowly conducting afferents activated by innocuous low temperature in human skin. J. Physiol. 2001;535:855–865.

Cao F, Hata R, Zhu P, Ma YJ, Tanaka J, Hanakawa Y, Hashimoto K, Niinobe M, Yoshikawa K, Sakanaka M.Overexpression of SOCS3 inhibits astrogliogenesis and promotes maintenance of neural stem cells. J Neurochem. 2006;98:459-70.

Calejesan AA, Kim SJ, Zhuo M. Descending facilitatory modulation of a behavioral nociceptive response by stimulation in the adult rat anterior cingulate cortex. Eur J Pain. 2000;4:83-96.

Carlson JD, Maire JJ, Martenson ME, Heinricher MM. Sensitization of pain-modulating neurons in the rostral ventromedial medulla after peripheral nerve injury. J Neurosci. 2007;27:13222–31.

Carlton SM. Peripheral excitatory amino acids. Curr Opin Pharmacol. 2001;1:52–6.

Choi Y, Yoon YW, Na HS, Kim SH, Chung JM. Behavioral signs of ongoing pain and cold allodynia in a rat model of neuropathic pain. Pain 1994;59:369–376.

Colburn RW, Lubin ML, Stone DJ, Wang Y, Lawrence D, D'Andrea MR, Brandt MR, Liu Y, Flores CM, Qin N. Attenuated Cold Sensitivity in TRPM8 Null Mice. Neuron 2007;54:379–386.

Craig AD, Chen K, Bandy D, Reiman EM. Thermosensory activation of insular cortex. Nat Neurosci. 2000;3:184-190.

Czéh B, Thomas Michaelis T, Watanabe T, Frahm J, Biurrun G, van Kampen M, Bartolomucci A, Fuchs E. Stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation are prevented by antidepressant treatment with tianeptine. PNAS 2001;98:12796–12801.

Davis M. Anatomic and physiologic substrates of emotion in an animal model. J. Clin. Neurophysiol. 1998;15:378–387.

Davis M, Whalen PJ. The amygdala: vigilance and emotion. Mol Psychiatry. 2001;6:13-34. Review.

Decosterd I, Woolf CJ. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain. 2000;87:149-58.

Dingledine R, Borges K, Bowie D, Traynelis SF. The glutamate receptor ion channels. Pharmacol Rev. 1999;51:7–61.

Dogrul A, Ossipov MH, Lai J, Malan Jr TP, Porreca F. Peripheral and spinal antihyperalgesic activity of SIB-1757, a metabotropic glutamate receptor (mGLUR(5)) antagonist, in experimental neuropathic pain in rats. Neurosci Lett. 2000;292:115–8.

Doyle KL, Khan M, Cunningham AM. Expression of the intermediate filament protein nestin by sustentacular cells in mature olfactory neuroepithelium. J Comp Neurol. 2001;437:186-95.

Farr SA, Uezu K, Creonte TA, Flood JF, John E. Morley JE. Modulation of memory processing in the cingulate cortex of mice, Pharmacology, Biochemistry, and Behavior 2000;65:363–368.

Fields HL. Is there a facilitating component to central pain modulation? APS J 1992;1:71–78.

Fields HL, Heinricher MM. Brainstem modulation of nociceptors driven withdrawal reflexes. Ann NY Acad Sci. 1989;563:34–44.

Fields HL, Heinricher MM, Mason P. Neurotransmitters in nociceptive modulatory circuits. Annu Rev Neurosci 1991;14:219–245.

Fields HL, Malick A, Burstein R. Dorsal horn projection targets of ON and OFF cells in the rostral ventromedial medulla. J Neurophysiol. 1995;74:1742-59.

Francis F, Koulakoff A, Boucher D, Chafey P, Schaar B, Vinet MC, Friocourt G, McDonnell N, Reiner O, Kahn A, McConnell SK, Berwald-Netter Y, Denoulet P, Chelly J. Doublecortin

is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. Neuron 1999;23:247–256

Frodl T, Meisenzahl E, Zetzsche T, Bottlender R, Born C, Groll C, Jäger M, Leinsinger G, Hahn K, Möller H-J. Enlargement of the amygdala in patients with a first episode of major depression. Biol. Psychiatry 2002;51:708–714.

Fundytus ME. Glutamate receptors and nociception: implications for the drug treatment of pain. CNS Drugs 2001;15:29–58.

Gabbott PL, Warner TA, Jays PR, Salway P, Busby SJ. Prefrontal cortex in the rat: projections to subcortical autonomic, motor, and limbic centers. J Comp Neurol. 2005;492:145-77.

Gauriau C, Bernard JF. Pain pathways and parabrachial circuits in the rat. Exp Physiol. 2002;87:251-8.

Gong X-Q, Frandsen A, Lu W-Y, Wan Y, Zabek RL, Pickering DS, Bai D. D-Aspartate and NMDA, but not L-aspartate, block AMPA receptors in rat hippocampal neurons British Journal of Pharmacology 2005;145:449–459.

Georgopoulos AP. Functional properties of primary afferent units probably related to pain mechanisms in primate glabrous skin. J. Neurophysiol. 1976;39:71–83.

Gleeson JG, Lin PT, Flanagan LA, Walsh CA. Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. Neuron 1999;23:257–271

Greenspan JD, Ohara S, Sarlani E, Lenz FA: Allodynia in patients with post-stroke central pain (CPSP) studied by statistical quantitative sensory testing within individuals. Pain 2004;109:357-366.

Han JS, Neugebauer V. Synaptic plasticity in the amygdala in a visceral pain model in rats. Neurosci Lett. 2004;361:254-7.

Han JS, Neugebauer V. mGluR1 and mGluR5 antagonists in the amygdala inhibit different components of audible and ultrasonic vocalizations in a model of arthritic pain. Pain. 2005;113:211-22.

Hasnie FS, Breuer J, Parker S, Wallace V, Blackbeard J, Lever I, Kinchington PR, Dickenson AH, Pheby T, Rice AS. Further characterisation of a rat model of varicella zoster virus (VZV)-associated pain: relationship between mechanical hypersensitivity and anxiety-related behaviour; and the influence of analgesic drugs. Neuroscience 2007;144:1495–1508.

Heinricher MM, Morgan MM, Fields HL. Direct and indirect actions of morphine on medullary neurons that modulate nociception. Neuroscience 1992;48:533–543.

Heinricher MM, Morgan MM, Tortorici V, Fields HL. Disinhibition of off-cells and antinociception produced by an opioid action within the rostral ventromedial medulla. Neuroscience 1994;63:279 –288.

Herrero JF, Laird JM, Lopez-Garcia JA. Wind-up of spinal cord neurones and pain sensation: much ado about something? Prog Neurobiol. 2000;61:169–203.

Hirayasu Y, Shenton ME, Salisbury DF, Kwon JS, Wible CG, Fischer IA, Yurgelun-Todd D, Zarate C, Kikinis R, Jolesz FA, McCarley RW. Subgenual cingulate cortex volume in first-episode psychosis. Am J Psychiatry. 1999;156:1091-1093.

Hollmann M, Heinemann S. Cloned glutamate receptors. Annu Rev Neurosci. 1994;17:31–108.

Huang J, Chang JY, Woodward DJ, Baccala LA, Han JS, Wang JY, Luo F. Dynamic neuronal responses in cortical and thalamic areas during different phases of formalin test in rats. Exp Neurol. 2006;200:124-34.

Hoover WB, Vertes RP. Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat. Brain Struct Funct. 2007;212:149-79.

Ikeda R, Takahashi Y, Inoue K, Kato F. NMDA receptor-independent synaptic plasticity in the central amygdala in the rat model of neuropathic pain. Pain. 2007;127:161-72.

Itoh T, Satou T, Nishida S, Hashimoto S, Ito H.Cultured rat astrocytes give rise to neural stem cells. Neurochem Res. 2006;31:1381-7.

Jasmin L, Burkey AR, Card JP, Basbaum AI. Transneuronal labeling of a nociceptive pathway, the spino-(trigemino-)parabrachio-amygdaloid, in the rat. J Neurosci. 1997;17:3751-3765.

Jensen TS, Gottrup H, Sindrup SH, Bach FW. The clinical picture of neuropathic pain. Eur J Pharmacol. 2001;429:1-11. Review.

Jensen TS, Baron R. Translation of symptoms and signs into mechanisms in neuropathic pain. Pain 2003;102:1–8.

Ji G, Zhuo S, Carlton SM. Intact Aδ-fibers up-regulate transient receptor potential A1 and contribute to cold hypersensitivity in neuropathic rats. Neuroscience 2008;154:1054–1066.

Johansen JP, Fields HL, Manning BH. The affective component of pain in rodents: direct evidence for a contribution of the anterior cingulate cortex. Proc Natl Acad Sci U S A. 2001; 98(14):8077-82.

Johansen JP, Fields HL. Glutamatergic activation of anterior cingulate cortex produces an aversive teaching signal. Nature Reviews Neuroscience 2004;7:398–403.

Jørum E, Warncke T, Stubhaug A Cold allodynia and hyperalgesia in neuropathic pain: the effect of N-methyl-d-aspartate (NMDA) receptor antagonist ketamine – a double-blind, cross-over comparison with alfentanil and placebo. Pain 2003;101:229–235.

Keller R, Rigardetto R, Vaccarino P, Maggioni M, Iannoccari G, Teriaca MJ. Screening anxious-depressive symptoms and pain in medical inpatients. Panminerva Med. 2008;50:217-20.

Kontinen VK, Kauppila T, Paananen S, Pertovaara A, Kalso E. Behavioural measures of depression and anxiety in rats with spinal nerve ligation-induced neuropathy. Pain. 1999;80:341-6.

Kovelowski CJ, Ossipov MH, Sun H, Lai J, Malan TP, Porreca F. Supraspinal cholecystokinin may drive tonic descending facilitation mechanisms to maintain neuropathic pain in the rat. Pain 2000;87:265–273.

Krämer HH, Stenner C, Seddigh S, Bauermann T, Birklein F, Maihöfner C. Illusion of pain: pre-existing knowledge determines brain activation of 'imagined allodynia'. The Journal of Pain 2008;9:543-551.

LaBuda CJ, Fuchs PN. A behavioral test paradigm to measure the aversive quality of inflammatory and neuropathic pain in rats. Exp Neurol. 2000;163:490-4.

LaBuda CJ, Fuchs PN. Attenuation of negative pain affect produced by unilateral spinal nerve injury in the rat following anterior cingulate cortex activation. Neuroscience. 2005;136:311-22.

LaGraize SC, Borzan J, Rinker MM, Kopp JL, Fuchs PN. Behavioral evidence for competing motivational drives of nociception and hunger. Neurosci Lett. 2004;372:30-4.

LeDoux JE. Emotion circuits in the brain. Annu Rev Neurosci. 2000;23:155-84. Review.

Lee CU, Shenton ME, Salisbury DF, Kasai K, Onitsuka T, Dickey CC, Yurgelun-Todd D, Kikinis R, Jolesz FA, McCarley RW. Fusiform gyrus volume reduction in first-episode schizophrenia: an MRI study. Arch Gen Psychiatry 2002;59:775-781.

LeDoux JE. Emotion circuits in the brain. Annu. Rev. Neurosci. 2000;23:155–184.

Lei LG, Sun S, Gao YJ, Zhao ZQ, Zhang YQ. NMDA receptors in the anterior cingulate cortex mediate pain-related aversion. Exp Neurol. 2004;189:413-21.

Li H, Chen A, Xing G, Wei ML, Rogawski MA. Kainate receptormediated heterosynaptic facilitation in the amygdala. Nat Neurosci. 2001;4:612–20.

Li W, Neugebauer V. Differential roles of mGluR1 and mGluR5 in brief and prolonged nociceptive processing in central amygdala neurons. J Neurophysiol. 2004;91:13-24.

Lu VB, Ballanyi K, Colmers WF, Smith PA. Neuron type-specific effects of brain-derived neurotrophic factor in rat superficial dorsal horn and their relevance to 'central sensitization'. J Physiol. 2007;584:543–563.

Macfarlane GJ, Morris S, Hunt IM, Benjamin S, McBeth J, Papageorgiou AC, Silman AJ. Chronic widespread pain in the community: the influence of psychological symptoms and mental disorder on healthcare seeking behaviour. J Rheumatol 1999;26:413–419.

Malenka RC, Nicoll RA. Long-term potentiation—a decade of progress? Science 1999;285:1870-4.

Malin EL, Ibrahim DY, Jessica W, Tu JW, James L. McGaugh JL. Involvement of the rostral anterior cingulate cortex in consolidation of inhibitory avoidance memory: Interaction with the basolateral amygdala. Neurobiology of Learning and Memory 2007;87:295-302.

Manning BH. A lateralized deficit in morphine antinociception after unilateral inactivation of the central amygdala. J Neurosci. 1998;18:9453-70.

Manning BH, Mayer DJ. The central nucleus of the amygdala contributes to the production of morphine antinociception in the formalin test. Pain. 1995;63:141-52.

Manning BH, Merin NM, Meng ID, Amaral DG. Reduction in opioid- and cannabinoid-induced antinociception in rhesus monkeys after bilateral lesions of the amygdaloid complex. J Neurosci. 2001;21:8238-8246.

Mason Jr DJ, Lowe J, Welch SP. Cannabinoid modulation of dynorphin A: correlation to cannabinoid-induced antinociception. Eur J Pharmacol. 1999;378:237–248.

McBeth J, Macfarlane GJ, Silman AJ. Does chronic pain predict future psychological distress? Pain 2002;96:239–245.

McDonald AJ. Cortical pathways to the mammalian amygdala. Prog Neurobiol. 1998;55:257-332. Review.

Miragall F, Kadmon G, Husmann M, Schachner M. Expression of cell adhesion molecules in the olfactory system of the adult mouse: presence of the embryonic form of N-CAM. Dev Biol. 1988;129:516-31.

Nacher J, Lanuza E, McEwen BS. Distribution of PSA-NCAM expression in the amygdala of the adult rat. Neuroscience. 2002;113:479-84.

Nakagawa T, Miyamoto O, Janjua NA, Auer RN, Nagao S, Itano T. Localization of nestin in amygdaloid kindled rat: an immunoelectron microscopic study. Can J Neurol Sci. 2004;31(4):514-9.

Neugebauer V. Metabotropic glutamate receptors: novel targets for pain relief. Expert Rev Neurotherapeutics 2001;1:207–224.

Neugebauer V. Metabotropic glutamate receptors: important modulators of nociception and pain behavior. Pain 2002;98:1–8.

Neugebauer V, Li W, Bird GC, Bhave G, Gereau RW. Synaptic plasticity in the amygdala in a model of arthritic pain: differential roles of metabotropic glutamate receptors 1 and 5, J. Neurosci. 2003;23:52–63.

Neugebauer V, Li W, Bird GC, Han JS. The amygdala and persistent pain. Neuroscientist. 2004;10:221-234. Review.

Ochoa JL, Yarnitsky D. The triple cold syndrome. Cold hyperalgesia, cold hypoaesthesia and cold skin in peripheral nerve disease. Brain 1994;117:185–197.

Ohayon MM, Schatzberg AF. Using chronic pain to predict depressive morbidity in the general population. Arch Gen Psychiatry. 2003;60:39-4.

Pan ZZ, Williams JT, Osborne PB. Opioid actions on single nucleus raphe magnus neurons from rat and guinea-pig in vitro. J Physiol (Lond). 1990;427:519 –532.

Paré D. Role of the basolateral amygdala in memory consolidation. Prog Neurobiol. 2003;70:409-420. Review.

Pedersen LH, Scheel-Kruger J, Blackburn-Munro G. Amygdala GABA-A receptor involvement in mediating sensory-discriminative and affective-motivational pain responses in a rat model of peripheral nerve injury. Pain 2007;724:127,17–26.

Pertovaara A. Plasticity in descending pain modulatory systems. Prog Brain Res. 2000;129:231-42. Review.

Pertovaara A, Wei H, Hamalainen MM. Lidocaine in the rostroventromedial medulla and the periaqueductal gray attenuates allodynia in neuropathic rats. Neurosci Lett. 1996;218:127–130.

Peveler R, Katona C, Wessely S, Dowrick C. Painful symptoms in depression: under-recognised and under-treated? Br J Psychiatry. 2006;188:202-3.

Porreca F, Burgess SE, Gardell LR, Vanderah TW, Malan TP Jr, Ossipov MH, Lappi DA, Lai J. Inhibition of Neuropathic Pain by Selective Ablation of Brainstem Medullary Cells Expressing the μ-Opioid Receptor. The Journal of Neuroscience 2001;21:5281-5288.

Porreca F, Ossipov MH, Gebhart GF. Chronic pain and medullary descending facilitation. Trends Neurosci. 2002;25:319-25.

Price DD, Dubner RR. Neurons that subserve the sensory-discriminative aspects of pain. Pain 1977;3:307–338.

Price DD, Barrell JJ, Gracely RH. A psychophysical analysis of experimental factors that selectively influence the affective dimension of pain. Pain 1980;8:137–149.

Rasmussen PV, Sindrup SH, Jensen TS, Bach FW. Symptoms and signs in patients with suspected neuropathic pain. Pain. 2004;110:461-469.

Roeska K, Doods H, KArndt K, Treede RD, Ceci A. Anxiety-like behaviour in rats with mononeuropathy is reduced by the analgesic drugs morphine and gabapentin. Pain 2008;139:349–357.

Sah P, Nicoll RA. Mechanisms underlying potentiation of synaptic transmission in rat anterior cingulated cortex in vitro. J. Physiol. 1991;433:615– 630.

Scadding JW, Koltzenburg M. Painful peripheral neuropathies. In McMahon, S.B. & Koltzenburg, M. (Eds), Wall and Melzack's Textbook of Pain, 5th edn. Elsevier, Location, China. 2006; pp. 973–999.

Schaible H-G, Ebersberger A, von Banchet GS. Mechanisms of pain in arthritis. Ann N Y Acad Sci. 2002;966:343–54.

Seifert F, Maihöfner C. Representation of cold allodynia in the human brain—A functional MRI study. NeuroImage 2007;35:1168–1180.

Shapiro LA, Ng K, Zhou QY, Ribak CE. Subventricular zone-derived, newly generated neurons populate several olfactory and limbic forebrain regions. Epilepsy Behav. 2009;14:74-80.

Suzuki T, Amata M, Sakaue G, Nishimura S, Inoue T, Shibata M, Mashimo T. Experimental Neuropathy in Mice Is Associated with Delayed Behavioral Changes Related to Anxiety and Depression. Anesth Analg. 2007;104:1570-1577.

Lee A, Shapiro LA, Ng K, Zhou Q-Y, Ribak CE. Subventricular zone-derived, newly generated neurons populate several olfactory and limbic forebrain regions. Epilepsy & Behavior 2009;14:74–80.

Seki T, Arai Y. Expression of highly polysialylated NCAM in the neocortex and piriform cortex of the developing and the adult rat. Anat Embryol (Berl). 1991a;184:395-401.

Seki T, Arai Y. The persistent expression of a highly polysialylated NCAM in the dentate gyrus of the adult rat. Neurosci Res. 1991b;12:503-13.

Sung B, Na HS, Kim YI, Yoon YW, Han HC, Nahm SH, Hong SK. Supraspinal involvement in the production of mechanical allodynia by spinal nerve injury in rats. Neurosci Lett 1998;246:117–119.

Suzuki R, Morcuende S, Webber M, Hunt SP, Dickenson AH. Superficial NK1-expressing neurons control spinal excitability through activation of descending pathways. Nat Neurosci. 2002;5:1319–26.

Tang J, Ko S, Ding HK, Qiu CS, Calejesan AA, Zhuo M. Pavlovian fear memory induced by activation in the anterior cingulate cortex. Mol Pain. 2005;1:6.

Tebartz van Elst L, Woermann F, Lemieux L, Trimble MR. Increased amygdala volumes in female and depressed humans. A quantitative magnetic resonance imaging study. Neurosci Lett. 2000;281:103-106.

Theodosis DT, Rougon G, Poulain DA. Retention of embryonic features by an adult neuronal system capable of plasticity: polysialylated neural cell adhesion molecule in the hypothalamo-neurohypophysial system. Proc Natl Acad Sci USA 1991;88:5494 –5498.

Tracey I, Becerra L, Chang I, Breiter H, Jenkins L, Borsook D, González RG. Noxious hot and cold stimulation produce common patterns of brain activation in humans: a functional magnetic resonance imaging study. Neurosci Lett. 2000;288:159-162.

Urban MO, Hama AT, Bradbury M, Anderson J, Varney MA, Bristow L. Role of metabotropic glutamate receptor subtype 5 (mGluR5) in the maintenance of cold hypersensitivity following a peripheral mononeuropathy in the rat. Neuropharmacology. 2003;44:983-993.

Vanegas H, Schaible HG. Descending control of persistent pain: inhibitory or facilitatory? Neurosci. Lett. 2000;281:103–106.

Vanderah TW, Suenaga NMH, Ossipov MH, Malan TP, Lai J, Porreca F. Tonic descending facilitation from the rostral ventromedial medulla mediates opioid-induced abnormal pain and antinociceptive tolerance. The Journal of Neuroscience 2001;21:279-286.

Varney MA, Gereau RW. Metabotropic glutamate receptor involvement in models of acute and persistent pain: prospects for the development of novel analgesics. Curr Drug Targets 2002;1:215–25.

Varty GB, Grilli M, Forlani A, Fredduzzi S, Grzelak ME, Guthrie DH, Hodgson RA, Lu SX, Nicolussi E, Pond AJ, Parker EM, Hunter JC, Higgins GA, Reggiani A, Bertorelli R. The antinociceptive and anxiolytic-like effects of the metabotropic glutamate receptor 5 (mGluR5) antagonists, MPEP and MTEP, and the mGluR1 antagonist, LY456236, in rodents: a comparison of efficacy and side-effect profiles. Psychopharmacology (Berl) 2005;179:207–17.

Verdugo R, Ochoa JL. Quantitative somatosensory thermotest. A key method for functional evaluation of small calibre afferent channels. Brain 1992;115:893-913.

Viisanen H, Pertovaara A. Influence of peripheral nerve injury on response properties of locus coeruleus neurons and coeruleospinal antinociception in the rat. Neuroscience 2007;146:1785–1794.

Vissers K, Meert T. A behavioral and pharmacological validation of the acetone spray test in gerbils with a chronic constriction injury. Anesth Analg. 2005;101:457–464.

Wahren LK, Torebjörk E. Quantitative sensory tests in patients with neuralgia 11–25 years after injury. Pain 1992;48:237–244.

Wallace VC, Blackbeard J, Segerdahl AR, Hasnie F, Pheby T, McMahon SB, Rice AS. Characterization of rodent models of HIVgp120 and anti-retroviral-associated neuropathic pain. Brain 2007;130:2688–702.

Watkins LR, Mayer DJ. Organization of endogenous opiate and nonopiate pain control systems. Science 1982;216:1185–1192.

Wei F, Li P, Zhuo M. Loss of synaptic depression mammalian anterior cingulate cortex after amputation. J. Neurosci. 1999;19:9346–9354.

Wei F, Wang GD, Kerchner GA, Kim SJ, Xu HM, Chen ZF, Zhuo M. Genetic enhancement of inflammatory pain by forebrain NR2B overexpression. Nat. Neurosci. 2001;4:164–169.

Xing H, Chen M, Ling J, Tan W, Gu JG. TRPM8 mechanism of cold allodynia after chronic nerve injury. J Neurosci 2007;27:13680–13690.

Yue F, Chen B, Wu D, Dong K, Zeng SE, Zhang Y. Biological properties of neural progenitor cells isolated from the hippocampus of adult cynomolgus monkeys. Chin Med J (Engl). 2006;119:110-6.

Zeng Q, Wang S, Lim G, Yang L, Mao J, Sung B, Chang Y, Lim JA, Guo G, Mao J. Exacerbated mechanical allodynia in rats with depression-like behavior. Brain Research 2008;1200:27-38.

Zhang L, Zhang Y, Zhao ZQ. Anterior cingulate cortex contributes to the descending facilitatory modulation of pain via dorsal reticular nucleus. Eur J Neurosci. 2005;22:1141-8.

Zhu CZ, Wilson SG, Mikusa JP, Wismer CT, Gauvin DM, Lynch JJ 3rd, Wade CL, Decker MW, Honore P. Assessing the role of metabotropic glutamate receptor 5 in multiple nociceptive modalities. Eur J Pharmacol. 2004;506:107–18.

Chapter 4

Conclusions and Future perspectives

With this work we have established that:

1. The activity of both RVM ON- and OFF-cells, located in a major centre of pain modulation is altered towards pronociception in neuropathic animals. However, ON-cells appear to be the main responsible for the increased hypersensitivity of SNI animals, as their activity increases greatly during hyperalgesia and allodynia.

2. Neuropathic pain induces changes in CeA and BLA, main afferent and efferent nuclei of a major area implicated in both emotional and pain processing. Neuroplasticity was present as an increased AMY volume resulting, at least in part, from the generation of newborn neurons. Importantly, AMY plasticity is paralleled with depressive-like behaviour.

3. Emotional pain-related behaviour increases in neuropathic animals. At the pharmacological level, this behaviour is mediated, at least in part, by the activation of NMDArs in the ACC and $mGluR_{1/5}$ in the CeA. The activation of $mGluR_1$ in the CeA induces an increase in RVM ON-cells activity and, therefore, an increase in hypersensitivity, which may constitute a mechanism contributing to emotional pain.

4. BLA and CeA neuronal basal activity are increased in neuropathic animals. Since these nuclei project to the RVM, this may contribute to the increase of basal hypersensitivity observed in animals with peripheral nerve injury.

Although we consider that this work has given answers to most of the questions that we had at the beginning of the project, this study also raised many new questions. Therefore, in the future, one of the priority goals is to verify if the newborn neurons also result from the proliferation of neural stem cells located outside the AMY. Additionally, we intend to evaluate if the administration of antidepressants to animals subjected to the SNI model reverses the neuropathic pain signs, the depressive-like behaviour, and/or the formation of new neurons and structural changes of the AMY. Finally, it will be very interesting to analyze if the expression of

different glutamate receptors is altered in, at least, the ACC, the AMY, and the RVM during neuropathy, and how it would be reverted with antidepressant therapy.