Neuropathic Pain and the Brain

Differential involvement of corticotropin-releasing factor and urocortin 1 in acute and chronic pain processing

Tom Rouwette

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Tom Pieter Hendrik Rouwette
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Promotores:	Prof. dr. K.C.P. Vissers
	Prof. dr. E.W. Roubos
Copromotores:	Dr. T.L. Kozicz
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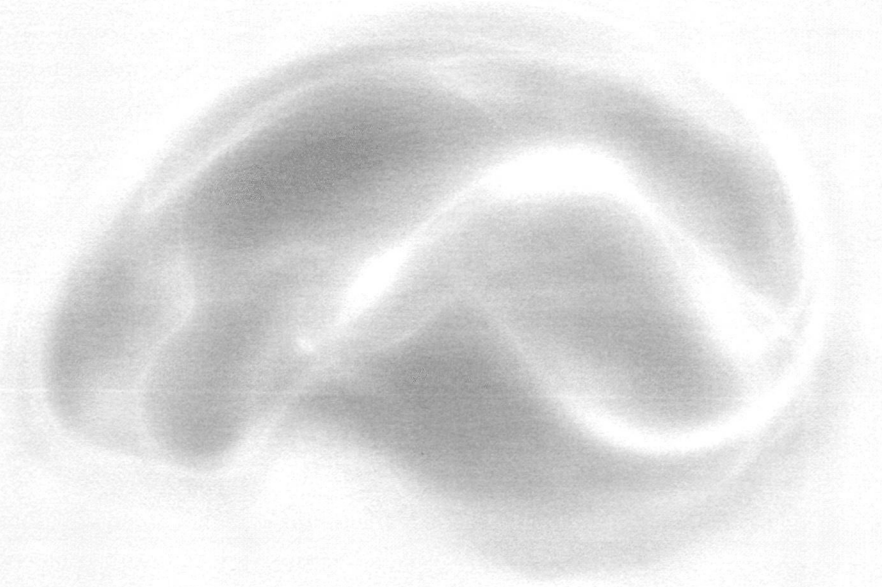
Chapter 1

General Introduction



Introducing hypotheses about neuropathic pain and their validation

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (Loeser and Treede, 2008). It is caused by stimulation of pain-signalling (nociceptive) nerve fibres or by damage or disease affecting the somatosensory nervous system (Treede et al., 2008). Generally, two types of pain are distinguished: acute (transient) and chronic (persistent) pain. The International Association for the Study of Pain (IASP) defines that chronic pain persists past the healing phase following injury (Turk and Okifuji, 2001), and in the common clinical practice pain is considered chronic when it lasts for at least 3 to 6 months after its onset (Apkarian et al., 2009). After surgery, approximately 10% of patients will experience chronic pain (Breivik and Stubhaug, 2008; Perkins and Kehlet, 2000) and this percentage may surpass 50% in case of severe interventions such as sternotomy and amputation of extremities (Kehlet et al., 2006; Macrae, 2008; Niraj and Rowbotham, 2011). Obviously, chronic pain is a major public health issue, affecting the quality of the social and working lives of patients and their family, and posing a heavy financial burden on society, with high costs of treatment and lost productivity (Breivik et al., 2006; Elliott et al., 1999).

Unfortunately, treatment of chronic pain is severely hampered by a lack of knowledge of the underlying neuronal mechanisms in the central nervous system (CNS), and therefore is largely based on the assessment of symptoms, which is a highly unreliable approach (Woolf, 2004). Therefore, better knowledge of the causative (neuronal) mechanisms that lead to chronic pain is necessary to improve clinical therapy including development of drugs that counter chronic pain at an early stage, with minimal side-effects. This holds in particular for neuropathic pain, which is a special form of pain caused by damage or disease affecting the central and/or peripheral somatosensory nervous system (Treede et al., 2008). Like pain in general, neuropathic pain can be divided in an acute and a chronic component. Acute neuropathic pain occurs after medical conditions that cause damage to the peripheral nervous system (PNS) or to the CNS, and may include sensations of sharp, burning or paroxysmal pain, altered pain sensation, hyperalgesia (increased pain sensitivity) and allodynia (pain in response to a non-nociceptive stimulus such as touch or vibration) (Gray, 2008). These sensations may be effectuated via the classical ascending and descending spinal pain pathways (Hunt and Mantyh, 2001). In addition to these averse sensory effects, chronic neuropathic pain (CNP) seems also to be associated or even to induce persistent stressful emotions and behavioural disorders like anxiety and major depression (Freynhagen et al., 2006). Based on this notion we have raised the hypothesis that CNP may affect the functioning of neural and neuroendocrine systems involved in the regulation of the stress response and of mood, such as the hypothalamo-pituitary-adrenal (HPA-) stress axis and components of the limbic

system, respectively. Moreover, since corticotropin-releasing factor (CRF) is the main and common neuronal messenger acting in these systems, we predict that this neuropeptide plays a crucial role in CNP-associated sensory and mood disorders. Furthermore, as several clinical studies have shown a relation between the development of post-operative chronic pain and the intensity of the acute pain experienced as a direct result of surgery (Kalso et al., 1992; Katz et al., 1996; Peters et al., 2007), we hypothesise that CNP-associated disorders are not (only) caused by the later chronic but (also) by the initial acute phase of neuropathic pain.

This thesis research aims to provide support for the hypotheses posed above, by studying in animal models (Sprague-Dawley, SD, rats) the effects of experimentally-induced CNP (by chronic constriction injury of the sciatic nerve, CCI) on the activity of various CRF- and CRF-related peptide-producing brain centres, and on pain sensitivity and the generation of anxiety and depressive-like behaviour. Furthermore, we have investigated if these rat brain centres are also affected by experimentally-evoked acute pain stress (formalin injection). Finally, attention has been paid to the potential of our rat CCI model to test the effectiveness of drugs to treat CNP-associated disorders.

Next in this General Introduction, we will deal in more detail with the theoretical backgrounds of the hypotheses presented above, and then briefly outline the contents of the chapters in which the respective studies to test these hypotheses have been described.

Clinical aspects of CNP

The most common causes of CNP are diabetic polyneuropathy, post-herpetic neuralgia, chemotherapy and post-traumatic and post-surgical nerve injury (Daousi et al., 2004; Davies et al., 2006; Farquhar-Smith, 2011; Finnerup et al., 2001; Galil et al., 1997). The exact incidence of CNP in the general population is not well-known, population-based studies reporting a wide range, from 1% to as high as 8% (Bouhassira et al., 2008; Dieleman et al., 2008; Torrance et al., 2006). In contrast, the strong impact of CNP on well-being is well established. Symptoms of CNP include spontaneous and provoked pain, the latter consisting of hyperalgesia and allodynia (Loeser and Treede, 2008). Furthermore, behavioural disorders may arise, such as anxiety and depression (Freynhagen et al., 2006). CNP is often the result of surgery involving peripheral nerve damage (Dworkin et al., 2003). Consequently, the site and kind of this damage can usually be well identified. Still, the same surgery can be very painful for some patients but less painful or even painless for others, and the reason for this individual pain sensitivity is largely unknown. Therefore, therapy of CNP patients is a cumbersome process of trial and error, based on symptoms rather than on insight into the causes of the disorder (Attal et al., 2008; Haanpaä and Treede, 2010). Moreover, although there are several tools for the clinical assessment of CNP-associated symptoms, such as laboratory testing, quantitative sensory testing, bedside examination and pain questionnaires,

all these approaches are prone to subjectivity (Cruccu and Truini, 2009). This urges the need for developing objective clinical parameters to measure neuropathic pain intensity and responsiveness for anti-CNP treatment. However, it is impossible to formulate such parameters solely on the basis of the aetiology, distribution and nature of the patient's pain symptoms. Therefore, the improvement of CNP treatment strategies requires better knowledge of the cellular and molecular mechanisms that underlie the initiation and progression of the disorder at the level where pain information is processed: the CNS. This knowledge can be best obtained from experimental studies on selected rodent models (Vissers, 2006; Woolf and Mannion, 1999). In this Introduction, such studies will be proposed, but first we will consider what is already known about the neuronal mechanisms underlying pain induction and processing, which actually apply to acute pain.

Pain mechanisms

Acute pain has a clear physiological function: it represents a dominant early warning signal for an adverse, stressful or harmful condition and urges the individual to escape from it or avoid its continuation (Millan, 1999). The two key systems in the processing of acute pain are the CNS, which includes the brain and the spinal cord, and the PNS. Pain stimuli perceived by peripheral nociceptors are transported via the PNS to the dorsal horn of the spinal cord, where reflexes are evoked that activate skeletal muscle to execute immediate action (Andersen, 2007). Meanwhile, the pain stimuli are also transported via the spinal cord to the brain, where ascending pathways carry the pain signal to dedicated brain centres for perception and cognitive processing. There are two main ascending pain pathways: the spinothalamic pathway, particularly concerned with the transport of sensory information to the somatosensory cortex, and the spinoparabrachial pathway, which projects to brain areas involved in the control of mood (Hunt and Mantyh, 2001). Ascending noradrenergic pathways activate internal organs, and especially the adrenal glands, via a powerful stimulatory input to the HPA-axis, to hormonally activate physiological processes essential to protect the organism from further damage (Ziegler and Herman, 2002). When these immediate actions of pain have been completed and, therefore, are no longer needed, pain signal generation will be blocked. This takes place via antinociceptive signals that mainly originate in the cerebral cortex, hypothalamus and amygdala, and reach the site of pain stimulus generation via descending pain pathways that run via the periaqueductal grey (PAG) and the lower brainstem (Fields, 2004; Hunt and Mantyh, 2001) (Fig. 1).

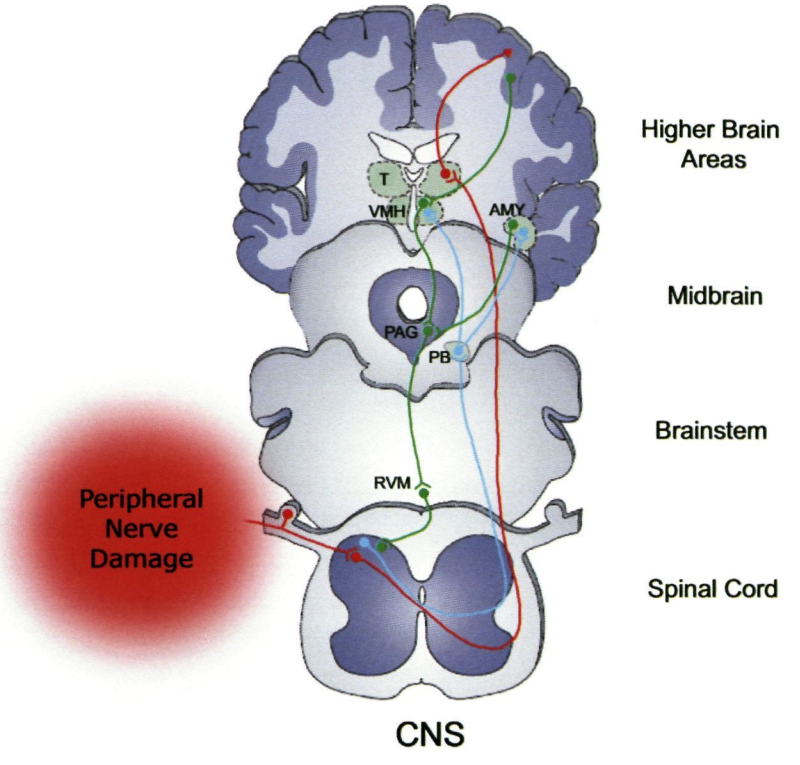


Figure 1. *The main ascending and descending pain pathways. From nociceptors in the peripheral nervous system pain signals are transported to the central nervous system (CNS) via the dorsal horn of the spinal cord. The CNS has two main ascending pain pathways: the spinothalamic pathway (red), which distributes nociceptive information to areas of the cortex concerned with sensory information, and the spinoparabrachial pathway (blue), originating in the superficial dorsal horn and projecting to brain centres involved in the control of mood. The descending pain pathway (green) processes information from the cerebral cortex, hypothalamus and amygdala to the periaqueductal grey (PAG). PAG neurons project to the lower brainstem to control antinociceptive and autonomic responses following noxious stimulation. AMY, amygdala; PB, parabrachial nucleus; RVM, rostroventral medulla; T, thalamus; VMH, ventromedial hypothalamus. Modified from Fields (2004).*

Pain and stress – (dys)regulation of the HPA-axis

Acute pain is not only important as an early warning signal triggering immediate physiological and psychological actions against actual or potentially damaging stimuli of transient nature (Woolf and Salter, 2000), it also stimulates the organism to adapt to the

situation in case the stressful situation persists over a longer period, by activating the major stress system, the HPA-axis (Tsigos and Chrousos, 2002). Peripheral nociceptors and the ascending pain pathways provide a powerful stimulatory input to this axis (Palkovits et al., 1999; Ziegler and Herman, 2002) by acting via various brain centres on the paraventricular nucleus of the hypothalamus (PVN). This regulator of the HPA-axis releases CRF from its neurohemal axon terminals located in the median eminence of the hypothalamus into the hypophyseal portal vessel system. In this way CRF, here acting as a neurohormone, reaches the corticotrope cells in the distal lobe of the pituitary gland to stimulate the secretion of adrenocorticotropic hormone (ACTH) into the general circulation. In turn, ACTH triggers the adrenal production and consequent secretion of glucocorticoids (corticosterone in rodents and cortisol in humans and fish). These steroid hormones have dual functions. First, they enable the body to adequately respond to the pain stressor by physiological changes, *e.g.* increasing heart rate and blood pressure, and activating energy metabolism. Glucocorticoids also exert a negative feedback to the brain including the PVN and the pituitary corticotrope cells, to decrease HPA-axis activity, in this way terminating the stress response and restoring physiological homeostasis (Fig. 2). However, when for some reason this process fails to be terminated, an excessive and prolonged glucocorticoid response suppresses immune responses (Mahbub et al., 2011) and may increase the risk of the occurrence of stress-related diseases of body and mind, such as anxiety and depression (Aguilera, 2011; de Kloet et al., 2008; Flandreau et al., 2012; Lightman, 2008; Pariante and Lightman, 2008). Such failure of the HPA-axis to inactivate upon negative adrenal corticosteroid feedback and the resulting homeostatic disturbance may have both genetic and environmental (*e.g.* early life events) causes (Veenema, 2009). Moreover, the permanently released corticosteroids may induce behavioural changes by acting directly on other brain centres including those of the limbic system (Roozendaal et al., 2009).

Whereas the above sketched activation by acute pain of HPA-axis activity is well known, the question (central to this thesis research) arises whether the axis also responds to CCI, and if such a response would lead to glucocorticoid-induced behavioural disorders. Different types of chronic pain can act as chronic, inescapable stressors, with increased cortisol/corticosterone responses (Blackburn-Munro and Blackburn-Munro, 2001). However, such actions have not been demonstrated specifically for CCI-induced CNP, which is a chronic pain (Bomholt et al., 2005; Ulrich-Lai et al., 2006).

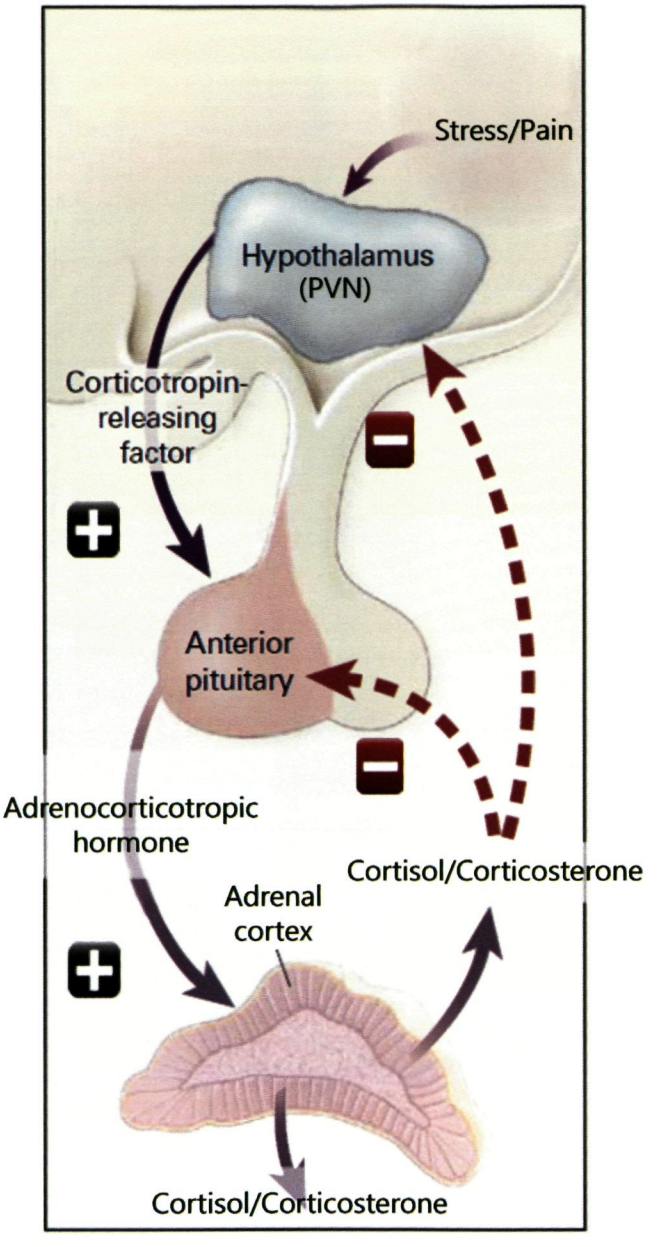


Figure 2. *Schematic overview of the classical HPA-axis. Brief or sustained periods of stress or pain are associated with an increased release of corticotropin-releasing factor (CRF) from the hypothalamus. In the distal (anterior) part of the pituitary gland, CRF stimulates the secretion of adrenocorticotropic hormone to trigger the secretion of cortisol or corticosterone from the adrenal gland. These corticosteroids cause physiological changes in the organism to restore homeostasis and provide negative feedback to the HPA-axis to eventually shut down the stress response. Modified from Yehuda (2002).*

The limbic system

Neuropathic pain is strongly associated with mood disorders in humans (Argoff, 2007; Hunt and Mantyh, 2001; Manas et al., 2011) and, similarly, with anxiety and depressive-like behaviours in rodents (Hu et al., 2009; Roeska et al., 2008, 2009). A prominent mediator in these disorders is CRF, a 41-amino acid residues-long peptide originally isolated from the ovine hypothalamus by Vale et al. (1981). It belongs to the CRF family of neuropeptides, and its major production site is the parvocellular subdivision of the PVN, but it is also present in extra-hypothalamic sites such as the hippocampus, thalamus, cerebellum and cerebral cortex (Merchenthaler et al., 1982; Palkovits et al., 1985). The major extra-hypothalamic site of CRF, however, is the limbic system and, more in particular, the mood controlling central amygdala (CeA) and bed nucleus of the stria terminalis (BST), which are involved in anxiety and in depressive-like behaviour, respectively (Davis and Shi, 1999; Regev et al., 2011). The CRF mRNA and CRF peptide contents of the BST and CeA are up-regulated by stressors and by neuropathic pain (Kim et al., 2010; Sterrenburg et al., 2012; Ulrich-Lai et al., 2006).

As to the possibility that the CeA is a target for CNP, it is interesting to note that this nucleus is part of each of the above-mentioned ascending and descending pain pathways. Moreover, when acute pain sensation is not properly terminated but, for some reason, persists, the amygdala reveals various biochemical and neuroplastic changes (Neugebauer et al., 2004), suggesting that the expression of (chronic) pain-induced mood changes is, at least in part, due to malfunctioning of this limbic nucleus.

As to the relation between pain, the limbic system and HPA-axis, the situation is complex and largely unclear. Whereas the BST and CeA provide important inputs to the HPA-axis, either directly or indirectly via projections to the PVN (Herman et al., 2003), the pain-induced changes in these brain centres do not need *per se* to change HPA-axis activity. Although normalisation of HPA-axis functioning after its stress-induced activation seems to be a prerequisite for successful treatment of these mood disorders, anxiety and depressive-like symptoms also occur in the absence of HPA-axis activation and at normal plasma cortisol/corticosterone titres, but in the presence of elevated CRF in extra-hypothalamic sites, especially in the BST and CeA (Bomholt et al., 2005; de Kloet et al., 2005; Ulrich-Lai et al., 2006). This indicates that these centres act independently of the HPA-axis. Indeed, the PVN receives inputs from various brain structures, *e.g.* inhibitory stimuli from the hippocampus and prefrontal cortex and stimulatory input from the amygdala (Herman et al., 2005), and pain-induced changes in the BST and amygdala may concern neurons that do not project to the HPA-axis. Moreover, as will be explained into more detail below, in its stress response the HPA-axis collaborates with another extra-hypothalamic stress-sensitive brain centre, the Edinger-Westphal centrally projecting neuron population (EWcp) in the midbrain. Therefore, the question arises if CNP exerts its actions on mood by acting either on the CeA

and BST or on the HPA-axis and the EWcp, or on all of these four areas together. This question has been investigated in this thesis research.

The CRF family member urocortin 1 in the Edinger-Westphal nucleus

After the identification of CRF, several other mammalian CRF-related peptides have been discovered, such as urotensin, sauvagine (Lovejoy and Balment, 1999), urocortin 1 (Ucn1; Vaughan et al., 1995), urocortin 2 (Ucn2; Hsu and Hsueh, 2001; Reyes et al., 2001) and urocortin 3 (Ucn3; Hsu and Hsueh, 2001; Lewis et al., 2001). This suggests that in addition to CRF, other CRF peptide family members may be involved in regulating the response to pain stimuli.

Ucn1 is a 40 amino acid peptide with a 45% sequence homology to CRF. It plays a main role in mood disorders like depression and anxiety, and Ucn1-deficient mice show increased anxiety behaviour in elevated plus maze and open field tests (Vetter et al., 2002). Remarkably, the majority of the brain's Ucn1 contents is present in the EWcp (Bittencourt et al., 1999; Kozicz et al., 1998, 2011). In this nucleus, the Ucn1-producing neurons are highly sensitive to acute (pain) and chronic stressors (Gaszner et al., 2004; Kozicz, 2001; Xu et al., 2010), suggesting that they also react to chronic pain stimuli like CCI. This idea is strengthened by the demonstration of a dramatic up-regulation of Ucn1 mRNA in the EWcp of suicide victims diagnosed with major depression (Kozicz et al., 2008). Being a CRF family member that is implicated in the stress response and involved in the regulation of mood, Ucn1 is a prominent candidate for playing a crucial role in CNP processing. The response of EWcp-Ucn1 neurons to acute stressors is very similar to that of the CRF neurons in the PVN, which show up-regulation of the neuronal activity marker cFos and of CRF mRNA (Imaki et al., 1992, 1995; Palkovits, 2008). However, in response to chronic stressors the EWcp markedly differs from the PVN: whereas chronic, repeated ether stress increases cFos in the EWcp in a similar way as the cFos response after acute ether stress (Korosi et al., 2005), chronic restraint stress causes a 3-fold lower cFos induction in the PVN than does acute stress (Viau and Sawchenko, 2002). Furthermore, in CRF-null mice, which lack the *Crf* gene, the Ucn1 mRNA content of the EWcp is increased (Weninger et al., 2000), whereas this content is decreased in the EWcp of mice over-expressing *Crf* (Kozicz et al., 2004). This suggests that the EWcp-Ucn1 and PVN-CRF neuronal circuits are anatomically distinct, though functionally interrelated systems, and may be co-ordinately regulated upon acute stimuli but inversely react to chronic stimuli (Kozicz, 2007; Kozicz et al., 2011). Whether this difference also holds for acute pain and chronic (neuropathic) pain, is also subject of this research.

Doublecortin-like kinase

Up to now, this General Introduction has been dealing with the (hypothetical) anatomical and cellular aspects of CNS responses to pain. However, the molecular pathways that are activated upon painful stimuli and may underlie pain-associated disorders are of great interest. Therefore, focus was on a recently discovered protein, doublecortin-like kinase (DCLK). As will be explained below, proteins of the DCLK family are involved in glucocorticoid signalling (Fitzsimons et al., 2008) and anxiety behaviour of mice (Schenk et al., 2010). This makes DCLK a good candidate marker for malfunctioning of neurons as a result of (pain) stress.

Several pain conditions lead to the activation of mitogen-activated protein kinases (MAPKs), a process that plays an important role in the induction and maintenance of neuronal plasticity during central and peripheral sensitisation (Ji et al., 2009). MAPKs phosphorylate proteins with a specific proline-threonine-serine-proline (PTSP) domain (David et al., 1995; Edelman et al., 2005; Engels et al., 2004; Ohmae et al., 2006). As members of the DCLK gene family possess a strongly conserved PTST-phosphorylation site, they have gained particular interest as intracellular signalling molecules (Boekhoorn et al., 2008; Fitzsimons et al., 2008; Francis et al., 1999; Friocourt et al., 2007). The *dclk* gene gives rise to three main splice variants: DCLK-short, DCLK-long and DCL (Engels et al., 2004; Omori et al., 1998; Sossey-Alaoui and Srivastava, 1999). Their phosphorylation as well as their ability to phosphorylate other proteins can lead to plastic changes in the brain (Schaar et al., 2004). An indication for their involvement in the stress response comes from the demonstration that DCL plays a role in the translocation of the activated glucocorticoid receptor to the nucleus of neuronal progenitor cells (Fitzsimons et al., 2008). Furthermore, mice over-expressing a truncated form of the DCLK-short transcript have been linked to anxiety behaviour, because they spend less time and move less on the open arms of the elevated plus maze (Schenk et al., 2010). Therefore, it is likely that DCLK proteins are players in (pain) stress-induced plasticity under conditions of adaptation or maladaptation like depression and anxiety. This idea has been studied in this thesis research.

Experimental animal models

In spite of the increasing number of pain-related human neuroimaging studies, complemented by techniques like quantitative sensory testing, microdialysis, and cell culture, and by physiological, epidemiological and genetic studies (Mogil et al., 2010), our knowledge of the cellular and molecular aspects of the pathogenesis of human neuropathic pain is still far from complete. In fact, animal studies are strongly needed, because they offer the possibility of characterizing neurochemistry and anatomy into great detail down to the subcellular and

gene levels, and allow accurate control of pain conditions and neuronal activity determinations with invasive methods that are impossible to perform in humans. Furthermore, animal models provide the opportunity to test the efficacy of potential new analgesic drugs empirically and on a large scale (Mogil et al., 2010). To study mechanisms involved in the response to acute and neuropathic pain, the research described in this thesis was conducted using two different rat models: formalin injection into the hind paw to induce acute pain stress (APS) and CCI to induce CNP. Below, these models are described in more detail.

The APS model

A widely used paradigm to study the effects of acute pain on neuroendocrine and neuronal systems is subcutaneous injection of formalin into a hind paw of a rodent. This activates the stress response by evoking a moderate pain generated by the injured tissue (Abbott et al., 1995; Tjølsen et al., 1992) and produces a biphasic behavioural effect, where the early phase reflects acute nociception and the following phase consists of tonic pain activation of sensory afferents and tissue damage as well as inflammation (Coderre et al., 1993; Cook et al., 1987; Jett et al., 1997; Woolf et al., 1994). Homeostatic responses like elevated heart rate and blood pressure (Culman et al., 1997), increase of plasma catecholamines and behavioural responses such as biting, licking, shaking and lifting of the injured hind paw, are often seen in this acute pain model. Since this type of pain stress activates the HPA-axis (Kant et al., 1982; Pacak et al., 1998; Vissers et al., 2004), the model is very suitable to study the effects of acute pain on this axis and on other stress-responsive areas in the brain functionally linked to the axis, such as the CeA, BST and EWcp.

The CCI model

Nerve injury models have proven to be very suitable for research on the central mechanisms of CNP, as they reveal typically human symptoms of increased pain sensation: hyperalgesia and allodynia (*e.g.* Daemen et al., 1998; Kim et al., 1997). One of these models is the CCI rat, where the sciatic nerve of the left hind paw is loosely constricted (Bennett and Xie, 1988). In our laboratory, the animal model of choice for CNP research is the SD rat, which reliably reveals allodynia to thermal stimulation and hyperalgesia to mechanical stimulation (Vissers et al., 2003a,b). In addition to these pain sensation phenomena, anxiety and depressive-like behaviour are observed in CCI rats as, compared to shams, these rats spend less time on the open arms of the elevated plus maze (Roeska et al., 2008, 2009) and stay more immobile in the forced swim test (Hu et al., 2009; Jesse et al., 2010). In the present research, we have

explored the development of increased pain sensitivity and behavioural changes in the SD rat as a result of CCI.

Cellular parameters for neuronal functioning

The expression of genes in the brain is regulated by multiple internal and external signals that induce discrete patterns of gene expression in the neurons of different brain nuclei. These unique expression patterns are involved in reshaping the functioning of these nuclei so that they can generate adaptive responses to changed external conditions. To study possible pain-induced changes in gene transcription and their subsequent translation into peptides/proteins in specific brain nuclei at the cellular level, we have used quantitative *in situ* hybridisation (to measure mRNA) and quantitative (single- and double-labelling) immunohistochemistry (to assess peptide/protein contents). Focus has been on the expression of the immediate early gene (IEG) encoding for the cFos protein and for the (genes of the) neuropeptides CRF and Ucn1.

IEGs are rapidly expressed upon cell stimulation, and predominantly encode for transcription factors that modify the expression of target genes. Subsequently, these gene expressions can alter the phenotype of the cell, *e.g.* by shifting the affinity or number of receptors or changing the rate of biosynthesis of enzymes and neurochemical messengers. The IEG *cFos* has a transcription level that is low under basal conditions but is experimentally elevated by a wide range of stimuli. The nuclear cFos protein appears within minutes after a physical or physiological challenge and induces gene activations in various brain areas. Therefore, this protein is very suitable as a marker for gene activations throughout the brain (Kovacs, 2008).

In this research, the mRNAs of CRF and Ucn1 have been measured to assess the neuronal production capacity of these stress neuropeptides, whereas CRF and Ucn1 peptide contents have been quantified to determine their storage inside neuronal cell bodies. Together, these methods are valuable tools to obtain an indication about several aspects of neuronal activity (gene expression, and the production, storage and release of neuropeptide messengers).

Drug testing

Developing novel drugs to specifically treat neuropathic pain with only minimal side-effects, is an ultimate goal of fundamental research on the central mechanisms underlying CNP. To date, recommended clinical first-line treatment of CNP patients includes antidepressants such as amitriptyline and gabapentin (Attal et al., 2010; Baastrup and Finnerup, 2008; Densmore et al., 2010), whereas second-line treatments are performed with opioid analgesics

like morphine (Dworkin et al., 2007b). Finally, paroxetine and minocycline are among drugs that come into play if patients do not respond to first- and second-line medications (Chen et al., 2010; Dworkin et al., 2007a). However, CNP is a complex disorder to treat and the administration of analgesics elicits differential responses in different patients, has rather unpredictable effectiveness, a slow analgesic onset, complicated dosing, and various side-effects, all factors impeding their use (Dworkin et al., 2007b). Therefore, the construct validity of these drugs is very low. Ideally, treatment of CNP should focus at eliminating the cause of the pain, but in practice treatment is largely based on symptoms, barely addressing the mechanisms underlying CNP development (Woolf, 2004). Therefore, to improve CNP treatment, more knowledge about these mechanisms is necessary (Vissers, 2006). In this research we have made an initial step into this direction by testing selected anti-neuropathic drugs in our SD CCI rat model.

Aims and outline of the thesis research

In the clinic, several surgical interventions lead to CNP, with anxiety and depression as co-morbid disorders on the long-term. These disorders have been linked to dysregulation of the stress response system of the brain, with CRF as a major player. Therefore, we hypothesise that severe, long-term acting stressors may disrupt the homeostatic functioning of this response system, with CNP and its associated disorders like increased pain sensitivity, anxiety and depression as a result. This disruption may take place at two different post-surgical time frames of neuropathic pain, viz. during the acute phase and during the chronic phase. To test this hypothesis, two pain paradigms have been used: the rat APS model mimicking the acute neuropathic phase following CCI-surgery, and the CCI rat as a model for the chronic phase of neuropathic pain.

Chapter 2 is concerned with a behavioural characterisation of the SD rat, as a model for our studies on CNP. First the possible effects of CCI on pain sensitivity, anxiety and depressive-like behaviour are described. Then, effects of clinically used anti-neuropathic drugs on these behavioural parameters are dealt with. **Chapter 3** reports whether CCI-surgery induces changes in the presence of CRF in the PVN, BST and CeA, and of Ucn1 in the EWcp. To investigate if such changes are related to the initial, acute phase of CNP, a similar study has been carried out with the APS rat, which was exposed to acute (formalin injection) pain stress (**Chapter 4**). **Chapter 5** describes the last experimental study of this thesis, in which the response to acute pain stress has been investigated in the PVN and the EWcp at the molecular level, assessing the phosphorylation of DCLK at its MAPK-PTSP domain and its involvement in the intracellular processing of the acute pain stimulus. On the basis of an extensive literature study, in **Chapter 6** a central mechanism for pain processing in the CNS is postulated, in which the amygdala plays a critical role in the transition from acute to

neuropathic pain. In the General Discussion (**Chapter 7**), the results and conclusions from this research will be discussed in view of the aims of this thesis research, and used to design an integrative model of the ways several brain centres and the HPA-axis together may generate CNP. Finally, suggestions have been made for future studies that may increase our understanding of the central mechanisms underlying CNP and improve the clinical treatment of this major disorder.

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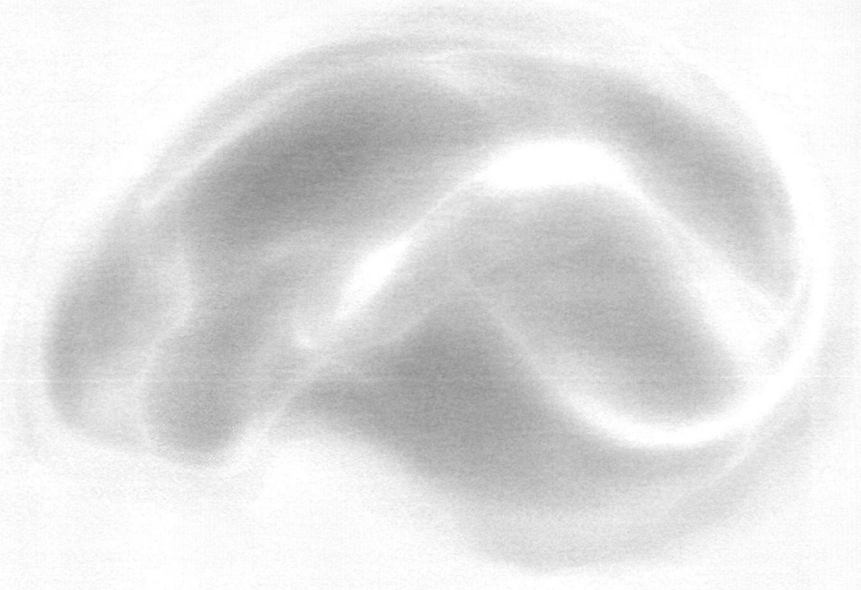
Chapter 2

Experimentally-induced neuropathy and the effect of chronic drug medication on Sprague-Dawley rats

Tom P.H. Rouwette, Pascal Vanelderen, Wim J.J.M. Scheenen, Francien van de Pol, Ilona van den Brink, Eric W. Roubos, Tamás L. Kozicz, Kris C.P. Vissers

Submitted



Abstract

Human neuropathic pain is associated with increased pain sensitivity and, often, with mood disorders like anxiety and depression, but its neuropathology is largely elusive. This makes treatment complex, with differential individual responses to medication accompanied by severe side-effects. To improve neuropathic pain treatment using drugs specifically designed to block pain signalling, the underlying mechanisms of the disorder should be elucidated. In this study, we have examined the temporal changes in pain sensitivity and in anxiety and depressive-like behaviour in Sprague-Dawley (SD) rats with neuropathy induced by chronic constriction injury (CCI) of the sciatic nerve of the left hind paw. In this way the hypothesis has been tested that CCI does not only lead to mechanical hyperalgesia and thermal allodynia, symptoms of human neuropathic pain, but is also associated with anxiety and depressive-like behaviour in this rat strain. Furthermore, is has been investigated if such symptoms can be reversed by chronic administration of drugs used to treat neuropathic patients. The results show that CCI causes allodynia and hyperalgesia for up to six weeks after surgery but, remarkably, has no effect on parameters for anxiety and depressive-like behaviour. Of the five drugs tested, morphine, gabapentin and paroxetine did not show any effect on CCI rats, whereas amitriptyline slightly reduced body growth and minocycline reduced thermal allodynia. Therefore, the SD CCI rat is a suitable model for investigating neuropathy-associated increased pain sensitivity in humans and for the effectiveness of chronic treatment of allodynia and hyperalgesia with anti-neuropathic drugs, but its face validity with respect to human neuropathy-associated anxiety and depression appears to be low.

Introduction

Neuropathic pain arises as a direct consequence of a lesion or disease affecting the somatosensory nervous system (Treede et al., 2008). Neuropathy patients suffer from various painful symptoms, including thermal allodynia and mechanical hyperalgesia, and their pain is often accompanied by anxiety and depression (Freynhagen et al., 2006). Neuropathic pain is a disorder difficult to treat because individual patients may respond differently to the same medication. Moreover, unpredictable effectiveness, delayed analgesic onset and the occurrence of various side-effects are confounding factors (Dworkin et al., 2007b). To better understand the underlying mechanisms of neuropathic pain and, thereby, improve its pharmacological treatment, basic research on animal models is necessary (Vissers, 2006).

Current recommended first-line treatment of neuropathic pain includes the use of antidepressants such as amitriptyline, gabapentin and pregabalin (Attal et al., 2010; Baastrup and Finnerup, 2008; Densmore et al., 2010). Opioid analgesics like morphine are recommended for second-line treatment or for first-line use in specific clinical situations (Dworkin et al., 2007a), while paroxetine is a selective serotonin reuptake inhibitor used as a third-line medication for patients who do not adequately respond to first- and second-line treatments (Dworkin et al., 2007b). The microglial inhibitor minocycline was recently shown to reduce the development of mechanical allodynia and thermal hyperalgesia in rodents (Guasti et al., 2009; Mika et al., 2007). Well-validated rodent models to study the two key symptoms of human neuropathic pain, *i.e.* allodynia and hyperalgesia, are the chronic constriction injury (CCI) model, where neuropathic pain has been induced by loosely constricting the sciatic nerve at the level of the (left) hind paw (Bennett and Xie, 1988; Rouwette et al., 2012; Vissers, 2006; Xu et al., 2001), and the (L5 and L6) spinal nerve ligation (SNL) model (Hu et al., 2009; Roeska et al., 2008, 2009; Xu et al., 2001). Neuropathic pain is often accompanied by mood disorders in humans (Argoff, 2007; Hunt and Mantyh, 2001; Manas et al., 2011) as well as in rodents exposed to CCI or SNL. More specifically, anxiety and depressive-like behaviour are displayed in CCI- and SNL-treated Wistar rats (Goncalves et al., 2008; Hu et al., 2009; Leite-Almeida et al., 2009; Roeska et al., 2008, 2009), but for Sprague-Dawley (SD) rats the effects of experimentally-induced nerve injury are inconsistent: in some studies no induction of anxiety and depressive-like behaviour was observed (Kontinen et al., 1999), whereas other SD rats revealed only anxiety (Pedersen and Blackburn-Munro, 2006) or only depressive-like behaviour (Hu et al., 2010).

This study has been designed to test the face and predictive validity of the CCI model for human neuropathy in SD rats. With regard to face validity, we first assessed CCI-induced changes in pain sensitivity, by testing for thermal allodynia and mechanical hyperalgesia. Next, to confirm the notion that neuropathic pain is often accompanied by co-morbid mood disorders like anxiety and depression, we assessed anxiety and depressive-like behaviour at

two different time points after CCI-surgery. Previously, several drugs effective in treating neuropathic pain in humans have been tested in CCI or SNL rodent models, but most of these studies focused on acute drug effects on behaviour (Densmore et al., 2010; Matsuzawa-Yanagida et al., 2008; Roeska et al., 2008), in spite of the fact that in the clinical practice of neuropathic pain treatment chronic administration is the standard. Therefore, to assess the predictive validity of the CCI model in the SD rat, we studied the effectiveness of chronic drug administration for two weeks after CCI. Five different treatments (amitriptyline, gabapentin, morphine, paroxetine and minocycline) were tested for their effectiveness to attenuate pain sensation (thermal allodynia and mechanical hyperalgesia) and anxiety and depressive-like behaviour induced by CCI.

Our results show that CCI induces in the SD rat chronic thermal allodynia and mechanical hyperalgesia (up to six weeks after injury), but not anxiety and depressive-like behaviour. Furthermore, we reveal that among the drugs tested only minocycline was able to substantially attenuate CCI-induced allodynia.

Materials & Methods

Animals

Male SD rats (Charles River Laboratories, Sulzfeld, Germany) were paired-housed in plastic cages (Makrolon type III; Tecniplast, Buguggiate, Italy), in a temperature- and humidity-controlled environment, on a 12/12 h light-dark cycle (lights on: 6:30 AM, light intensity: 200 lux), with *ad libitum* access to tap water and soy-free rodent chow. Rats were acclimatised during one week before pre-operative testing and surgery. All measures were taken to minimise the number of animals and their suffering, and the experiments were conducted following the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and had been approved by the Animal Ethics Committee of Radboud University Nijmegen.

Chronic constriction injury

Chronic constriction of the sciatic nerve was performed by surgery as described elsewhere (Bennett and Xie, 1988). Briefly, under isoflurane anaesthesia (induction 5%, maintenance 2.5%) combined with oxygen (30%) and nitrous oxide (65-67.5%), the sciatic nerve of the left hind paw was exposed at the mid-thigh level proximal to the sciatic trifurcation, by blunt incision of the biceps femoris muscle. Four chromic catgut 5-0 ligatures (Catgut GmbH, Markneukirchen, Germany) were tied loosely around the nerve, 1 mm apart. The overlying muscle was sutured with vicryl 5-0 (Johnson & Johnson, St. Stevens-Voluwe, Belgium) and the skin closed with autoclips (7.5 x 1.75 mm; Rudolf Medizintechnik, Fridingen, Germany).

Sham rats underwent the same surgery, where the sciatic nerve was exposed but not ligated, whereas control rats did not undergo any surgery.

Thermal allodynia - cold plate test

Rats were tested for thermal allodynia on a cold plate apparatus (Ugo Basile, Comerio, Italy) consisting of an acrylic cylinder (diameter 19 cm, height 31 cm) fixed to a stainless steel plate which temperature was maintained at 4 °C. Upon placing a rat on the cold plate the duration of lifting of the left as well as of the right hind paw was measured with a digital hand chronometer during a 5 min period. Rats were considered neuropathic if the total lifting time of the left, operated hind paw exceeded 20 s.

Mechanical allodynia - Von Frey test

Sensitivity to mechanical stimuli was assayed using a series of force-calibrated Von Frey filaments (Somedic AS, Stockholm, Sweden). Rats were placed individually on a mesh-wire floor of a plastic box (20 x 16 x 14 cm), and allowed to habituate for 30 min. Filaments had been applied perpendicularly to the plantar surface of a hind paw with sufficient force to bend. Brisk withdrawal or paw flinching were considered as positive responses. In the absence of a response, the filament of the next greater force was applied. Each monofilament was used 5 times on the same hind paw.

Anxiety - elevated plus maze test

Anxiety was evaluated with an elevated plus maze consisting of two open and two closed arms (50 x 10 x 50 cm each arm) and a central area (10 x 10 cm) that was elevated 50 cm from the floor. Upon placing the rat in the centre of the maze, facing an open arm, the times spent in the open and closed arms were recorded during a 5 min period. Room light intensity was 10 lux and the plus maze was cleaned with 70% ethanol before testing a next animal.

Depressive-like behaviour - forced swim test

Depressive-like behaviour was evaluated with the forced swim test using a glass cylinder (diameter 20 cm, height 50 cm) containing 30 cm of water (25 ± 0.5 °C). Rats were individually placed into the cylinder and left to swim for 15 min, after which they were removed from the water, towel-dried and returned to their home cages. Twenty-four hours later, rats were tested under the same conditions for 5 min (test session). They were considered to be immobile as long as both hind legs were not moving.

Experiment 1 - Development of neuropathic pain-like behaviour

CCI and sham rats were prepared as described above. Thirty-six animals were divided into four groups, according to Table 1. They were tested for pain sensitivity one day prior to surgery and at days 7, 14 and 21 for all groups, and additionally at days 28, 35, 42, 49 and 56 for groups 3 and 4. Behavioural testing was performed at days 19 and 20 for groups 1 and 2, and at days 54 and 55 for groups 3 and 4.

Table 1. Experimental set-up of experiment 1

Group	Surgery	Experiment length
1	Sham (n = 12)	3 weeks
2	CCI (n = 12)	3 weeks
3	Sham (n = 6)	8 weeks
4	CCI (n = 6)	8 weeks

Experiment 2 – Drug effects

Forty-eight rats were divided in 8 equal groups, according to Table 2. They were tested for pain sensitivity one day prior to surgery and at days 7, 14 and 21. Behavioural testing was performed at days 19 and 20. Drug treatment started at day 7 after testing for thermal allodynia and mechanical hyperalgesia, and lasted till the day before perfusing the rats. Drugs were dissolved in 0.9% NaCl and administered twice a day, at 7.00 AM and 7.00 PM. Body weight was monitored daily throughout the experiment.

Table 2. Experimental set-up of experiment 2 (n = 6 per group). s.c., subcutaneous.

Group	Surgery	Drug	Drug supplier
1	Control	saline s.c.	B. Braun, Melsungen, Germany
2	Sham	saline s.c.	B. Braun, Melsungen, Germany
3	CCI	saline s.c.	B. Braun, Melsungen, Germany
4	CCI	morphine s.c. (3 mg/kg)	Centrafarm, Etten-Leur, The Netherlands
5	CCI	gabapentin s.c. (50 mg/kg)	Pfizer, Capelle a/d IJssel, The Netherlands
6	CCI	amitriptyline s.c. (5 mg/kg)	Sigma-Aldrich, St. Louis, MO, USA
7	CCI	paroxetine s.c. (5 mg/kg)	Campro Scientific, Bristol, MA, USA
8	CCI	minocycline oral (25 mg/kg)	Sigma-Aldrich, St. Louis, MO, USA

Statistics

Data on body weight, allodynia, hyperalgesia, anxiety and depressive-like behaviour were tested by analysis of variance with repeated measures (ANOVA), followed by Bonferroni's *post-hoc* test. All analyses were preceded by tests for normality (Shapiro and Wilk, 1965) and for homogeneity of variance (Snedecor and Cochran, 1989), using GraphPad Prism 5.02 (GraphPad Software, San Diego, CA, USA) (α = 5%). Data are presented as means and the standard error of the mean (SEM).

Results

Experiment 1 - Development of neuropathic pain-like behaviour

Body weight

Prior to surgery, no difference was present in mean body weight between CCI rats (324.1 ± 5.3 g; n = 18) and shams (330.2 ± 3.8 g; n = 18; P > 0.05). During the first three weeks after surgery, both shams and CCI rats increased body weight with similar speeds, as can be seen from the steadily increasing cumulative body weight gain (Fig. 1A), but CCI rats revealed an initial, transient drop in weight gain (-5.6 ± 0.9 g at day 1), so that during the first three weeks, their average weight gain was 9.0% lower than that of shams ($F_{(1,34)}$ = 38.20, P < 0.001). However, from day 35 post-surgery onwards, CCI rats gained as much weight as shams.

Mechanical hyperalgesia and thermal allodynia

Following surgery, CCI rats developed marked cold allodynia in the operated, left hind paw, whereas the contralateral, non-operated paw rarely or not showed allodynia, and neither did the left and right hind paws of shams ($F_{(1,34)}$ = 6.85, P < 0.001; Fig. 1B). Lifting duration of the left hind paw of CCI rats strongly increased after surgery, reaching a maximum at day 14 (66.1 ± 7.9 s) to gradually decrease again till almost zero at the end of the experiment.

There was no difference between left and right hind paw withdrawal thresholds in response to Von Frey hair stimulation between the groups prior to surgery (Fig. 1C). However, CCI rats developed marked mechanical hyperalgesia in the operated left hind paw compared to the right hind paw and compared to the hind paws of shams, as appeared from a clearly decreased withdrawal threshold ($F_{(1,34)}$ = 2.99, P < 0.01). Hyperalgesia was maximal after two weeks and then gradually disappeared so that 6 weeks after CCI-surgery the withdrawal threshold had returned to the level of the shams.

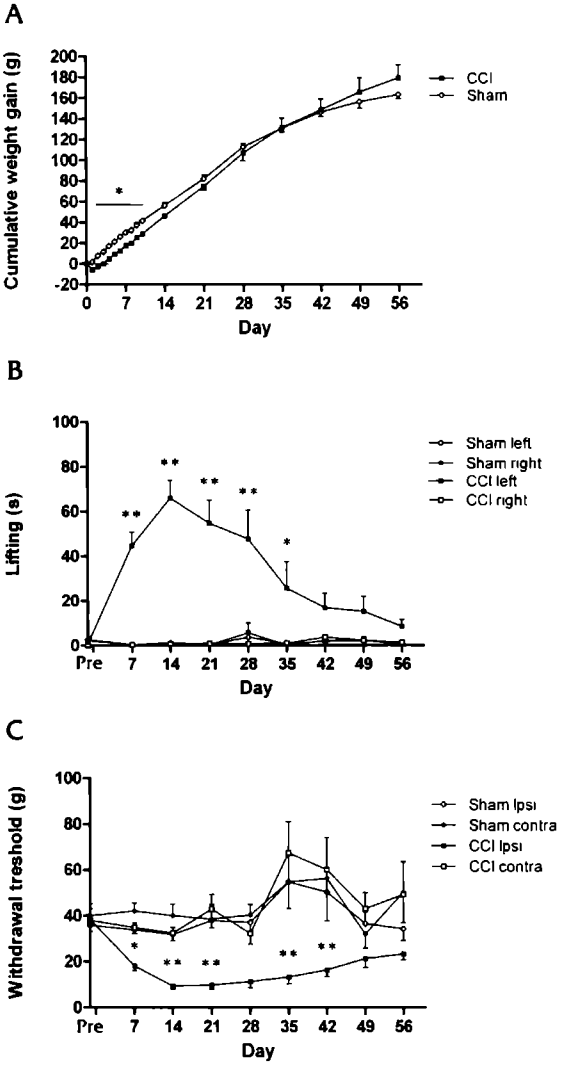


Figure 1. *Chronic nerve constriction (CCI) reduces body weight gain and induces thermal allodynia and mechanical hyperalgesia in the SD rat. (A) Relative to shams (open symbols), CCI rats (solid symbols) show negative cumulative body weight gain during the first two days after surgery. Until day 35, the weight gain of shams is higher than of CCI rats. After day 35, CCI rats gain more weight. (B) CCI causes cold allodynia, as seen from the total time the injured ipsilateral hind paw was lifted on a cold plate over a 5-min period. Thermal allodynia appears within the first week after surgery, and persists until day 35, after which there is no difference in paw lifting times between shams and CCI rats. (C) CCI results in mechanical hyperalgesia in the ipsilateral hind paw, evidenced by a lower withdrawal threshold of the injured paw in response to Von Frey hair stimulation. After day 42, withdrawal thresholds between groups do not differ. n = 18 rats per group until day 21, n = 6 rats per group from day 28 onwards. Pre, pre-operative testing. * $P < 0.05$ and ** $P < 0.001$.*

Anxiety and depressive-like behaviour

In the forced swim test, all rats showed similar periods of active behaviour (swimming and diving for more than 60% of the time) and inactive behaviour (floating and sinking), so that no effect of CCI was seen, neither at day 25 nor at day 55 ($F_{(3,32)} = 0.01$, $P > 0.05$; Fig. 2A). Furthermore, in the elevated plus maze, no significant differences were present between CCI rats and shams as to the period animals stayed on the open arms ($F_{(3,32)} = 1.27$, $P > 0.05$; Fig. 2B) or the number of closed arm entries ($F_{(3,32)} = 0.47$, $P > 0.05$; Fig. 2C).

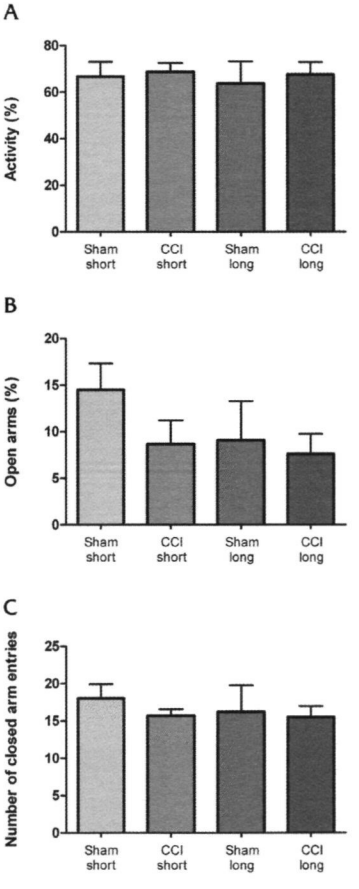


Figure 2. *CCI does not change anxiety and depressive-like behaviour in SD rats. (A) CCI rats and shams, both tested 20 days ("short" groups) and 55 days ("long" groups) after surgery show similar percentages of active behaviour in the forced swim test. (B) The percentage time spent on the open arms of the elevated plus maze (EPM), as a measure for anxiety, does not differ between groups. (C) Rats of the four groups have similar numbers of closed arm entries of the EPM as a measure for total activity. n = 18 for short groups, n = 6 for long groups.*

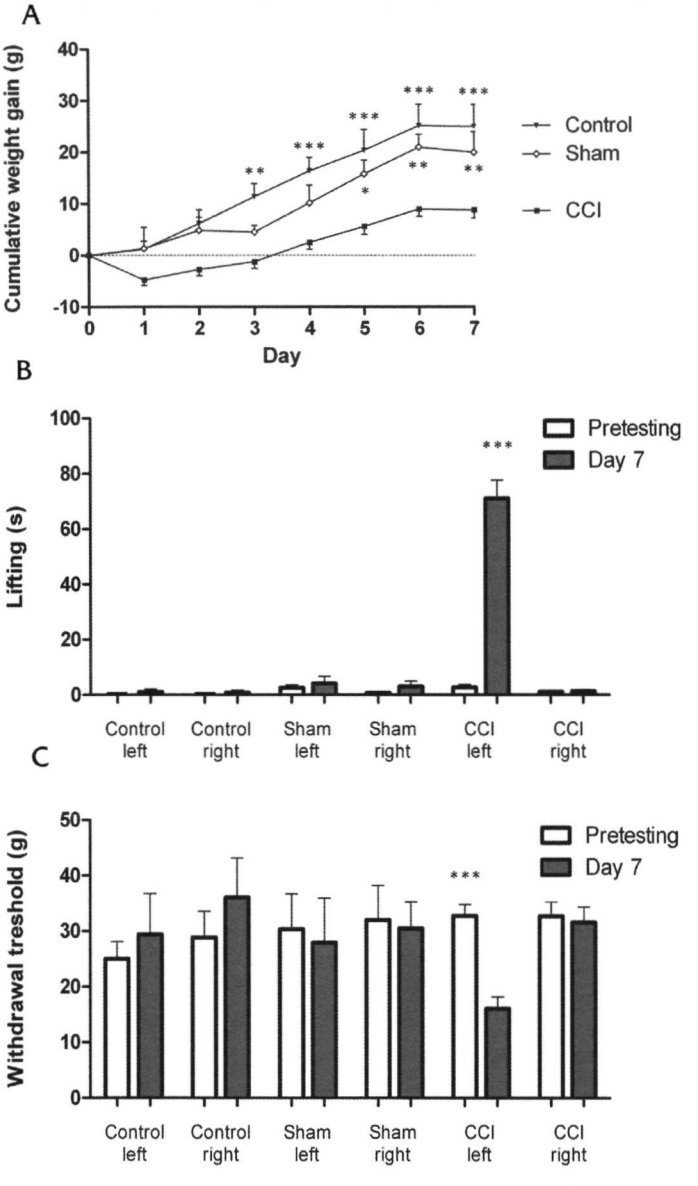


Figure 3. *Validation of CCI paradigm in the SD rat. (A) Cumulative weight gain of controls, shams and CCI rats. Compared to controls and shams, cumulative body weight gain of CCI rats is negative during the first three days after surgery, and remains lower during the first week. (B) CCI causes cold allodynia, as seen from the total time the injured left hind paw is lifted on a cold plate over a 5 min period. (C) During the first week after surgery, CCI rats show mechanical hyperalgesia in their injured left hind paw. n = 6 rats per group for controls and shams; n = 36 rats per group for CCI rats. * P < 0.05, ** P < 0.01, and *** P < 0.001.*

Experiment 2 - Drug effects

Body weight

Prior to surgery, there was no difference in cumulative body weight between control rats (331.2 ± 11.4 g; n = 6), shams (341.3 ± 8.6 g; n = 6) and CCI rats (340.7 ± 2.0 g; n = 36). Where controls and shams gained weight following sham-surgery, CCI rats experienced a transient drop in cumulative body weight gain following CCI-surgery at day 1 (-4.8 ± 1.0 g). During the first week, the average cumulative weight gain of controls and shams was higher than that of CCI rats ($F_{(2,45)} = 52.77$, $P < 0.001$; Fig. 3A).

From day 7 till day 21 after surgery, CCI rats received medication twice a day. ANOVA indicated an overall effect of medication on weight gain during this 2-week period ($F_{(5,30)} = 54.51$, $P < 0.001$; Fig. 4). *Post-hoc* analysis showed that compared to CCI rats injected with saline, amitriptyline-treated animals gained slightly less body weight ($P < 0.001$), whereas an inhibitory morphine effect was only seen at the end of the 2-week period ($P < 0.001$). Minocycline, gabapentin and paroxetine had no effect on body weight gain.

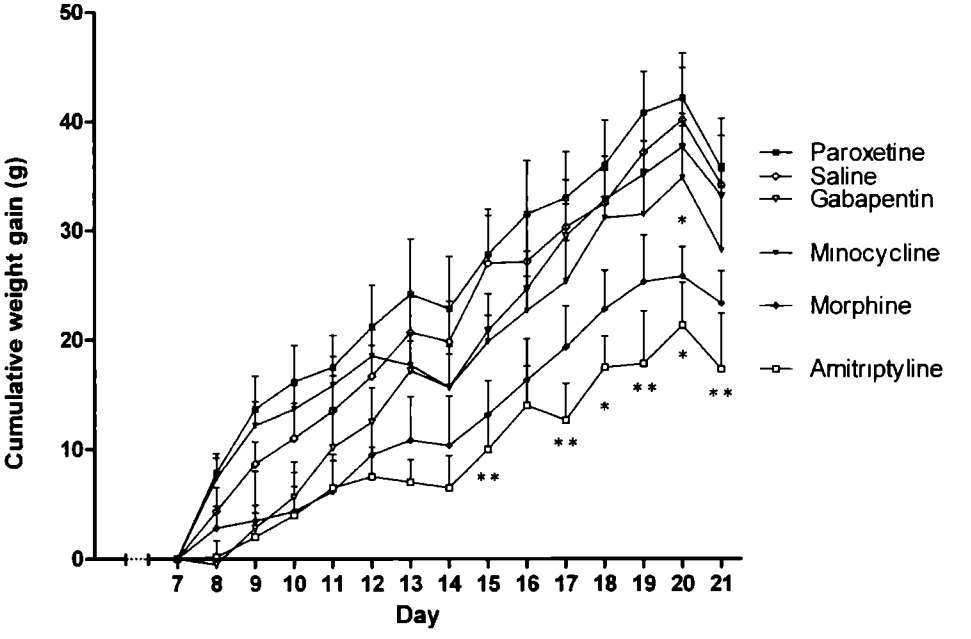


Figure 4. *Cumulative body weight gain of CCI rats following drug injection. Compared to saline-injected rats, morphine- and amitriptyline-injected rats gain less weight. n = 6 rats per group. * P < 0.05 and ** P < 0.01 compared to saline-injected rats.*

Allodynia and hyperalgesia

Following CCI-surgery, rats developed marked cold allodynia in the operated, left hind paw, with a lifting time of 71.1 ± 6.6 s at day 7, whereas controls and shams did not show any lifting throughout the experiment ($F_{(2,45)} = 18.30$, $P < 0.001$; Fig. 3B). Drug treatments had no effect on lifting time in CCI rats except for minocycline ($F_{(5,30)} = 3.74$, $P < 0.01$; Fig. 5A), which markedly reduced lifting time at day 14, by 38.5%, whereas CCI saline-injected controls revealed an increase in lifting time at day 14 by 48.5% compared to day 7 ($P < 0.05$; Fig. 5A).

Like in experiment 1, there was a clear and strong decrease in withdrawal threshold in response to mechanical stimulation in CCI rats at day 7, by 42.8% compared to shams ($P < 0.001$; Fig. 3C). None of the drugs significantly changed this threshold ($F_{(5,30)} = 0.31$, $P = 0.91$; Fig. 5B). Since experiment 1 had not shown any effect of CCI as to thermal allodynia or mechanical hyperalgesia in the right hind paw, in experiment 2 data were only collected for the left hind paw.

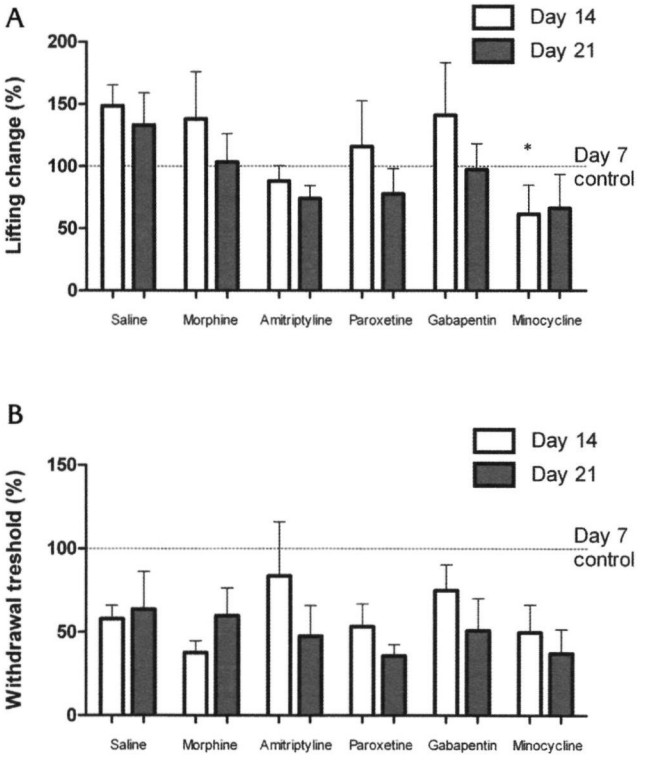


Figure 5. *Effect of drugs on thermal allodynia and mechanical hyperalgesia in CCI rats. (A) Drug treatment decreases lifting on the cold plate in all groups, but only minocycline causes a significant decrease at day 14. (B) Drug treatment does not significantly affect the left hind paw withdrawal threshold to Von Frey stimulation. n = 6 rats per group. * P < 0.05 compared to saline-injected rats.*

Anxiety and depressive-like behaviour

As in experiment 1, in the forced swim test all rats showed more active than inactive behaviour ($F_{(5,30)}$ = 119.45, P < 0.001), but no effects of drug treatment on CCI rats were detected ($F_{(5,30)}$ = 0.00, P = 1.00; Fig. 6A).

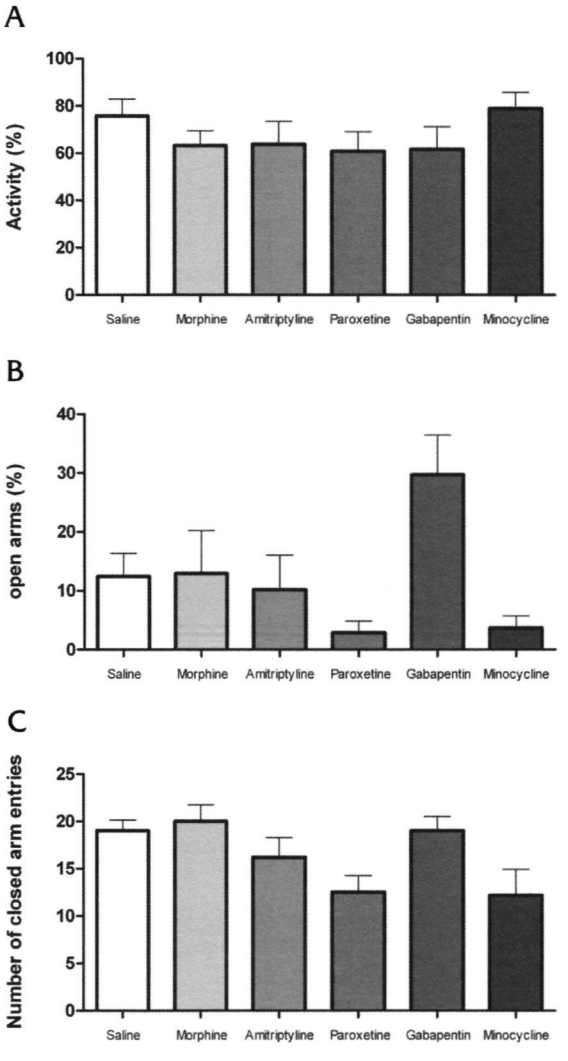


Figure 6. *Behavioural testing following drug treatment. (A) The forced swim test does not reveal any difference in activity between drug- and saline-injected groups. (B) Compared to saline controls, none of the CCI groups demonstrates altered anxiety behaviour or (C) altered general activity on the elevated plus maze. n = 6 rats per group.*

As to CCI rats on the elevated plus maze, there was an overall effect of treatment between groups in the percentage time rats spent on the open arms ($F_{(5,30)} = 3.33$, $P < 0.01$). *Post-hoc* analysis revealed that this effect was due to a difference between the high percentage of the gabapentin group on the one hand, and the low percentages of the paroxetine and minocycline groups on the other ($P < 0.01$; Fig. 6B). However, no difference was found for any drug treatment compared to the saline-injected CCI control group. General activity of the CCI rats, indicated by the number of closed arm entries, did not differ between any of the groups (Fig. 6C).

Discussion

General remarks

This study on the SD rat had three aims: 1) to determine the long-term effect of CCI-surgery on thermal allodynia and mechanical hyperalgesia, 2) to assess whether CCI leads to the development of anxiety and depressive-like behaviour, and 3) to conclude whether drugs used in human neuropathic pain treatment are also effective to attenuate neuropathic pain-associated behaviour in SD CCI rats. Our results show that CCI does result in the development of chronic (up to six weeks) allodynia and hyperalgesia, but that it does not induce anxiety and depressive-like behaviour. Furthermore, four out of the five drugs tested in this study failed to attenuate CCI-induced allodynia or hyperalgesia, or to influence anxiety and depressive-like behaviour. Interestingly, the tetracyclic drug, minocycline, clearly attenuated CCI-induced allodynia. These conclusions and their consequences for our insight into neuropathic pain-associated behaviour and its treatment will be discussed below.

The SD CCI rat model

At the start of each experiment, we assessed body weight changes in the respective experimental groups to obtain insight into the physiological condition of the animals. In general, rats in all groups gained body weight throughout the experiments. CCI rats revealed a specific effect of surgery during the first week, as they initially lost weight and only later caught up with the controls. This observation, in line with our previous results (Rouwette et al., 2012), indicates that CCI-surgery may exert an initial adverse health effect, and/or a changed energy flow that is more directed to wound healing. At the same time, this initial weight loss was an indication that CCI-surgery had been successful. Furthermore, our study confirms previous findings that CCI rats have a lower cumulative weight gain during the first weeks after surgery (Ulrich-Lai et al., 2006), but we did not observe an effect of CCI on long-term weight gain. Also, we here confirm our earlier conclusion that CCI-surgery of the SD rat has clear effects in the cold plate (Rouwette et al., 2012) and Von Frey tests, indicating,

respectively, that CCI induces marked thermal allodynia and mechanical hyperalgesia, main symptoms of human neuropathy. We also show that allodynia and hyperalgesia lasted up to six weeks, after which no differences were observed between groups. The same pattern was seen in other studies, where pain sensitivity following CCI-surgery peaked after about three weeks and then returned to pre-operative values (Bennett and Xie, 1988; Roeska et al., 2009; Ulrich-Lai et al., 2006). Other models for neuropathic pain, such as rats with (partial) SNL, show similar responses to spontaneous and evoked pain (Kim et al., 1997). Taken these data together, we conclude that the SD CCI rat is an animal model with a high face validity to study underlying mechanisms of allodynia and hyperalgesia, symptoms seen in human neuropathy.

However, as to the anxiety and depressive-like behaviour assumed to be associated with human neuropathy (Argoff, 2007; Hunt and Mantyh, 2001; Manas et al., 2011), the situation for the SD rat seems to be different. In contrast to other rat models for peripheral mononeuropathy (CCI and SNL Wistar rats), we did not observe any effect of CCI-surgery on SD rats in the forced swim and elevated plus maze tests. This means that, at least under our experimental conditions, in the SD rat CCI-induced neuropathy is not concomitant with anxiety and depressive-like behaviour. This finding on SD CCI rats is in accordance with the observations by Kontinen et al. (1999) using SNL SD rats, showing that SNL does induce allodynia and hyperalgesia but not anxiety and depressive-like behaviour. Therefore, we conclude that the SD CCI rat has a high face validity to study specifically the underlying mechanisms of human neuropathy-related allodynia and hyperalgesia, whereas the animal's face validity with respect to human neuropathy-associated mood disorders probably is low.

Drug effects

In general, we found a limited effectiveness of chronic drug treatment on body weight, allodynia, hyperalgesia, anxiety and depressive-like behaviour. However, CCI rats injected with amitriptyline showed a clearly lower body weight gain than saline-injected CCI controls. This indicates a decreased well-being of rats treated with this drug. Indeed, amitriptyline-injected rats revealed more piloerection, worse skin condition, less locomotor activity, and more signs of inflammation at the site of injection than rats from all other groups (T.P.H. Rouwette, unpubl. res.). Estebe and Myers (2004) reported that amitriptyline has central neurotoxic effects, leading to loss of motor function and increased flinching and biting/licking behaviour. Therefore, these authors strongly recommended not using amitriptyline as an anaesthetic agent. Such neurotoxicity might explain the lower body weight gain and decreased well-being of our amitriptyline-treated rats.

Furthermore, minocycline-injected CCI rats revealed less thermal allodynia than saline-injected CCI rats. The mechanism by which this tetracyclic drug exerts this apparently

therapeutic effect is speculative. Minocycline is known to have pleiotropic effects, acting as an anti-inflammatory agent on various organs such as the respiratory system and intestine, but also has inhibitory effects on microglia in the central nervous system (Amin et al., 1996; Yrjanheikki et al., 1998). Whether the reducing effect of minocycline on allodynia is due to an anti-inflammatory action and/or to a central action (possibly on microglia) remains to be determined.

With regard to morphine, gabapentin and amitriptyline, it may seem surprising that drugs well-known for their analgesic action (Attal et al., 2010; Dworkin et al., 2007b) did not exert an analgesic effect on our SD CCI rat. Morphine and gabapentin attenuate pain symptoms in Wistar rats (Roeska et al., 2008) and amitriptyline reduces mechanical allodynia in mice (Jesse et al., 2010). However, these studies involved acute drug application with higher doses and intraperitoneal or oral instead of subcutaneous (as in our study) application. Hu et al. (2010) found that chronic daily administration of amitriptyline ameliorated mechanical allodynia and depressive-related behaviour, but their study involved the use of SNL to induce neuropathic pain-like behaviour whereas we used the CCI paradigm.

Taken together, the absence of drug effects in our study would seem to indicate that the SD CCI rat has low predictive validity for the drugs we tested and are often used in the clinical practice to treat neuropathic pain. However, we choose drug concentrations on the basis of the available literature (Brunton et al., 2010) and of our empirical data on treatment of human neuropathic pain (K.C.P. Vissers, unpubl. res.). Possibly, protocols involving other concentrations and durations of drug administration will yield data that fit better the described clinical effectiveness of these drugs.

Conclusions

We have provided evidence that the SD rat, like the Wistar rat, experiences clearly increased pain sensitivity upon CCI-surgery, but, in contrast, does not show changes in mood as a result of experimentally-induced neuropathy. This indicates that the SD CCI rat is a good model for elucidating mechanisms of neuropathic pain induction but has low face validity for studying co-morbid anxiety and/or depression as revealed by neuropathy patients. Furthermore, although the predictive validity of the SD CCI model in testing the therapeutical potential for neuropathic pain of amitriptyline, gabapentin, morphine and paroxetine is doubtful, the shown effectiveness of minocycline to attenuate CCI-induced allodynia is intriguing and the underlying mechanisms of this action deserve further exploration.

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Chapter 3

Experimental neuropathy increases limbic forebrain corticotropin-releasing factor

Tom P.H. Rouwette, Pascal Vanelderen, Marjolein de Reus, Nicola F.M. Olde Loohuis, Janneke Giele, Jan van Egmond, Wim J.J.M. Scheenen, Gert Jan Scheffer, Eric W. Roubos, Kris C.P. Vissers, Tamás L. Kozicz

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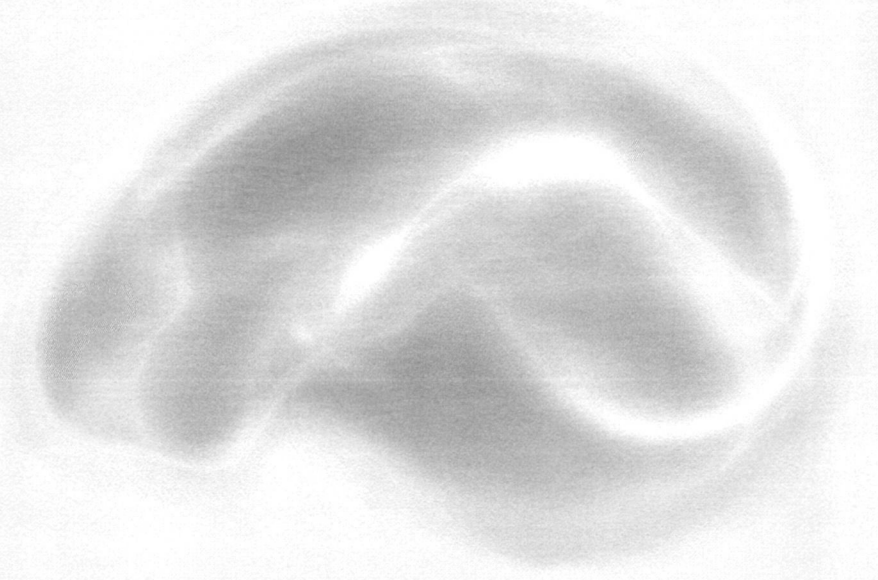

Abstract

Neuropathic pain in humans is often accompanied by stress, anxiety and depression. Although there is evidence for involvement of corticotropin-releasing factor (CRF), the detailed neuronal basis of these pain-related mood alterations is unknown. This rodent study shows that peripheral mononeuropathy was accompanied by changes in limbic forebrain CRF but did not lead to changes in the functioning of the hypothalamo-pituitary-adrenal (HPA-) axis and the midbrain Edinger-Westphal centrally projecting neuron population (EWcp), which play main roles in the organism's response to acute pain. Twenty-four days after chronic constriction injury (CCI) of the rat sciatic nerve, the oval bed nucleus of the stria terminalis (BSTov) contained substantially more CRF mRNA as did the central amygdala (CeA), which, in addition, possessed more CRF peptide. In contrast, CRF mRNA and CRF contents of the hypothalamic paraventricular nucleus (PVN) were unaffected by CCI. Similarly, EWcp neurons, producing the CRF family member urocortin 1 (Ucn1) and constitutively activated by various stressors including acute pain, did not show an effect of CCI on Ucn1 mRNA or Ucn1. Also, the immediate early gene products cFos and deltaFosB in the EWcp were unaffected by CCI. These results indicate that neuropathic pain does not act via the HPA-axis or the EWcp, but includes a main action of CRF in the limbic system, which is in clear contrast to stressors like acute and chronic pain, which primarily act on the PVN and the EWcp.

Introduction

Corticotropin-releasing factor (CRF) is essential in stress adaptation, evoking physiological stress responses via the hypothalamic paraventricular nucleus (PVN; *e.g.* de Kloet et al., 2005) and behavioural responses via extra-hypothalamic centres, especially the limbic oval bed nucleus of the stria terminalis (BSTov) and central amygdala (CeA; Deyama et al., 2007; Ji and Neugebauer, 2008). Chronic pain often disturbs hypothalamo-pituitary-adrenal (HPA-) axis activity, and is highly co-morbid with anxiety and depression (Blackburn-Munro and Blackburn-Munro, 2001), mood disorders frequently associated with dysfunctioning of limbic CRF (Arborelius et al., 1999; Davis and Shi, 1999; Regev et al., 2011). Another extra-hypothalamic centre implicated in regulating stress responses and the pathogenesis of anxiety and depression (Moreau et al., 1997) is the Edinger-Westphal centrally projecting neuron population (EWcp), located in the rostroventral periaqueductal grey (PAG) and the brain's richest source of the CRF neuropeptide family member urocortin 1 (Ucn1; Kozicz, 2007; Kozicz et al., 2011). Acute pain stress up-regulates Ucn1 mRNA and Ucn1 peptide in the EWcp (Rouwette et al., 2011), whereas chronic stress evokes a habituating response of Ucn1 mRNA (Korosi et al., 2005). Furthermore, Ucn1 in the EWcp co-localises with cholecystokinin (May et al., 2008), which, in the PAG, plays a central role in anxiety-induced and peripheral neuropathy-associated hyperalgesia (Lovick, 2008). Therefore, the EWcp might be another target for pain signals to interfere with mood. To support our hypothesis that chronic pain exerts its negative effects on mood via the CRF-producing limbic nuclei and/or Ucn1-producing EWcp, in the present study we have tested if peripheral mononeuropathy changes the activity of these brain areas.

In the adult male Sprague-Dawley rat, neuropathic pain-like behaviour can be induced by loosely constricting the sciatic nerve (Bennett and Xie, 1988). This chronic constriction injury (CCI) is a well-established experimental paradigm for inducing neuropathic pain-like behaviour in rodents, evoking key symptoms of human neuropathic pain, *i.e.*, allodynia and hyperalgesia. It was previously shown that CCI does not affect indices of basal or restraint stress-induced HPA-axis activity, nor does it change CRF mRNA expression in the PVN. It does induce, however, CRF mRNA expression in the CeA (Bomholt et al., 2005; Ulrich-Lai et al., 2006). These results indicate that neuropathic pain is associated with changed neuronal activity in the limbic system but not in the PVN.

We have investigated the effect of CCI on different aspects of the activity of CRF neurons in the PVN, BSTov and CeA, and of Ucn1 neurons in the EWcp. CRF and Ucn1 contents were determined by quantitative immunohistochemistry, whereas the capacity to synthesise these neuropeptides was assessed by measuring their respective neuronal mRNA contents with quantitative *in situ* hybridisation. In the EWcp, cFos and deltaFosB contents

were measured to determine the degree of immediate early gene expression. The results indicate that peripheral neuropathy in the rat is associated with changes in neuronal activity in CRF-producing brain areas primarily involved in control of mood (CeA and BSTov), whereas areas regulating fast physiological processes (PVN and EWcp) do not show such activity changes.

Materials & Methods

Animals

Male Sprague-Dawley rats (Harlan Animal Laboratories, Gannat, France), weighing 200-250 g, were paired-housed in plastic cages (Makrolon type III; Tecniplast, Buguggiate, Italy), in a temperature- and humidity-controlled environment, on a 12/12 h light-dark cycle (lights on: 6:30 AM, light intensity: 200 lux), with *ad libitum* access to tap water and soy-free rodent chow. Body weight was monitored on days 0-10, 14 and 21. Rats were acclimatised during one week before pre-operative testing and surgery. All measures were taken to minimise the number of animals used and their suffering, and the experiments were conducted following the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and had been approved by the Animal Ethics Committee of Radboud University Nijmegen.

Chronic constriction injury

CCI of the sciatic nerve was performed by surgery as described elsewhere (Bennett and Xie, 1988). Briefly, under isoflurane anaesthesia (induction 5%, maintenance 2.5%) combined with oxygen (30%) and nitrous oxide (65-67.5%), the sciatic nerve of the left hind paw was exposed at the mid-thigh level proximal to the sciatic trifurcation, by blunt incision of the biceps femoris muscle. Four chromic catgut 5-0 ligatures (Catgut, Markneukirchen, Germany) were tied loosely around the nerve, about 1 mm apart. The overlying muscle was sutured with Vicryl 5-0 (Johnson & Johnson, St. Stevens-Voluwe, Belgium) and the skin closed with autoclips (7.5 x 1.75 mm; Rudolf Medizintechnik, Fridingen, Germany). CCI rats were tested for thermal allodynia one day before CCI and on days 7, 14 and 21 after CCI, on a cold plate apparatus (Ugo Basile, Comerio, Italy) consisting of an acrylic cylinder (diameter 19 cm, height 31 cm) fixed to a stainless steel plate which temperature was maintained at 4 °C. Rats were placed on the cold plate and the durations of lifting of the left as well the right hind paw measured with a digital hand chronometer during a 5 min period. They were considered neuropathic if the total lifting time of the left operated hind paw exceeded 20 s. In addition to 6 neuropathic CCI rats, 6 sham-operated rats were included in the study. These rats underwent the same surgery, where the sciatic nerve was exposed but not ligated.

Tissue fixation and sectioning

At day 24, three days after the last cold plate testing, rats were perfused transcardially under deep isoflurane anaesthesia, with 100 ml 0.1 M sodium phosphate-buffered saline (PBS; pH 7.4) for 10 min, followed by 200 ml 4% ice-cold paraformaldehyde in PBS, for 20 min. Then, rats were decapitated, and their brains removed, post-fixed in fresh paraformaldehyde, for 16 h, transferred into 30% sucrose in PBS, for 48 h, and frozen on dry ice. Coronal 25 µm sections, 125 µm interspaced, were made through the medial part of a brain nucleus with a freezing microtome (Microm, Walldorf, Germany), and stored in sterile antifreeze solution (0.05 M PBS, 30% ethylene glycol, 20% glycerol) at -20 °C, till further use.

In situ hybridisation

CRF mRNA and Ucn1 mRNA were detected using antisense cRNA probes transcribed from linearised CRF cDNA and Ucn1 cDNA, respectively (probes kindly provided by Dr. W.W. Vale, The Salk Institute, San Diego, CA, USA). Sense probes served as controls (no hybridisation signal was seen). All probes were labelled with digoxigenin (DIG)-11-UTP (Roche Molecular Biochemicals, Basel, Switzerland) and hybridisations were carried out at 20 °C unless stated otherwise. Sections were rinsed in PBS, for 4 x 15 min, and fixed in the paraformaldehyde fixative, at 4 °C, for 30 min (Ucn1 mRNA) or for 18 h (CRF mRNA). Then, they were rinsed 4 x 7 min in PBS followed by pre-incubation in proteinase K medium containing 0.1 M Tris/HCl, 0.05 M EDTA and 10 µg/ml proteinase K (Invitrogen, Carlsbad, CA, USA), for 10 min at 37 °C. After rinsing in autoclaved MQ-water, acetylation was performed with 0.25% acetic acid anhydride in 0.1 M tri-ethanolamine buffer (pH 8.0), for 10 min, followed by 2 rinses in 2x concentrated standard saline citrate buffer (2xSSC; pH 7.0), for 5 min. Hybridisation mixture (50% deionised formamide, 0.3 M NaCl, 0.001 M EDTA, Denhardt's solution, 10% dextran sulphate) together with 0.5 mg/ml tRNA (Roche) and the mRNA DIG-probe (*ca.* 40 ng/ml), was placed in a water bath, for 5 min at 80 °C, and then on ice for another 5 min. Sections were incubated in hybridisation solution, for 18 h at 58 °C, rinsed 4 x 7 min with 4xSSC, incubated for 30 min at 37 °C in preheated RNAse medium (0.5 M NaCl, 0.01 M Tris/HCl, 1 mM EDTA; pH 8.0) containing 0.01 mg/ml RNAse A (Roche) that had been added just before the start of incubation, and stringently rinsed in decreasing SSC concentrations (2x, 1x, 0.5x). Next, the sections were incubated in 0.1xSSC, for 30 min at 58 °C. To detect DIG-label, the alkaline phosphatase method with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate and toluidine salt (NBT/BCIP; Roche) as a substrate, was used. Briefly, after rinsing 4 x 5 min with buffer A (0.1 M Tris/HCl, 0.15 M NaCl; pH 7.5), sections were pre-incubated in buffer A containing 0.5% blocking agent (Roche), for 1 h, followed by 3 h incubation with sheep anti-DIG-AP (Roche; 1:5,000)

in buffer A containing 0.5% blocking agent. Subsequently, sections were rinsed 4 x 5 min in buffer A, followed by 2 x 5 min rinsing in buffer B (0.1 M Tris/HCl, 0.15 M NaCl, 0.05 M $MgCl_2$; pH 9.5). After 6 h incubation in NBT/BCIP mixture consisting of 10 ml buffer B, 2.4 mg levamisole (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 175 µl NBT/BCIP (Roche) in a light-tight box, the reaction was stopped by placing the sections in buffer C (0.1 M Tris/HCl, 0.01 M EDTA; pH 8.0). After rinsing for 2 x 5 min, sections were mounted on gelatine-coated glass slides, dried for 16 h at 37 °C, dehydrated, cleared in xylene, cover-slipped with Entellan (Merck, Darmstadt, Germany) and studied with a DMRBE microscope connected to a DC 500 digital camera (Leica Microsystems, Wetzlar, Germany). For each probe, all sections were processed in the same assay to exclude inter-experimental error.

Immunohistochemistry

For immunolabelling of CRF, cFos and deltaFosB, sections were first rinsed 4 x 15 min in 0.1 M PBS, and then treated with 0.5% Triton X-100 (Sigma-Aldrich) in 0.1 M PBS, for 30 min. After three additional 15 min rinses in PBS, they were incubated for 45 min in a quenching mixture of PBS and 1% H_2O_2, rinsed 3 x 15 min in PBS and incubated in 2% normal goat serum (NGS; Jackson Immunoresearch Labs, West Grove, PA, USA) in PBS, for 30 min. Then, sections were incubated in polyclonal (rabbit) CRF antiserum (1:2,000; kindly provided by Dr. W.W. Vale), polyclonal (rabbit) cFos antiserum (1:4,000; sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or polyclonal (rabbit) deltaFosB antiserum (1:4,000; sc-48, Santa Cruz) in 2% NGS, for 18 h, followed by 3 x 15 min rinses in PBS, and incubated in biotinylated goat anti-rabbit serum (Vector ABC Elite Kit; PK-6101; Vector Labs, Burlingame, CA, USA), for 1 h. Then, after 3 x 15 min rinses in PBS, they were incubated in ABC reagent supplied with the ABC Elite Kits (Vector Labs), for 1 h. Immunostaining was visualised with 10 mg diaminobenzidine (DAB; D5637; Sigma-Aldrich) in 50 ml Tris buffer (pH 7.6), for 10 min. Sections were mounted on gelatine-coated glass slides, embedded in Entellan, and studied with the DMRBE microscope.

For immunofluorescent labelling of Ucn1, sections were rinsed 4 x 15 min in PBS, and incubated in 0.5% Triton X-100 in PBS, for 30 min, in 2% normal donkey serum in PBS, for 30 min, and in polyclonal goat anti-Ucn1 (R-20; sc-1825; Santa Cruz) at 1:250 in 2% normal donkey serum, for 18 h. Then, incubation followed in secondary Cy^2-conjugated donkey anti-rabbit IgG (1:100; Jackson Immunoresearch Labs) in PBS, for 2.5 h. Following several rinses in PBS, sections were mounted on gelatine-coated glass slides, cover-slipped with FluorSave (Merck) and studied with a TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems).

Image analysis

The PVN, CeA, BSTov and EWcp were identified in the sections on the basis of the co-ordinates given by Paxinos and Watson (1997). Per rat, quantifications were made in 3 sections of the PVN and BSTov and in 5 sections of the CeA and EWcp, of which digital images were taken with the DMRBE microscope at 1,200 x 1,600 dpi, for *in situ* hybridisation and NiDAB immunohistochemistry, or with the TCS SP2 AOBS confocal laser scanning microscope at 1,024 x 1,024 dpi, for fluorescent immunohistochemistry. Images were analysed with Scion Image software (version 3.0b; NIH, Bethesda, MD, USA), according to a double-blind protocol, studying for each brain area two parameters: 1) the number of stained neurons and 2) the strength of the staining of a neuron (specific signal density, SSD), which was averaged over 10 randomly taken neurons and corrected for background density outside the brain area in the same section. To increase statistical power, no distinction was made between left and right parts of a given brain nucleus because we have never found indications for lateralisation with respect to any of the four brain areas or their peptide (mRNA) contents (Gaszner et al., 2004; T.L. Kozicz et al., unpubl. res.). Outcomes were averaged over the sections, providing for each parameter and brain centre one value per animal, which was used for statistical analysis of possible differences between the CCI and the sham rats.

Statistics

Body weights and behavioural parameters were tested by analysis of variance (ANOVA) while neuron measurements were analysed using Student's two-tailed t-test. All analyses were preceded by tests for normality (Shapiro and Wilk, 1965) and for homogeneity of variance (Bartlett's Chi-square test; Snedecor and Cochran, 1989), and performed with Microsoft Excel software and Statistica (StatSoft, Tulsa, OK; α=5%). Data are presented as means and standard error of the mean (SEM).

Results

Body weight and allodynia

Prior to surgery, there was no difference in cumulative body weight between CCI rats (310.7 $\pm$ 12.7 g; n = 6) and shams (293.5 $\pm$ 9.7 g; n = 6; $F_{(1,10)} = 1.2$, $P > 0.05$; Fig. 1A). However, 21 days post-surgery CCI rats had gained 39.3 $\pm$ 8.9 g, which was substantially less than the weight gain of shams (+62.5 $\pm$ 7.8 g; $F_{(1,10)} = 30.0$, $P < 0.001$). This difference in overall weight gain was due to a transient drop in body weight of the CCI rats during the first week post-surgery (-8.7 $\pm$ 2.0 g at day 1). During this period, sham rats had an average weight gain of 20.8 $\pm$ 3.6 g, whereas CCI rats had a slightly negative weight gain (-0.67 $\pm$ 2.1 g; $F_{(1,10)} = 44.3$, P

< 0.001). From day 7 on, CCI and sham rats did not differ in body weight gain (2.9 ± 0.5 *vs.* 3.0 ± 0.5 g/day, respectively; $F_{(1,10)} = 0.08$, $P = 0.78$).

Following surgery, CCI-operated rats developed marked cold allodynia in the operated paw, with lifting times of 123.3 ± 24.4, 129.7 ± 17.7 and 95.8 ± 15.4 s for 7, 14 and 21 days post-surgery, respectively. The contralateral, non-operated hind paw did not show any lifting (Fig. 1B). In contrast, shams hardly showed lifting, neither in the ipsilateral (0.5 ± 0.3, 0.2 ± 0.2 and 0.3 ± 0.3 s) nor in the contralateral (0.0 ± 0.0, 0.3 ± 0.3 and 0.2 ± 0.2 s) paw (treatment effect: $F_{(1,10)} = 67.1$, $P < 0.001$).

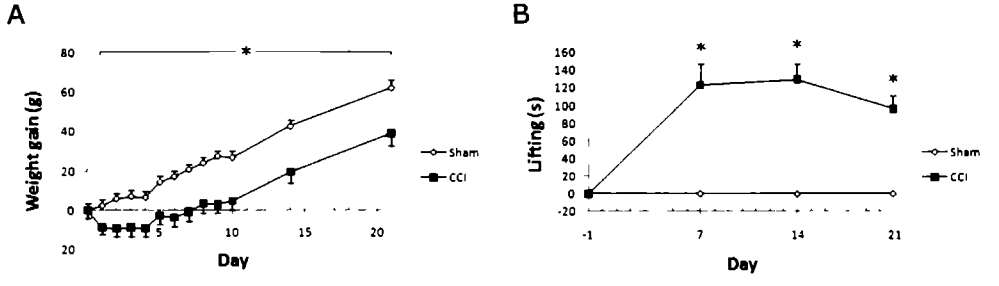

Figure 1. *CCI reduces cumulative body weight gain and induces thermal allodynia. (A) Compared to shams (open symbols), CCI rats (solid symbols) show negative body weight gain during the first days after surgery. From day 7 on, however, weight gain of the CCI rats is similar to that of sham-operated rats. (B) CCI causes cold allodynia, as seen from the total time the injured ipsilateral hind paw is lifted on a cold plate over a 5 min period. Thermal allodynia appears within the first week after surgery, and persists during the next two weeks. The untreated contralateral paw does not show any lifting on the cold plate, neither do both hind paws of sham-operated rats. *P < 0.01 compared to shams.*

In situ hybridisation and immunohistochemistry

In the CeA, BSTov and PVN individual neurons revealed clear, positive *in situ* hybridisation signals for CRF mRNA (BSTov: see Fig. 2A) and strong CRF staining with immunohistochemistry (CeA: see Fig. 2B). Similarly, in the EWcp, neurons were strongly stained for both Ucn1 mRNA and Ucn1 peptide. Immunostaining of cFos (Fig. 2C) and deltaFosB (Fig. 2D) was obvious in the nuclei of EWcp neurons. No signals were seen when using sense probes (*in situ* hybridisation), or when primary antisera were preabsorbed with their respective synthetic peptides or when the first antiserum was omitted (immunohistochemistry), all indicating the high specificity of the reactions.

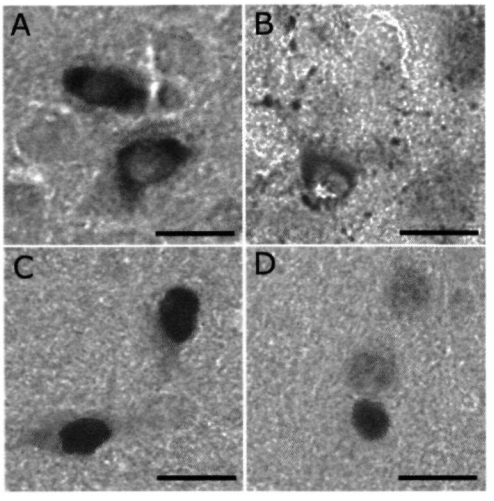

Figure 2. *CRF mRNA hybridisation of neurons of the oval bed nucleus of the stria terminalis (BSTov; A), CRF-immunoreactive neuronal cell bodies in the central amygdala (CeA; B), and nuclei of neurons immunoreactive to cFos (C) and deltaFosB (D), in the Edinger-Westphal centrally projecting nucleus. Scale bars = 25 μm.*

CRF mRNA and CRF in the PVN, BSTov and CeA

In the PVN, CRF mRNA expression (Fig. 3A, B) and CRF-immunoreactivity (Fig. 3C, D) occurred throughout the parvocellular area in both sham- and CCI rats. No difference in CRF mRNA expression or CRF-immunoreactivity (Fig. 3E, F) was seen between the groups, either as to the number of stained neurons (*in situ* hybridisation: P = 0.47, immunohistochemistry: P = 0.42) or as to the SSD of these neurons (*in situ* hybridisation: P = 0.27, immunohistochemistry: P = 0.26).

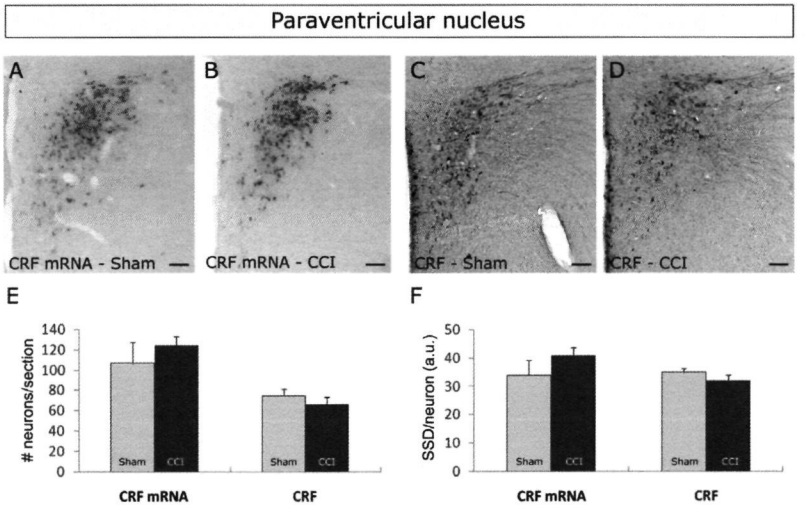

Figure 3. *CCI-surgery does not affect CRF mRNA expression (A, B) and CRF-immunoreactivity (C, D) in the PVN as to the number of positive neurons per section (E) and their SSD (F). n = 6 per group. Scale bars = 100 μm.*

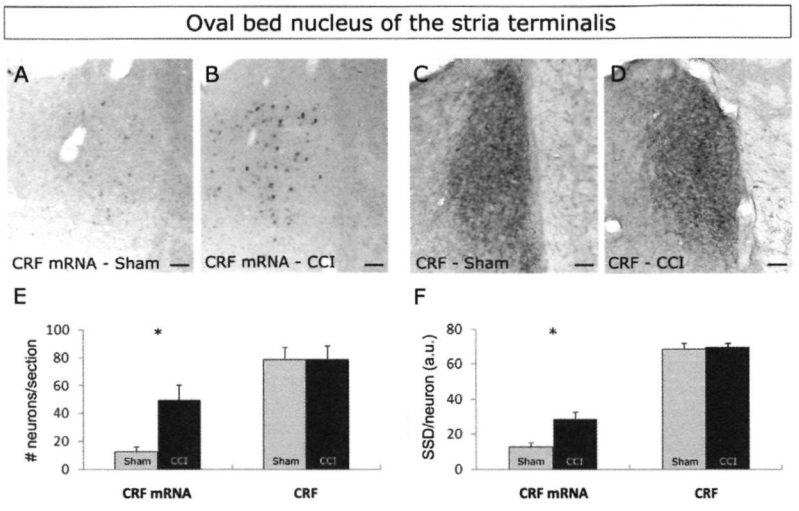

Figure 4. *CCI-surgery evokes higher CRF mRNA expression (A, B), but not CRF-immunoreactivity (C, D) in the BSTov, evident from a higher number of CRF mRNA-positive neurons per section (E) and higher SSD per neuron (F). CCI does not change the number of CRF-positive neurons per section (E) or neuronal SSD (F). n = 6 per group, * P < 0.05. Scale bars = 100 μm.*

In the BSTov, the number of CRF mRNA-positive neurons was much higher (4 times) in CCI rats than in shams ($P < 0.02$; Fig. 4A, B, E), whereas the SSD of the hybridisation signal per neuron was more than twice as high ($P < 0.02$; Fig. 4C, D, F). No effect of CCI-surgery was detected on the number of CRF-positive neurons in the BSTov ($P = 0.99$; Fig. 4E) or on the degree of the CRF-staining (SSD: $P = 0.76$; Fig. 4F).

In the CeA, CCI rats showed dramatically more (9 times) CRF mRNA-positive neurons than shams ($P < 0.001$; Fig. 5A, B, E). The degree of hybridisation per neuron, as expressed by the SSD of the *in situ* hybridisation staining, tended to be larger in CCI rats than in shams, but this difference was not statistically significant, probably due to inter-animal variability ($P = 0.09$; Fig. 5F). As to CRF-immunoreactivity, CCI rats showed 54% more positively stained neurons than shams ($P < 0.01$; Fig. 5C, D, E) while the SSD did not reveal a difference ($P = 0.93$; Fig. 5F).

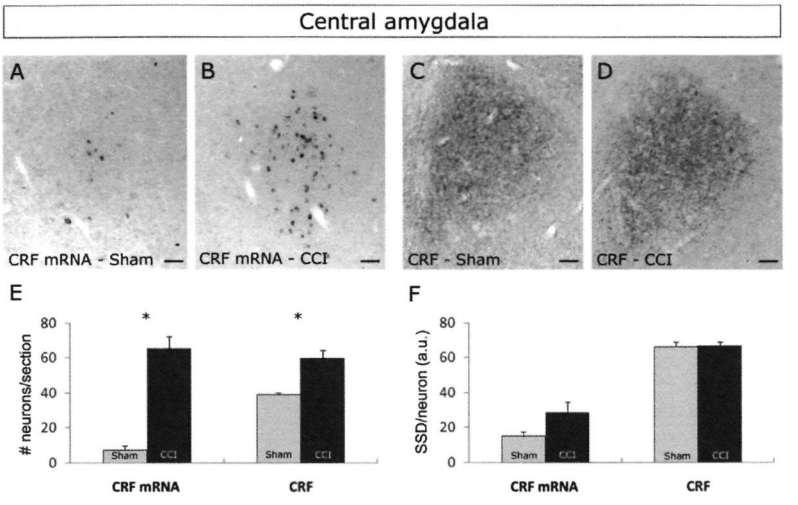

Figure 5. *CCI-surgery increases CRF mRNA expression (A, B) and CRF-immunoreactivity (C, D) in the CeA, as appears from higher numbers of stained neurons per section (E). Staining intensities of individual neurons (SSD) do not significantly differ (F). n = 6 per group, * P < 0.05. Scale bars = 100 μm.*

Ucn1 mRNA and Ucn1 in the EWcp

Ucn1 mRNA expression and Ucn1-immunoreactivity occurred throughout the EWcp in both shams and CCI rats (Fig. 6). No effect of CCI was seen on the number of Ucn1 mRNA-positive neurons (P = 0.06) or on their SSD (P = 0.60). Also, Ucn1-immunoreactivity, as determined by the number of positive neurons (P = 0.69) and their SSD (*P* = 0.80), did not differ between CCI rats and shams.

cFos and deltaFosB in the EWcp

Since this is the first study concerning the reaction of the EWcp to a neuropathic pain stimulus, and the Ucn1 mRNA and Ucn1 peptide contents in this nucleus appeared to be unaffected by CCI (see above), we decided to study the possible effect of peripheral mononeuropathy on the presence of immediate early gene products in this nucleus. No difference was observed between CCI rats and shams in the numbers of positively stained neurons for cFos (P = 0.73) or deltaFosB (P = 0.56; Fig. 7).

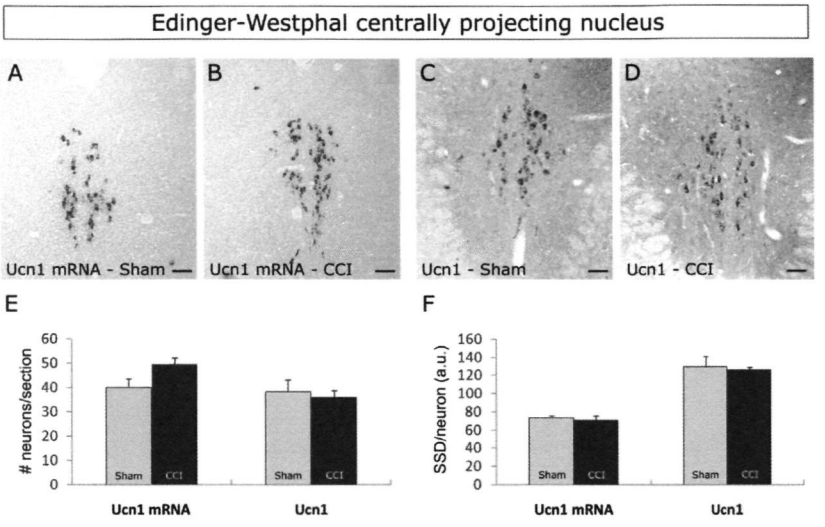

Figure 6. *CCI-surgery does not affect Ucn1 mRNA expression (A, B) and Ucn1-immunoreactivity (C, D) in the EWcp, as appears from similar numbers of positive neurons per section (E) and SSD (F). n = 6 per group. Scale bars = 100 μm.*

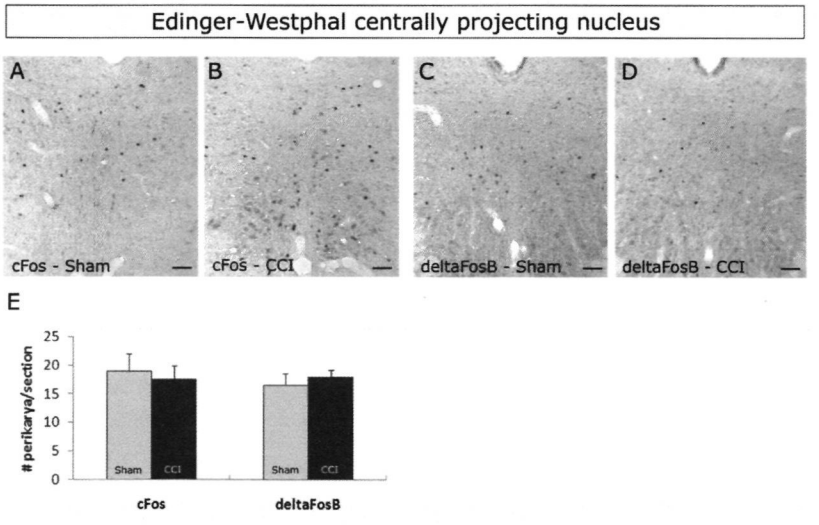

Figure 7. *CCI-surgery does not affect cFos- (A, B) and deltaFosB- (C, D) immunoreactivity in the EWcp, as no differences in the number of cFos- and deltaFosB-positive neurons per section are seen (E). n = 6 per group. Scale bars = 100 μm.*

Discussion

In the present study, we have tested our hypothesis that peripheral mononeuropathy alters different aspects of neuronal activity in the rat hypothalamic PVN, limbic BSTov and CeA, and midbrain EWcp, with particular attention to CRF and its related peptide Ucn1. The results do not show an effect of CCI, as a model for peripheral mononeuropathy, on the PVN and the EWcp, two brain nuclei clearly affected by acute pain, but do reveal clear CCI effects on the functioning of the CRF-producing CeA and BSTov. The latter nuclei are not only implicated in HPA-axis regulation but also in the control of anxiety and depression. Consequently, the observed activity changes of these limbic brain centres might be related to the mood changes known to be associated with chronic pain as induced by peripheral neuropathies. Below, these conclusions and considerations will be discussed in detail.

Nociceptive testing and weight gain

Nociceptive testing clearly revealed cold allodynia in the operated left hind paw of CCI rats, but not in the contralateral, non-operated paw. Sham-operated rats did not show any sign of cold allodynia. These data are in agreement with previous studies using CCI as a method for inducing neuropathic pain-like behaviour (Bennett and Xie, 1988; Kontinen et al., 2003), supporting the suitability of the CCI rat as a model for studying the effect of peripheral mononeuropathy on (brain) physiology. We also found that CCI rats decreased in body weight during the first few days following injury. This suggests a decrease in well-being of these rats, possibly as a result of a compromised autonomic/endocrine system. Apparently, this effect was only transient, since afterwards, and at the time we performed our neuronal measurements (three weeks after CCI-surgery), weight gains of shams and CCI rats were similar.

The PVN

Whereas chronic pain stress has been supposed to affect HPA-axis functioning (Blackburn-Munro and Blackburn-Munro, 2001), Bomholt et al. (2005), studying overall CRF mRNA contents by radioautographic *in situ* hybridisation, could not show an effect of CCI on the PVN. This suggests that CCI should not be considered as a general pain stressor, but rather as a specific, neuropathic pain stimulus that does not act via the PVN/HPA-axis. In the present study we have investigated in detail the possible effect of CCI at the level of individual PVN neurons, by measuring not only the overall amount of CRF mRNA in this nucleus, but also the amounts of CRF mRNA and CRF peptide in individual PVN neurons, using immunohistochemical signal detection methods. We clearly show that CCI does not influence CRF mRNA and CRF contents of individual neurons or the number of PVN

neurons that express these messenger molecules. These data strengthen the idea that CCI does not change the activity of the PVN, and help to clarify why previous studies failed to reveal an effect of CCI on plasma adrenocorticotropic hormone and corticosterone titres (Bomholt et al., 2005; Ulrich-Lai et al., 2006; Vissers et al., 2003). Moreover, they strongly indicate that CCI should not be considered a chronic stressor that acts via the HPA-axis, but exerts its chronic neuropathic effect via (a) target(s) located elsewhere in the brain.

Peripheral mononeuropathy increases limbic forebrain CRF

Neuropathic pain is strongly associated with changes in mood, both in humans (Argoff, 2007; Hunt and Mantyh, 2001; Manas et al., 2011) and rodents (Hu et al., 2009; Jesse et al., 2010; Roeska et al., 2008, 2009). CRF plays a prominent role in these disorders, because over-expression of this neuropeptide in the mouse BSTov and CeA affects depressive-like and anxiety behaviour, respectively (Regev et al., 2011), and peripheral mononeuropathy evokes an increase in the amount of CRF mRNA in the rat CeA (Ulrich-Lai et al., 2006). Here, we extend this evidence, as we not only show that CCI increases the amount of CRF mRNA in the CeA, but also reveal that this increase consists of two components: a rise in the number of mRNA-positive neurons and an increase in mRNA contents of individual neurons. These results indicate that CCI stimulates the capacity of the CeA to synthesise CRF, in two ways: recruiting more neurons to produce the peptide, and enhancing the rate of this production in the individual neuron.

Previously, no effect of CCI could be detected on the CRF mRNA amount in the BSTov (Ulrich-Lai et al., 2006). In the present study, however, measuring individual neurons with DIG-label *in situ* hybridisation, we observed in the BSTov, like in the CeA, a clear, CCI-induced increase in CRF mRNA, as appeared from both the number of CRF mRNA-containing neurons and the degree (SSD) of CRF mRNA hybridisation in individual neurons. Since CRF mRNA in the BSTov is higher in CCI rats than in shams, we propose that, as for the CeA, CCI increases the capacity of BSTov neurons to synthesise CRF. No effect of CCI on the amount of CRF in BSTov neurons was observed, and therefore we assume that CRF production and outward CRF transport (from the cell body to the axon terminals) are stimulated to the same degree, leaving the net amount of CRF in the cell body unchanged. A higher amount of axonal CRF might serve increased CRF secretion, but to date testing this assumption by measuring synaptic CRF release is technically impossible.

The CeA and BSTov are both involved in the regulation of emotional aspects of pain (Morano et al., 2008; Neugebauer et al., 2004) and also control negative affective components of visceral and somatic pain (Deyama et al., 2007; Han and Neugebauer, 2005). The present study clearly shows that CCI induces neuronal plasticity in the limbic forebrain as reflected by changes in *Crf* gene transcription and translation in the CeA and BSTov. Taken together, it

seems that CRF in the CeA and BSTov plays an important role in mediating neuropathy-induced changes in mood, an intriguing notion currently being tested in our laboratory.

In humans, there is increasing evidence from preclinical and clinical studies, that CRF plays a main role in the control of mood disorders like depression and anxiety (for review see Binder and Nemeroff, 2010). These disorders are often associated with increased activity of the CeA (Anand and Shekhar, 2003; Shekhar et al., 2005). As to the BSTov, several sub-regions contribute to the integration of neuroendocrine and autonomic signals, but their precise roles in this regulation are not clear (Ulrich-Lai and Herman, 2009). Since we show that peripheral neuropathy affects these centres in the CCI rat, this animal would seem to be a suitable model with a clear face validity to explore neuronal correlates of neuropathic pain-induced changes in mood and body homeostasis in humans.

The EWcp

Although CRF and Ucn1 belong to the same neuropeptide family, their mRNA expressions are strictly separated, with Ucn1 mRNA being present in the EWcp, which does not contain CRF, and absent from the CRF-producing PVN and limbic forebrain centres (Bittencourt et al., 1999). While the present study shows a clear up-regulation of CRF mRNA in the CeA and BSTov, no effect of CCI was seen on the numbers of EWcp neurons containing Ucn1 mRNA and Ucn1 peptide or on the amounts of this mRNA and peptide in individual EWcp neurons. Therefore, we conclude that CCI does not influence the neurons' capacity to produce and process Ucn1. To test whether CCI could have an effect on the expression of (immediate early) genes, we also studied the amount of cFos and deltaFosB in these neurons. Prolonged expression of cFos is not uncommon in challenged neural cells, as it was shown before in long-term activated endocrine melanotrope cells of *Xenopus laevis* (Ubink et al., 1997) and upon kainic acid-induced neuronal damage in the adult rat limbic system (Schreiber et al., 1993). In the EWcp, increased cFos-immunoreactivity was observed in rats exposed to a chronic mild variable stress paradigm (Xu et al., 2010). DeltaFosB, once induced, persists for prolonged periods in the brain, which is due to its high stability (Nestler et al., 1999). Furthermore, deltaFosB is up-regulated in the PVN, BSTov and CeA by chronic stress paradigms (Nunez et al., 2010) and 2 weeks of chronic mild variable stress increase both cFos- and deltaFosB-immunoreactivities in the EWcp and the PVN (T.L. Kozicz, unpubl. res.). Since our data do not reveal any effect of CCI on the number of EWcp neurons that contain these immediate early genes, nor on the strengths of the respective immunostainings, CCI does not seem to affect *cFos*-related gene expressions in these neurons. Our notion that a neuropathic pain condition does not affect the expression of *cFos* and *Ucn1* genes, whereas stressors like acute pain and chronic variable mild stress do change both cFos and Ucn1 and their mRNA contents in this nucleus (Rouwette et al., 2010, 2011; T.L. Kozicz, unpubl. res.),

points at CCI as a neuropathic stimulus affecting brain centres involved in mood control rather than in stress adaptation.

Conclusions

We have shown that peripheral mononeuropathy induced by CCI does not result in long-term changes in activities of the hypothalamic PVN and midbrain EWcp, but persistently recruits CRF neurons in the CeA and BSTov. Therefore, we assume that changes in the brain following peripheral mononeuropathy do not (primarily) involve HPA-axis activity, but rather the functioning of the limbic CeA and BSTov, with alterations at the level of CRF mRNA transcription and CRF peptide translation. We suggest that peripheral neuropathy-related alterations in the expression of CRF in the CeA and BSTov could contribute to the pathobiology of peripheral mononeuropathy-related mood disorders such as anxiety and depression.

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Chapter 4

Differential responses of corticotropin-releasing factor and urocortin 1 to acute pain stress in the rat brain

Tom P.H. Rouwette, Koen Klemann, Balázs Gaszner, Gert Jan Scheffer, Eric W. Roubos, Wim J.J.M. Scheenen, Kris C.P. Vissers, Tamás L. Kozicz

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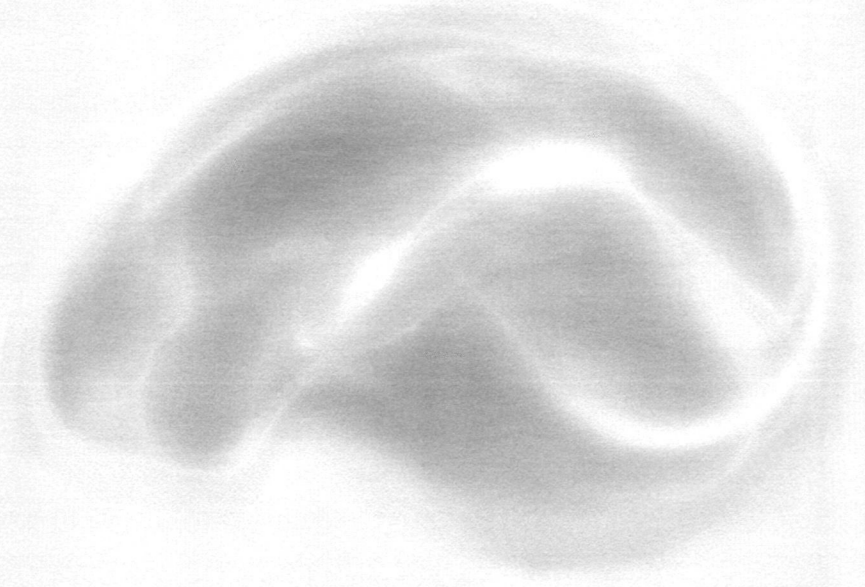

Abstract

It has been hypothesised that corticotropin-releasing factor (CRF) and its related neuropeptide urocortin 1 (Ucn1) play different roles in the initiation and adaptive phases of the stress response, which implies different temporal dynamics of these neuropeptides in response to stressors. We have tested the hypothesis that acute pain stress (APS) differentially changes the dynamics of CRF expression in the paraventricular nucleus of the hypothalamus (PVN), oval subdivision of the bed nucleus of the stria terminalis (BSTov) and central amygdala (CeA), and the dynamics of Ucn1 expression in the midbrain Edinger-Westphal centrally projecting neuron population (EWcp). Thirty minutes after APS, induced by a formalin injection into the left hind paw, PVN, BSTov, CeA and EWcp all showed a peak in cFos mRNA expression that was followed by a robust increase in cFos protein-immunoreactivity, indicating a rapid increase in (immediate early) gene expression in all four brain nuclei. CRF dynamics, however, were affected by APS in a brain nucleus-specific way: in the PVN, CRF-immunoreactivity was minimal at 60 min after APS and concomitant with a marked increase in plasma corticosterone, whereas in the BSTov not CRF peptide but CRF mRNA peaked at 60 min, and in the CeA a surge of CRF peptide occurred as late as 240 min after APS. The EWcp differed from the other centres, as Ucn1 mRNA and Ucn1 peptide peaked at 120 min. These results support our hypothesis that each of the four brain centres responds to APS with CRF/Ucn1 dynamics that are specific as to nature and timing. In particular, we propose that CRF in the PVN plays a major role in the initiation phase, whereas Ucn1 in the EWcp may act in the later, termination phase of the adaptation response to APS.

Introduction

The sensation of pain acts as an early warning signal to alert the organism for the presence of actual or potentially damaging stimuli (Woolf and Salter, 2000). Acute pain triggers the individual to avoid the harmful situation (Millan, 1999) and initiates, via the release of corticotropin-releasing factor (CRF) from the hypothalamic paraventricular nucleus (PVN), the activation of the hypothalamo-pituitary-adrenal (HPA)-axis (Imaki et al., 1992; Johnston et al., 1985; Kozicz, 2001; Viau and Sawchenko, 2002). Besides the PVN, many other brain centres play a role in autonomic, endocrine and behavioural responses to pain. In the present study we focus on those stress-associated brain centres that use, like the PVN, CRF as their (main) neurotransmitter, viz. the oval subdivision of the bed nucleus of the stria terminalis (BSTov) and the central amygdala (CeA). Moreover, we pay attention to the midbrain Edinger-Westphal centrally projecting neuron population (EWcp), which contains the brain's largest amount of the CRF family member, urocortin 1 (Ucn1). Both CRF and Ucn1 have been strongly implicated in pain and stress responses (Bale and Vale, 2004; Kozicz, 2001, 2007). Upon activation of the PVN, CRF induces in pituitary corticotropes the synthesis of pro-opiomelanocortin, which is processed to adrenocorticotropic hormone that subsequently stimulates the adrenal cortex to secrete corticosteroids. These hormones feed back to the brain and stimulate neuronal networks to evoke some aspects of adaptation behaviour, other aspects being elicited by centrally acting CRF (de Kloet, 2000, 2005; Greisen et al., 1999; Makara et al., 1969; Taylor et al., 1998). The PVN contains several subpopulations of neurons that respond to a variety of physical and psychological stressors including pain, as is evident from stress-induced expression of the immediate early gene *cFos* and the *Crf* gene (Imaki et al., 1992, 1995; Palkovits, 2008; Viau and Sawchenko, 2002). The PVN receives strong input from centres in the limbic system (Herman et al., 2005) including the CRF-producing CeA and bed nucleus of the stria terminalis (BSTov). The CeA plays a key role in HPA-axis regulation and stress-related behaviours (Davis and Shi, 1999), especially in response to stressors that threat to disturb homeostasis (Xu et al., 1999). The CeA activates the PVN indirectly, especially via the BSTov (Jankord and Herman, 2008; Ulrich-Lai and Herman, 2009). The CeA and BSTov are major sites of extra-hypothalamic expression of CRF and CRF receptors (Merchenthaler et al., 1982; Van Pett et al., 2000) and their CRF mRNA and CRF contents are up-regulated by stressors and pain stimuli (Kim et al., 2010; Ulrich-Lai et al., 2006).

The Ucn1-producing EWcp changes its activity in response to a variety of acute and chronic stressors (Gaszner et al., 2004; Kozicz, 2001; Weninger et al., 2000). While the PVN has a fast response to acute stimuli, with cFos peaking within an hour (Cullinan et al., 1995; Viau and Sawchenko, 2002), the EWcp responds very slowly to acute (pain) stress, with cFos and Ucn1 contents peaking only 4 hours after stress initiation (Kozicz, 2001; Weninger et al.,

2000). In line with this notion we hypothesise that CRF in the PVN, acting via CRF receptor 1 (CRFR1), plays a major role in the stress adaptation response initiation, whereas Ucn1 in the EWcp would rather be involved in the later, termination phase of the adaptation response, acting on CRFR2 (Coste et al., 2000; de Kloet et al., 2005). In the present study we have tested this hypothesis by comparing responses to an acute pain stress paradigm (APS) by the PVN, BSTov, CeA and EWcp.

A frequently used method to induce APS in rat is injecting formalin into the hind paw (Dubuisson and Dennis, 1977; Mravec et al., 2007; Vissers et al., 2004). This procedure causes moderate, continuous pain and evokes a complex behavioural response consisting of licking, biting and shaking of the affected limb, for a period of about one hour after injection (Abbott et al., 1995; Tjølsen et al., 1992). Using this paradigm, we have studied the temporal dynamics of pain stress-related changes in (immediate early) gene expression (cFos mRNA and cFos protein as markers) and in mRNA and peptide contents of CRF (in the PVN, CeA and BSTov) and of Ucn1 (in the EWcp), using quantitative *in situ* hybridisation and immunohistochemistry, respectively.

Materials & Methods

Animals and stress paradigm

Twenty-five male albino Wistar-R Amsterdam rats, 12-14 weeks old, bred in the Animal Facility of the Department of Anatomy, Pécs, Hungary, were paired-housed in standard plastic cages (40 x 25 x 20 cm) in a temperature- and humidity-controlled environment. They were kept on a 12 h light/12 h dark cycle (lights on at 6:00 AM, light intensity 200 lux) and were allowed access *ad libitum* to rodent chow and tap water throughout the experiment. Rats were acclimatised to these conditions for one week before starting the experiments. Fifty µl 4% paraformaldehyde in pyrogen-free saline (PFA; Sigma Chemical, St. Louis, MO, USA) were injected subcutaneously into the left hind paw of an APS animal (n = 5 per group). Immediately after injection, rats were put back in their home cages, and sacrificed 30, 60, 120 or 240 minutes later by anaesthetisation and decapitation, as described below. Control animals (n = 5) were treated in the same way, but had not been injected. All efforts were made to minimise the number of animals used and their suffering, and all procedures were conducted in accordance with the Declaration of Helsinki and the animal use guidelines based on the law of 1998, XXVIII, for animal care and use in Hungary, and approved by the Medical Faculty Advisory Committee for Animal Resources of Pécs University. Chemicals were obtained from Merck (Darmstadt, Germany) unless stated otherwise.

Tissue fixation and sectioning

Rats were perfused transcardially under deep anaesthesia with pentobarbital (nembutal, Sanofi-Synthélabo, Budapest, Hungary; 100 mg/kg body weight) with 50 ml 0.1 M sodium phosphate-buffered saline (PBS; pH 7.4), followed by 250 ml of 4% ice cold PFA in PBS, for 20 min. Then they were rapidly decapitated, and their brains post-fixed in fresh 4% PFA, transferred into 30% sucrose in PBS, and when completely submerged, frozen on dry ice. Twenty-five μm thick coronal slices of the forebrain, midbrain and of the periventricular zone of the hypothalamus were cut on a freezing microtome (Microm, Walldorf, Germany), and stored in sterile antifreeze solution (0.05 M PBS, 30% ethylene glycol, 20% glycerol) at -20 °C.

Corticosterone radioimmunoassay

From each rat, a 3 ml blood sample was taken from the left ventricle prior to insertion of the perfusion needle, and collected into a pre-cooled vial with 150 μl 7.5% EDTA, and centrifuged at 3,000 g for 10 min. Plasma aliquots were assayed for corticosterone by radioimmunoassay as described previously (Gaszner et al., 2004), using [^{3}H] corticosterone (12,000 cpm; 90-120 Ci/mmol, NET-399; Perkin-Elmer, Boston, MA, USA) and CS-RCS-57 antiserum (Jozsa et al., 2005). The inter- and intra-assay coefficients of variation were 9.2 and 6.4%, respectively.

Antiserum characterisation

Goat polyclonal antiserum against Ucn1 IgG (sc-1825; 1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA) generated against a peptide mapping at the C-terminus of rat Ucn1, has high specificity, as reported previously (Bachtell et al., 2003a,b). Rabbit polyclonal cFos antiserum (sc-52; 1:4,000; Santa Cruz Biotechnology) had been raised against the epitope corresponding to residues 3-16 of human cFos. The high specificities of these antisera have been previously confirmed by their pre-absorption with the synthetic peptides to which they have been raised, which abolished staining in all cases (Gaszner et al., 2004, 2009). Rabbit polyclonal CRF antiserum (1:2,000; kind gift from Dr. W.W. Vale, The Salk Institute, San Diego, CA, USA) had been raised against rat CRF and its high specificity was described earlier on the basis of pre-absorption with the homologous synthetic peptide (Rivier et al., 1983; Sawchenko et al., 1984). In our studies, no staining was observed after omission of first antisera or their pre-absorption with respective antigens.

In situ hybridisation

CRF mRNA, Ucn1 mRNA and cFos mRNA were detected using antisense cRNA probes transcribed from their respective linearised cDNA (GenBank accession codes AY128673.

U33935 and X06769.1, respectively; probes kindly provided by Dr. W.W. Vale). Sense cRNA probes served as controls (no hybridisation signal was seen). Probes were labelled with digoxigenin (DIG)-11-UTP (Roche Molecular Biochemicals, Basel, Switzerland). Hybridisations were carried out at 20 °C unless stated otherwise. Sections were rinsed in PBS, for 4 x 15 min, and fixed in 4% ice-cold PFA, at 4 °C for 30 min. Subsequently, they were rinsed 4 x 7 min in PBS followed by pre-incubation for 10 min at 37 °C in proteinase K medium containing 0.1 M Tris/HCl, 0.05 M EDTA and 10 µg/ml proteinase K (Invitrogen, Carlsbad, CA, USA). After rinsing in autoclaved MQ-water, acetylation was performed with 0.25% acetic acid anhydride in 0.1 M tri-ethanolamine buffer (pH 8.0), for 10 min, followed by a rinse in 2x concentrated standard saline citrate buffer (2xSSC; pH 7.0), for 5 min. Hybridisation mixture (50% deionised formamide, 0.3 M NaCl, 0.001 M EDTA, 1xDenhardt's solution, 10% dextran sulphate) together with 0.5 mg/ml tRNA (Roche) and the mRNA-DIG probe (*ca.* 40 ng/ml of Ucn1 probe or of cFos probe, or 5 ng/ml of CRF probe) was placed in a water bath, for 5 min at 80 °C, and then on ice for another 5 min. Sections were incubated in hybridisation solution, for 16 h at 58 °C, rinsed 4 x 7 min with 4xSSC, incubated for 30 min at 37 °C in preheated RNAse medium (0.5 M NaCl, 0.01 M Tris/HCl, 1 mM EDTA; pH 8.0) containing 0.01 mg/ml RNAse A (Roche) that had been added just before the start of incubation, and stringently rinsed in decreasing SSC concentrations (2x, 1x, 0.5x). Afterwards the sections were incubated in 0.1xSSC, for 30 min at 58 °C. To detect DIG-labelling, the alkaline phosphatase method with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate and toluidine salt (NBT/BCIP; Roche) as a substrate, was used. After rinsing 4 x 5 min with buffer A (0.1 M Tris/HCl, 0.15 M NaCl; pH 7.5), sections were pre-incubated for 1 h in buffer A containing 0.5% blocking agent (Roche), followed by incubation with sheep anti-DIG-AP (Roche; 1:5,000) in buffer A containing 0.5% blocking agent, for 3 h. Then, sections were rinsed 4 x 5 min in buffer A, followed by 2 x 5 min rinsing in buffer B (0.1 M Tris/HCl, 0.15 M NaCl, 0.05 M $MgCl_2$; pH 9.5). After incubation in NBT/BCIP mixture consisting of 10 ml buffer B, 2.4 mg levamisole (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 175 µl NBT/BCIP (Roche) in a light-tight box, for 6 h, the reaction was stopped by rinsing the sections in buffer C (0.1 M Tris/HCl, 0.01 M EDTA; pH 8.0), for 2 x 5 min. Then, sections were mounted on gelatine-coated glass slides, dried for 16 h at 37 °C, dehydrated, cleared in xylene, cover-slipped with Entellan and studied with a DMRBE microscope connected to a DC 500 digital camera (Leica Microsystems, Wetzlar, Germany). For each probe, all sections were processed in a single assay to minimise inter-experimental error.

Immunohistochemistry

Immunofluorescent labelling was performed as described previously (Gaszner et al., 2007). In short, sections were washed in PBS, treated with 0.5% Triton X-100 (Sigma Chemical) in PBS, pre-incubated in PBS with 2% normal donkey serum (NDS), and incubated in fresh PBS with 2% NDS and primary antiserum (see above), for 16 h. Following rinses in PBS, incubation was in PBS with 2% NDS and the secondary antiserum, for 2 h. Secondary antisera were Cy^3-conjugated donkey anti-rabbit/goat IgG and Cy^5-conjugated donkey anti-rabbit IgG (both diluted 1:100; Jackson Immunoresearch Laboratories, West Grove, PA, USA). After several rinses in PBS, sections were mounted on gelatine-coated glass slides, cover-slipped with FluorSave, and studied with a TCS SP2 AOBS confocal microscope (Leica Microsystems).

Image analysis

Although unilateral injection of formalin may lead to hemispheric lateralisation of extracellular-regulated kinase (Carrasquillo and Gereau, 2008) and to pain-related plasticity (Ji and Neugebauer, 2009) in the right amygdala, pilot studies (T.L. Kozicz et al., unpubl. res.) did not show lateralisation as to cFos or CRF/Ucn1 responses in this nucleus nor in the PVN, BST or EWcp upon acute stress. This is in accordance with a recent study on the PVN using the same pain-evoking stress stimulus (Palkovits, 2008), indicating a cross-over of pain-conducting fibres between the spinal cord and hypothalamus to cause bilateral cFos mRNA increase (Pacak and Palkovits, 2001; Palkovits et al., 1999). Therefore, to increase accuracy of the measurements and statistical power, the expression of mRNAs and the degree of immunoreactivity were assessed bilaterally, without distinguishing left and right nucleus parts, in the PVN, BSTov, CeA and centrally in the EWcp. The nuclei were identified on the basis of the coordinates given by Paxinos and Watson (1997). Per rat, quantifications were made in coronal sections interspaced by 150 μm, medially through a nucleus, 3 from the PVN and BSTov and 5 from the CeA and EWcp, using digital images taken with the DMRBE microscope (at 1,200 x 1,600 dpi, for *in situ* hybridisation) or with the confocal laser scanning microscope (at 1,024 x 1,024 dpi, for fluorescent immunohistochemistry). Images were analyzed with ImageJ software (NIH, Bethesda, MD, USA), determining the number of immunostained neurons per section, and the specific signal staining density per neuron (SSD), the latter parameter expressed as the average of 10 randomly taken neurons, corrected for background density in the same section outside the brain area. Outcomes were averaged per section, providing for each parameter and brain nucleus one value per animal.

Statistics

Parameter values per animal were expressed as means and standard error of the mean (SEM) and analysed by one-way ANOVA to test for statistical differences between the mean values at different time points, and with Tukey's test for *post hoc* analysis after testing for normality (Shapiro and Wilk, 1965) and for homogeneity of variance (Bartlett's Chi-square test; Snedecor and Cochran, 1989) ($\alpha = 5\%$).

Results

Hormonal responses following acute pain stress

Following APS, the plasma corticosterone titre steadily increased, showing a peak at 60 min after APS (Fig. 1; control *vs.* 60 min, $P < 0.001$). Four hours after APS, the plasma corticosterone titre had returned to non-APS control level. This titre is somewhat higher than in fully unstressed rats, since anaesthesia with pentobarbital has a stimulatory effect on the HPA-axis (Vahl et al., 2005).

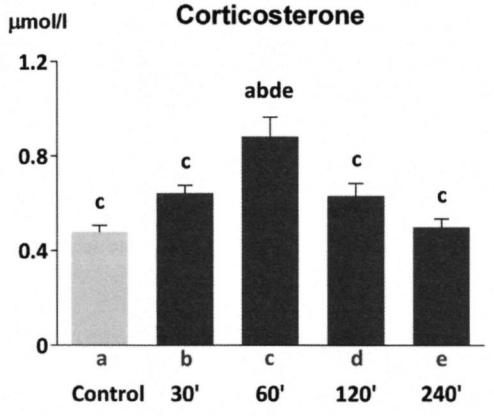

Figure 1. *Corticosterone plasma titre of control rats and of rats exposed for different periods (in minutes) to acute pain stress (APS). n = 5 rats per group. Top letters indicate groups with which a significant difference exists (P < 0.05).*

Effect of APS on cFos mRNA

To assess the degree of induction of immediate early gene expression, the number of cFos mRNA-positive perikarya and the SSD of the hybridisation signal per perikaryon were determined. Upon APS, strong cFos mRNA expression was observed in all four brain nuclei (Fig. 2), and a significant effect of time was found for each parameter (PVN, number: $F_{4,20} = 42.97$, $P < 0.0001$ and SSD: $F_{4,20} = 119.3$, $P < 0.0001$; BSTov, number: $F_{4,20} = 22.30$, $P < 0.0001$ and SSD: $F_{4,20} = 38.09$, $P < 0.0001$; CeA, number: $F_{4,20} = 13.92$, $P < 0.0001$ and SSD: $F_{4,20} = 46.70$, $P < 0.0001$; EWcp, number: $F_{4,20} = 18.12$, $P < 0.0001$ and SSD: $F_{4,20} = 22.22$, $P < 0.0001$).

In all nuclei cFos mRNA expression peaked already at 30 min ($P < 0.001$ *vs.* control) and subsequently dropped to basal value ($P > 0.05$ *vs.* control), with the SSD in the CeA even below basal value ($P < 0.01$). The EWcp showed cFos mRNA around control value at 120 min, with a significant increase at 240 min ($P < 0.01$). Thirty minutes after APS, the PVN revealed the strongest reaction, with a 32x (number of positive perikarya) and 4.2x (SSD) increase in cFos mRNA, followed by the BSTov (number: 18x, SSD: 2.8x), EWcp (number: 11x, SSD: 3.5x) and CeA (number: 10x, SSD: 1.8x) (Fig. 2).

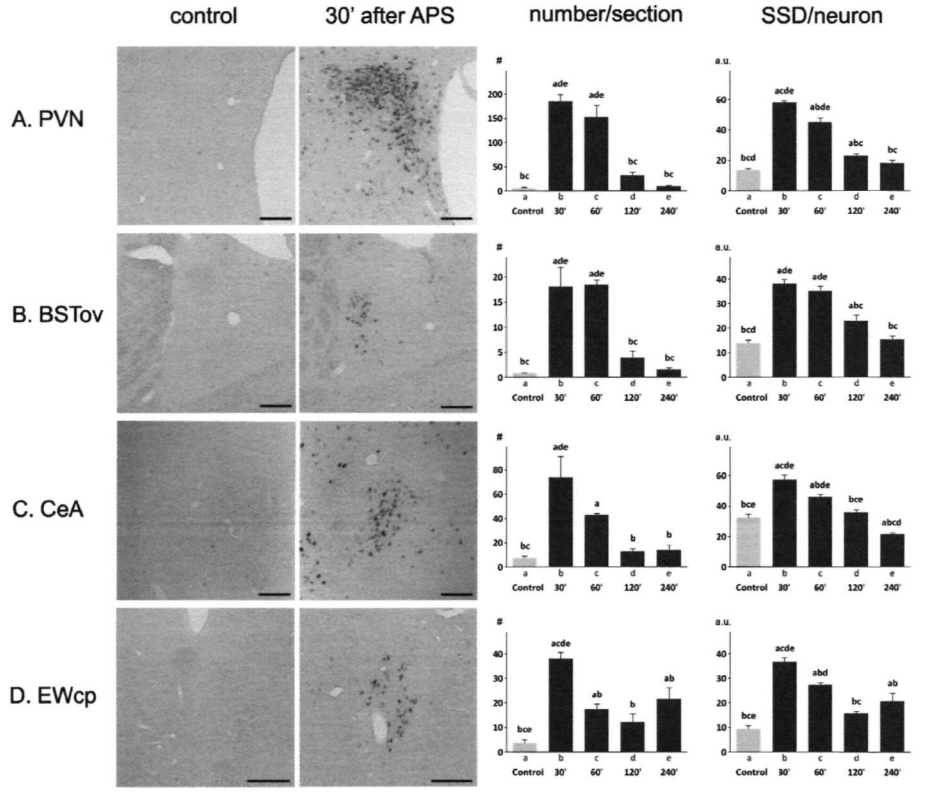

Figure 2. *In situ hybridisation of cFos mRNA, showing representative images, neuron numbers per section, and specific signal density per neuron (SSD; in arbitrary units, a.u.) of (A) the paraventricular nucleus of the hypothalamus (PVN), (B) the oval bed nucleus of the stria terminalis (BSTov), (C) the central amygdala (CeA) and (D) the Edinger-Westphal centrally projecting neuron population (EWcp), of control rats and of rats exposed for different periods (in minutes) to APS. Horizontal bars = 100 µm. n = 5 per group, top letters indicate groups with which a significant difference exists ($P < 0.05$).*

Effect of APS on cFos protein

To determine the amount of cFos produced as a result of APS, the degree of cFos-immunoreactivity was measured in the four brain nuclei, at five time points. Clear nuclear

cFos-presence was observed in all nuclei (Fig. 3), together with a time-dependent increase in the number of cFos-positive neurons in stressed rats (PVN: $F_{4,20} = 21.20$, $P < 0.0001$; BSTov: $F_{4,20} = 8.70$, $P < 0.001$; CeA: $F_{4,20} = 10.15$, $P < 0.001$; EWcp: $F_{4,20} = 14.82$, $P < 0.0001$). Compared to non-stressed controls the strongest increase in number was seen in the PVN (18.2x; $P < 0.001$, 120 min *vs.* control). Less dramatic but still substantially higher numbers were found in the BSTov (4.1x; $P < 0.01$, 120 min *vs.* control), CeA (3.3x; $P < 0.001$, 60 min *vs.* control) and EWcp (2.7x; $P < 0.001$, 60 min *vs.* control; Fig. 3). At 240 min, the numbers of cFos-positive neurons in all nuclei were again at control levels ($P > 0.05$ *vs.* control for all four brain nuclei).

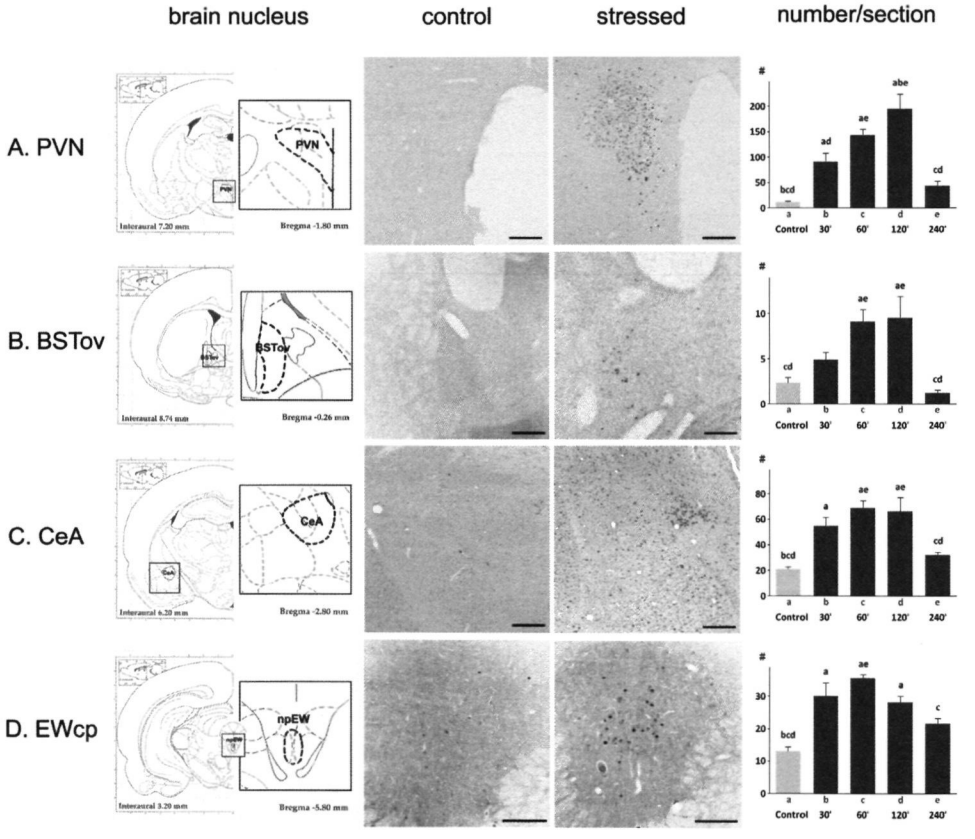

Figure 3. *Locations (on the basis of Paxinos and Watson, 1997), representative images, and numbers per section of cFos-immunoreactive neurons, of (A) PVN, (B) BSTov, (C) CeA and (D) EWcp, in control and APS rats. Horizontal bars = 100 µm. n = 5 per group, top letters indicate groups with which a significant difference exists (P < 0.05).*

Effect of acute pain stress on CRF/Ucn1 mRNA

Quantitative *in situ* hybridisation demonstrated intense CRF mRNA expression in neurons of the PVN, BSTov and CeA and strong Ucn1 mRNA expression in the EWcp (Fig. 4). In the

PVN, the number of CRF mRNA-positive perikarya was significantly increased 240 min after APS ($P < 0.05$), but the SSD revealed no effect of the stressor. In the BSTov no difference in the number of positive perikarya and SSD was found compared to controls. The significant increase 60 min after APS compared to the 30 min time point ($P < 0.05$) was only transient. In contrast, the CeA did not show an effect of APS, neither in the number of positive perikarya nor in the SSD. In the EWcp, the SSD of Ucn1 mRNA-hybridizing perikarya was increased 60 and 120 min after APS initiation ($P < 0.05$) and had returned to baseline control values at 240 min. The number of these perikarya initially remained unchanged during the first 120 min after APS initiation, after which it decreased ($P < 0.05$).

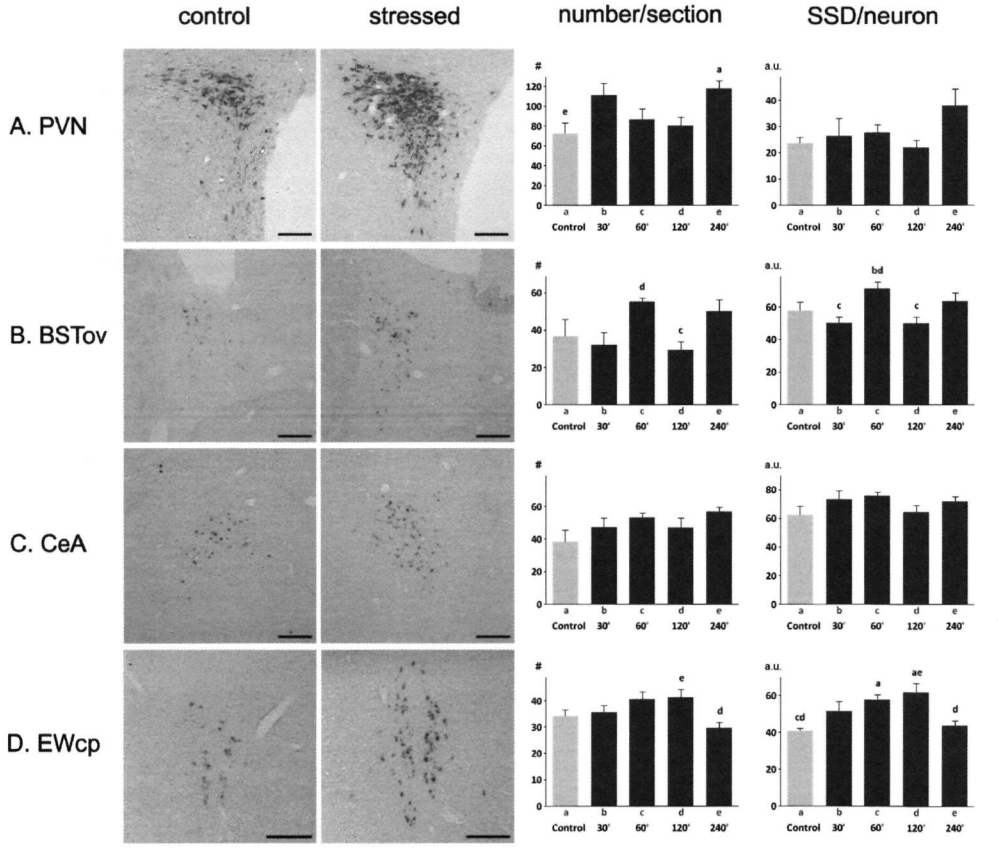

Figure 4. *In situ hybridisation of CRF mRNA in (A) PVN, (B) BSTov and (C) CeA, and in situ hybridisation of Ucn1 mRNA in the EWcp (D), with representative images and neuron numbers per section and SSD, of control rats and of rats exposed for different periods (in minutes) to APS. Horizontal bars = 100 μm. n = 5 per group, top letters indicate groups with which a significant difference exists ($P < 0.05$).*

Effect of acute pain stress on CRF/Ucn1 peptide

In both controls and APS rats, the PVN, BSTov and CeA revealed strong neuronal CRF-staining, whereas neurons in the EWcp were intensively Ucn1-immunoreactive (Fig. 5). In the PVN, no effect of APS on the number of CRF-positive neurons was observed, but their SSD had clearly decreased 60 min after APS (40% decrease; $P < 0.05$ *vs.* control) to increase again thereafter. The BSTov did not reveal an effect of APS on the number of neurons and their SSD. In contrast, in the CeA the neuron numbers, but not the SSD, were higher after APS, especially at 240 min (2.5x increase *vs.* control). In response to APS, the Ucn1 contents of the EWcp increased, both as to the number of positive neurons (54% increase; 60 min *vs.* control: $P < 0.05$) and their SSD (50% increase; 120 min *vs.* control: $P < 0.01$).

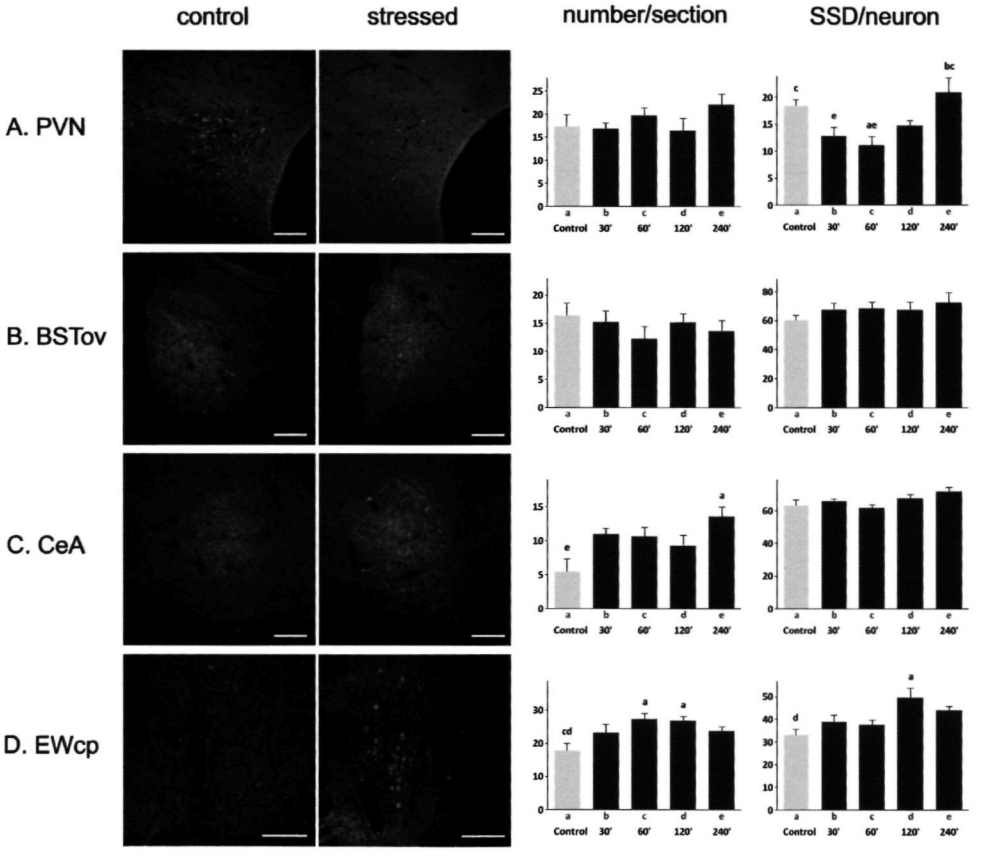

Figure 5. *Immunohistochemistry of CRF in (A) PVN, (B) BSTov and (C) CeA, and of Ucn1 in the EWcp (D), with representative images, neuron numbers per section and SSD, of control and APS rats. Horizontal bars = 100 µm. n = 5 per group, top letters indicate groups with which a significant difference exists ($P < 0.05$).*

Discussion

We have tested our hypothesis that brain nuclei (PVN, BSTov, CeA and EWcp) involved in the stress response by producing CRF or the CRF-related peptide Ucn1, react to an acute pain stimulus, and do so with different temporal dynamics. The robust induction of cFos mRNA and cFos protein after APS clearly shows that acute pain stress indeed activates all four brain nuclei, whereas the dynamics of their APS-induced CRF/Ucn1 mRNA and CRF/Ucn1 peptide responses confirm that the nuclei differ from each other in their temporal mRNA and peptide contents. Below we will discuss these conclusions in detail.

Corticosterone responses to APS

Subcutaneous injection of formalin into a rodent's hind paw is routinely used as an acute stress paradigm and induces homeostatic adaptive responses, such as an increase in heart rate and in blood pressure (Culman et al., 1997), an elevated plasma catecholamine titre, and behavioural responses reflecting local pain, such as shaking, lifting, licking or biting of the paw (Abbott et al., 1995; McCall et al., 1996). The APS paradigm also activates the central stress response, with an increase of adrenocorticotropic hormone release from the anterior lobe of the pituitary gland and of corticosterone from the adrenal cortex (Kant et al., 1982; Pacak et al., 1998; Vissers et al., 2004). In line with these findings, we here show that the corticosterone blood titre increases following APS, with a peak at 60 min. This increase was only transient, since 120 min after APS, the corticosterone titre returned to non-APS control level, which is in agreement with previous studies (Imaki et al., 1995; Mravec et al., 2007). Our data are consistent with the idea that APS acts as an acute stressor, inducing a stress adaptation response consisting of a fast initiation phase (rising corticosterone titre) followed by a termination phase (corticosterone titre returns to baseline value) (de Kloet et al., 2005). Although the acute pain stressor used in this study evokes a stressor-specific response, we cannot dissociate possible stress effects from specific pain processing-related effects.

Activation of stress-responsive brain nuclei by APS - cFos response

The immediate early gene *cFos* is a well-established marker for neuronal activation (Kovacs, 2008). In response to various acute pain stressors, cFos protein is induced in several brain regions, including the limbic forebrain and hypothalamus (Ohtori et al., 2000; Palkovits, 2008). Recently, we observed pain stress-induced expression of this protein in the midbrain EWcp and hypothalamic PVN (Rouwette et al., 2010). Although the effects of acute stress on either cFos mRNA or protein dynamics in the PVN have been extensively investigated (Imaki et al., 1992, 1995; Palkovits, 2008; Viau and Sawchenko, 2002), the present study is the first to

reveal the temporal changes of both cFos mRNA and cFos protein in the PVN as well as in the BSTov, CeA and EWcp in response to an acute pain stressor, within the same experiment.

Assessing the responses of cFos mRNA and protein during the first four hours after APS, we observed that the four brain nuclei reacted with very similar temporal patterns. Also, we found, as expected, that the increases in cFos mRNA preceded similar increases in cFos protein, a process that, again, showed similar dynamics in all four nuclei. These data indicate that all nuclei react to APS with the same dynamics of cFos production. However, the four brain nuclei have different functions, the PVN being involved in control of the HPA-axis, possibly in concert with the EWcp (Kozicz, 2007) and the BSTov and CeA rather playing roles in stress-related control of mood (Davis and Shi, 1999; Regev et al., 2011).

Effect of acute pain stress on CRF/Ucn1 dynamics

Since in the PVN, cFos strongly co-localises with CRF (Loughlin et al., 2006), it should be no surprise that our immunohistochemical study has demonstrated that APS does not only rapidly activate PVN neurons, as indicated by their increased cFos production, but also rapidly affects the PVN's CRF content, which drops 30 min after APS, reaches a minimum at 60 min, and returns to control value from 120 min onwards. This temporal pattern, suggesting emptying and subsequent repletion of the neuronal cell bodies with CRF, neatly matches the temporal rise in corticosterone titre, which peaks at 60 min, and therefore likely reflects increased CRF secretion from the PVN into the portal circulation. Similar patterns of CRF and cortisol release occur in sheep following acute predator stress (Cook, 2004), supporting our conclusion that APS leads to a rapid but transient stress response by the HPA-axis. However, despite a clear decrease in CRF contents of PVN neurons, there are no significant changes in CRF mRNA within the first 2 hours after APS. Although there is a rise in the number of positive neurons after 30 min, possibly due to inter-animal variation, this difference is not statistically different. Subcutaneous injection of nicotine gives a similar non-significant increase in CRF mRNA in the PVN 30 min after injection, whereas cFos mRNA expression reveals a clear activation of the nucleus (Loughlin et al., 2006). On the other hand, these data are in contrast with restraint- and foot shock-induced stress, which reveal a strong increase in CRF mRNA 2 hours after application of the stressor (Cespedes et al., 2010). This clearly implicates that different stressors have different effects on CRF expression in the PVN.

Whereas APS induces a transient drop in CRF content of the PVN, the response of the BSTov to APS shows a different pattern. While CRF mRNA was markedly higher 60 min after APS and returned to basal values thereafter, the amount of CRF peptide present in the BSTov did not change upon APS, indicating that an increased production of CRF around 60 min is likely compensated by increased export of CRF from the cell body to be released from the axons, but confirmation of this idea would need direct measurement of CRF release, which is

beyond the scope of this study. Meanwhile, it should be noted that in contrast to the PVN, in our study of the BSTov, CRF dynamics cannot be directly linked to cFos dynamics, because stress-induced cFos expression in the BSTov seems mainly to occur in enkephalin-producing neurons instead of in CRF neurons (Day et al., 1999).

Like in the BSTov, CRF-containing neurons in the CeA hardly show cFos induction upon stress (Day et al., 1999; Loughlin et al., 2006). Studying the effect of APS on CRF expression in this nucleus, however, reveals that, unlike in the BSTov, APS does not induce a change in CRF mRNA contents of the CeA. Yet, APS does increase the number of CRF-positive neurons in this nucleus. This finding suggests that APS affects mRNA translation rather than DNA transcription. Since this effect is only evident 4 hours after APS, the CeA does not seem to play a role in the initiation phase of the stress response but rather in the later, (termination of the) adaptive phase.

The EWcp differs not only from the other three brain nuclei in that it does not produce CRF but the related peptide Ucn1, but also its reaction to APS is different. Ucn1 highly co-localises with cFos in the EWcp in response to a variety of acute stressors (Gaszner et al., 2004). Following APS, Ucn1 peptide content of this nucleus peaked at 60-120 min, and returned to control value at 240 min after APS. A similar pattern was found for the changes in the nucleus' Ucn1 mRNA content, with a peak 120 min after APS. These results indicate that APS stimulates both *Ucn1* gene transcription and Ucn1 mRNA translation. In line with the idea that CRF is involved in the initiation of the stress response and Ucn1 in its termination, the EWcp-Ucn1 response to APS is delayed compared to that of the PVN (and, to a certain degree, to that of the BSTov). The notion that Ucn1 is essential for termination of the stress response receives support from a recent study on Ucn1-deficient mice that appear to be unable to properly recover from acute stress (Neufeld-Cohen et al., 2010).

Conclusions

Taken together, our data show that acute pain stress induces gene expression in the PVN, BSTov, CeA and EWcp. In the BSTov and CeA, this activation, indicated by APS-induced recruitment of the immediate early gene *cFos*, is not necessarily coupled to CRF dynamics. In the PVN and EWcp, however, cFos induction may be related to increased *de novo* peptide production and release (CRF and Ucn1, respectively). It is well documented that in the rat, the formalin injection-induced pain response lasts for only about one hour after injection (Tjølsen et al., 1992; Abbott et al., 1995). As we show, during this period the corticosterone titre rises (initiation phase of the stress response) and the PVN is activated. This observation supports the idea that the PVN is involved in the initiation phase. We further demonstrate that the EWcp becomes active later, when the corticosterone titre is decreasing. Therefore, we propose that this nucleus is involved in the termination phase of the stress response.

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Chapter 5

Acute pain increases phosphorylation of doublecortin-like kinase-long in the Edinger-Westphal nucleus but not in the hypothalamic paraventricular nucleus of the rat

Tom P.H. Rouwette, Tamás L. Kozicz, Nicola F.M. Olde Loohuis, Balázs Gaszner, Erno Vreugdenhil, Gert Jan Scheffer, Eric W. Roubos, Kris C.P. Vissers, Wim J.J.M. Scheenen

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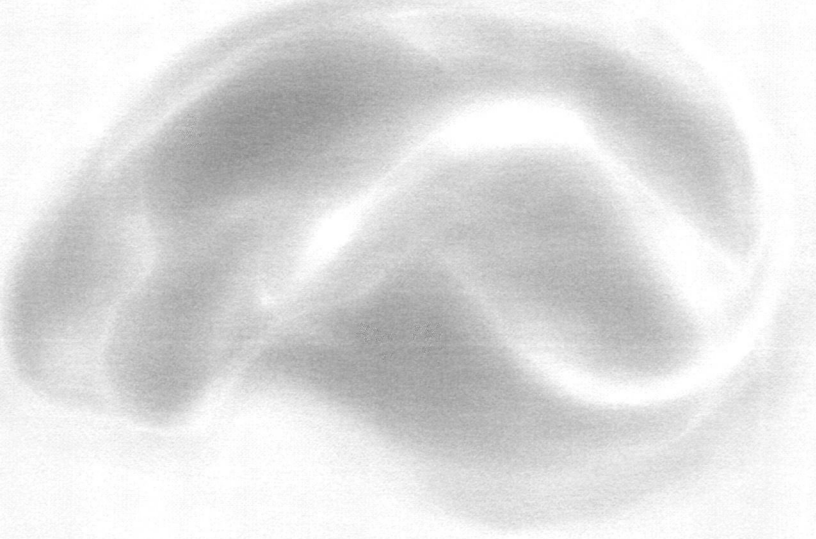

Abstract

Doublecortin-like kinase (DCLK) is crucially involved in neuronal plasticity and microtubule-guided retrograde transport of signalling molecules. We have explored the possibility that DCLK is involved in pain-induced signalling events in adult male Wistar rats. Our results show that both DCLK-short and DCLK-long splice variants are present in the cell body and proximal dendrites of neurons in stress-related nuclei, i.e., the paraventricular nucleus of the hypothalamus (PVN) and the Edinger-Westphal centrally projecting neuron population (EWcp) in the rostroventral periaqueductal grey. We found that DCLK-long but not DCLK-short is phosphorylated in its serine/proline-rich domain. Furthermore, it is demonstrated that phosphorylation of DCLK-long in the EWcp is increased by acute pain, whereas DCLK-long phosphorylation in the PVN remains unaffected. This is the first report revealing that DCLK isoforms in the PVN and EWcp occur in the adult mammalian brain and that pain differentially affects DCLK-long-mediated neuronal plasticity in these two stress-sensitive brain centres.

Introduction

Pain is considered as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (Bonica, 1979). Pain is a burden for society and the individual, and although the mechanisms underlying pain are relatively well-known, we know less about the mechanisms underlying pain treatment. It is known that acute pain triggers an organism to avoid harmful situations (Millan, 1999) and coincides with an activation of the hypothalamo-pituitary-adrenal (HPA)-axis, as revealed by increased blood titres of adrenocotricotropic hormone (ACTH) and cortisol or corticosterone (Greisen et al., 1999; Makara et al., 1969; Taylor et al., 1998). Different pain stressors, such as acute pain stress (APS), lead to activation of mitogen-activated protein kinases (MAPKs; Ji and Woolf, 2001). MAPK activation plays an important role in the induction and maintenance of neuronal plasticity, including peripheral and central sensitisation underlying increased pain sensitivity after injuries (Ji et al., 2009). MAPK activity phosphorylates proteins that have a specific proline-threonine-serine-proline (PTSP)-domain (David et al., 1995; Edelman et al., 2005; Engels et al., 2004; Ohmae et al., 2006). One family of proteins with a strongly conserved MAPK-phosphorylation site that recently gained particular interest as intracellular signalling molecules is the doublecortin-like kinase (DCLK) family (Boekhoorn et al., 2008; Fitzsimons et al., 2008; Francis et al., 1999; Friocourt et al., 2007). The *dclk* gene gives rise to short and long forms of DCLK, viz. DCLK-short and DCLK-long, and to a doublecortin-like splice variant, DCL (Engels et al., 2004; Omori et al., 1998; Sossey-Alaoui and Srivastava, 1999). In the developing brain DCL has been implicated in regulating microtubule dynamics (Feng and Walsh, 2001; Fitzsimons et al., 2008; Vreugdenhil et al., 2007). The functions of DCL, DCLK-short and DCLK-long in the adult brain are largely unknown. Phosphorylation and dephosphorylation regulate the function and localisation of doublecortin (DCX), a protein with a high homology to DCL, and result in plastic changes (Schaar et al., 2004). DCX phosphorylation lowers its affinity to microtubules *in vitro*, reduces its effect on polymerisation, and displaces it from microtubules in cultured neurons (Bielas et al., 2007; Tanaka et al., 2004). Since all *dclk* products contain a conserved MAPK-PTSP phosphorylation site, and pain activates MAPK pathways, we raised the question whether acute pain changes the phosphorylation of the PTSP-motif of DCLK.

A frequently used animal paradigm to study the effects of acute pain on neuronal and neuroendocrine systems is subcutaneous injection of formalin into a rat's hind paw, which also activates the stress response (Dubuisson and Dennis, 1977; Mravec et al., 2007; Tjolsen et al., 1992; Vissers et al., 2003, 2004). Activation of the stress response involves the secretion of corticotropin-releasing factor (CRF) from the paraventricular nucleus (PVN) of the hypothalamus (de Kloet, 2000, 2005). The neuropeptide urocortin 1 (Ucn1), a member of the CRF peptide family, is also involved in stress adaptation. Its main expression site is the

Edinger-Westphal centrally projecting neuron population (EWcp), located in the rostroventral periaqueductal grey. The activity of the EWcp changes in response to acute and chronic stressors (Kozicz, 2001; Weninger et al., 2000). Both the PVN and EWcp are activated by acute pain (Kozicz, 2001; Palkovits, 2008). These nuclei show similar responses to acute stressors but opposite responses to long-lasting, chronic stressors (Kozicz et al., 2004; Weninger et al., 2000). This implies that, although both brain nuclei respond to a variety of stressors, different signalling cascades may lead to their eventual stress-mediated activation. Although the response of the PVN and the EWcp to acute pain has been documented, the intracellular signalling pathways by which acute pain activates these stress-sensitive centres are unknown.

In this study we have tested our hypothesis that acute pain changes the phosphorylation of the PTSP-motif of DCLK, by determining (1) which DCLK splice variants are present in the PVN and EWcp, and (2) whether phosphorylation of the PTSP-motif is differentially regulated by acute pain. Our results reveal the presence of DCLK-short and DCLK-long isoforms in both brain centres, and show that acute pain leads to up-regulation of phosphorylation of the PTSP-motif of DCLK-long in the EWcp but not in the PVN.

Materials & Methods

Animal husbandry and stress paradigm

Albino male Wistar-R Amsterdam rats, bred in-house (Animal Facility of the Department of Anatomy, Pécs University, Hungary), 12-14 weeks old, were housed in standard plastic cages (40 x 25 x 20 cm) in a temperature- and humidity-controlled environment. They were maintained on a 12 h light/12 h dark cycle (lights on at 6:00 AM, light intensity 200 lux) and were allowed *ad libitum* access to tap water and rodent chow throughout the experiment. Rats were acclimatised to these housing conditions for one week before starting the experiments.

APS animals (n = 5 per group) were given a subcutaneous injection in the left hind paw of 50 µl 4% paraformaldehyde in pyrogen-free saline (PFA; Sigma Chemical, St. Louis, MO, USA). Immediately after injection, animals were put back to their home cages, and sacrificed 2 h later by anaesthetisation and decapitation, as described below. The 2 hours time point was chosen since PVN and EWcp show strong activation 2 hours after the initiation of an acute stressor (Gaszner et al., 2004; Kozicz, 2001; Viau and Sawchenko, 2002). Control animals (n = 5 per group) were treated in the same way, but had not been injected. Since this study concerns the effects of acute pain on two stress-responsive brain centres, no post-operative analgesia was applied.

All measures were taken to minimise the number of animals used and their suffering, and all procedures were conducted in accordance with the animal use guidelines approved by

the Medical Faculty Advisory Committee for Animal Resources of Pécs University, based on the law of 1998, XXVIII, for animal care and use in Hungary. Chemicals were obtained from Merck (Darmstadt, Germany) unless stated otherwise

Antisera

Goat polyclonal antiserum against Ucn1 IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) had been generated against a peptide mapping at the C-terminus of rat Ucn1 Its high specificity has been reported by Bachtell et al (2003a,b) Rabbit cFos antiserum (Santa Cruz Biotechnology) had been raised against the epitope corresponding to residues 3-16 of human cFos The high specificities of these antisera have been previously confirmed by their pre-absorption with the synthetic peptides to which they had been raised, which abolished immunostaining in all cases (Gaszner et al , 2009). Guinea pig polyclonal antiserum against CRF (Bachem Peninsula, San Carlos, CA, USA) was collected from guinea pigs immunised with a synthetic peptide raised against rat CRF (Rivier et al., 1983) The high specificity of this serum was shown by comparing immunolabellings of CRF wildtype and CRF-null mice (Treweek et al., 2009) Highly specific mouse candidate plasticity-related gene (CPG16) antiserum (BD Biosciences, San Jose, CA, USA) had been generated against the C-terminal 330-424 kinase part of rat CPG16 and its specificity described before (Fitzsimons et al , 2008) The rabbit polyclonal DCLK antiserum was produced by injection of a 55-amino acid-long synthetic peptide immunogen corresponding to the N-terminal domain of DCLK-short, designated CARP, which high specificity was previously described (Boekhoorn et al , 2008, Fitzsimons et al , 2008, Kruidering et al., 2001, Vreugdenhil et al , 2007). The rabbit polyclonal pDCLK antiserum was raised against a SPSSPTSPGSLRKQR synthetic peptide with the underlined serine in the PTSP-domain of the SP-rich region phosphorylated. This serum specifically detects multiple DCLK splice variants in their PTSP-phosphorylated form (T F Dijkmans, unpubl res)

Immunohistochemistry

For immunohistochemistry, rats were perfused transcardially under deep anaesthesia with pentobarbital (nembutal, Sanofi-Synthélabo, Budapest, Hungary, 100 mg/kg body weight) with 50 ml 0.1 M sodium phosphate-buffered saline (PBS, pH 7.4), followed by 250 ml of 4% ice-cold PFA in PBS After perfusion, animals were rapidly decapitated, and their brains removed, post-fixed in fresh 4% PFA, and stored at 4 °C Forty-eight hours before sectioning, brains were transferred into 30% sucrose in PBS, and when completely submerged, frozen on dry ice. Twenty-five µm thick coronal slices of the midbrain and of the periventricular zone of

the hypothalamus were cut on a freezing microtome (Microm, Walldorf, Germany), and stored in sterile antifreeze solution (0.05 M PBS, 30% ethylene glycol, 20% glycerol) at -20 °C.

Free-floating nickel-diaminobenzidine (DAB) immunohistochemistry for phosphorylated DCLK (pDCLK) and for cFos was carried out as described previously (Korosi et al., 2005.) In short, after washing sections in PBS to remove antifreeze solution and incubation in 0.5% Triton X-100 (Sigma Chemical) in PBS for 30 min to enhance antibody penetration, they were incubated in a quenching mixture of PBS and 1% H_2O_2 (1:1 v/v), washed again in PBS and pre-incubated in blocking buffer (2% normal goat serum, NGS, in PBS) to block non-specific binding sites. Then they were incubated in polyclonal rabbit anti-cFos antiserum (1:4,000) or in polyclonal rabbit-anti pDCLK antiserum (1:1,000), in PBS with 2% NGS, for 18 h. Next, sections were rinsed in PBS, and incubated with biotinylated goat anti-rabbit IgG (1:200) in PBS containing 2% NGS, for 1 h. After rinsing in PBS, incubation was in avidin-biotin complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 1 h, and rinsing in PBS. Immunostaining was performed in Tris buffer containing 0.02% DAB (Sigma Chemical). After stopping the reaction in this buffer, sections were mounted on gelatine-coated glass slides, embedded in Entellan, and studied with a Leica DMRBE microscope (Leica Microsystems, Wetzlar, Germany).

Single and double immunofluorescent labellings were performed as described previously (Gaszner et al., 2007). In short, sections were washed in PBS treated with 0.5% Triton X-100 (Sigma Chemical) in PBS, pre-incubated in PBS with 2% normal donkey serum (NDS), and incubated in fresh PBS with 2% NDS and rabbit polyclonal anti-pDCLK serum (1:1,000), for 18 h. Incubation followed in secondary Cy^3-conjugated donkey anti-rabbit IgG (1:100; Jackson Immunoresearch Laboratories, West Grove, PA, USA) in PBS, for 2.5 h. After several washes in PBS, sections were mounted on gelatine-coated glass slides, cover-slipped with FluorSave, and studied with a Leica TCS SP2 AOBS confocal microscope. For double immunofluorescent labelling, sections were incubated in a mixture of rabbit anti-pDCLK (1:1,000) and goat anti-Ucn1 (1:250) or in a mixture of rabbit anti-pDCLK (1:1,000) and guinea pig anti-CRF (1:250), for 18 h. After washing in PBS, a secondary antiserum cocktail (Cy^3-conjugated anti-rabbit IgG and Cy^2-conjugated anti-goat or anti-guinea pig IgG; all 1:100) was applied for 2.5 h. Next, sections were treated and studied as described above for single immunohistochemistry.

Protein extraction and Western blotting

For Western blotting, rats were anaesthetised, perfused with 50 ml 0.1 M PBS (pH 7.4), followed by 0.1 M sodium fluoride in PBS to prevent dephosphorylation of proteins of interest, decapitated, and their brains removed as described above. Using a brain matrix (Ted Pella, Redding, CA, USA), 2 mm thick coronal brain slices were cut with 2 razor blades. Slices

were placed on a chilled plastic mat and the EWcp or PVN punched out with a Harris Unicore Hole 1.5 mm puncher (Ted Pella). Punches were solubilised with lysis buffer containing 50 mM HEPES (pH 7.2), 140 mM NaCl, 0.1% Triton X-100, 1.0% Tween-20, 1 mM EDTA and 0.1% deoxycholate, and supplemented with a protease inhibitor cocktail of 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 2 μg/ml PMSF, 100 μg/ml benzamidine (Sigma Chemical), 8 μg/ml calpain I and 8 μg/ml calpain II (Boehringer Mannheim, Mannheim, Germany). Next, punches were homogenised, kept on ice for 2 h, and centrifuged at 16,000 g, for 30 min at 4 °C. Equal volumes of lysate were separated by SDS-PAGE and transferred to immobilon-P PVDF (Millipore, Bedford, MA, USA). Blots were blocked for 1 h with blocking buffer consisting of Tris-buffered saline with 0.2% Tween (TBST) and 5% milk, incubated with rabbit anti-DCLK (1:1,000), rabbit anti-pDCLK (1:1,000) or mouse anti-CPG16 (1:500) in blocking buffer, for 1.5 h, washed 5 x 10 min with TBST, incubated in swine anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated antisera (Dako, Glostrup, Denmark) in blocking buffer, for 1 h, washed for 10 min in TBST and 4 x 10 min in Tris-buffered saline. Antibody binding was detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). A lysate of COS-1 cells over-expressing recombinant rat DCL was used as a DCL-positive control for anti-DCLK.

Microscopy, digital imaging and morphometry

Digital images were taken in 5 serial coronal sections per animal at the midlevel of the EWcp (Bregma -5.3 to -6.5 mm) and in 3 serial coronal sections per animal at the level of the PVN (Bregma -1.3 to -2.1 mm), sections interspaced by 125 μm. Images were taken at a resolution of 1,200 x 1,600 pixels, with a Leica DC 500 digital camera mounted on the Leica DMRBE microscope, or at a resolution of 1,024 x 1,024 pixels with the Leica TCS confocal microscope. Since the mathematical correction factor for section thickness according to Floderus (1944) was nearly 1 (0.965), no correction for this thickness was applied. The same sections and neurons were used to quantify the amount of pDCLK per neuron, by determining with the ImageJ software (NIH, Bethesda, MA, USA) the specific signal density (SSD) of 10 randomly taken neurons, which was corrected for background density present in the same section. Counts were averaged per section, and SSD first per neuron and then per section, providing for each parameter one value per animal.

Statistical analysis

Random selection procedures were maintained throughout the experiment. Per parameter values of each experimental group (n = 5) were expressed as means and standard error of the mean (SEM) and statistically analysed by Student's t-test (α = 5%), after testing for normality

(Shapiro and Wilk, 1965) and for homogeneity of variance (Bartlett's Chi-square test; Snedecor and Cochran, 1989) using Microsoft Excel software and Statistica (StatSoft, Tulsa, OK, USA).

Results

Effect of acute pain on cFos

With the cFos antiserum nuclei of neurons in both the PVN and EWcp were intensely stained (Fig. 1). Acute pain strongly increased the number of these cFos-immunoreactive neurons in these brain centres. In the PVN, the number of cFos-immunoreactive cells in APS animals was 109 ± 12, *i.e.*, 3.9 times higher than in the control animals (28 ± 7; $P < 0.001$; Fig. 1). In the EWcp, APS animals revealed 2.5x more cFos-immunoreactive neurons than controls (28 ± 2 *vs.* 11 ± 3; $P < 0.01$; Fig. 1).

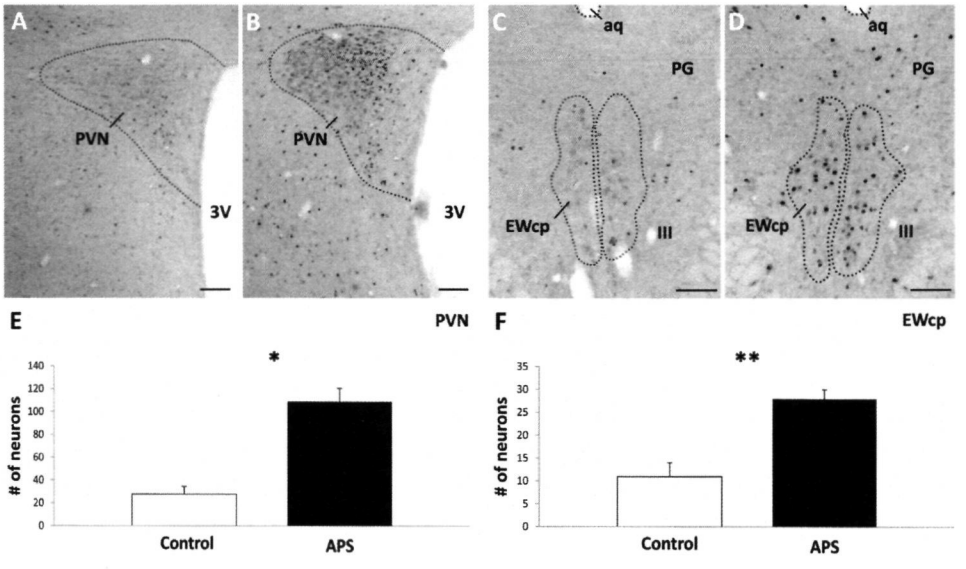

Figure 1. *Immunohistochemistry for cFos in control and acute pain stressed (APS) animals in the paraventricular nucleus of the hypothalamus (PVN; A, B) and the Edinger-Westphal centrally projecting neuron population (EWcp; C, D). Numbers of cFos-positive neurons in the PVN (E) and the EWcp (F). n = 5 per group. * $P < 0.001$, ** $P < 0.01$. 3V, third ventricle; aq, cerebral aqueduct; PG, periaqueductal grey; III, oculomotor nucleus. Scale bars = 100 µm.*

DCLK

Qualitative Western blot analysis demonstrated the presence of multiple DCLK splice variants in lysates containing the PVN or the EWcp (Fig. 2). In these brain areas, the DCLK antiserum, which recognises both DCLK-short and DCL (Boekhoorn et al., 2008; Kruidering et al., 2001), showed three clear bands around 50 kDa in both control and stressed animals, named DCLK-short A, B, and C (Fig. 2). DCL is likely not expressed in the PVN or EWcp, because a 40 kDa band, present in the DCL-positive COS-1 control, was not detected. To test if DCLK-long was present in our samples, we performed a Western blot with the CPG16 antiserum, which recognises DCLKs by binding to their calmodulin kinase domain. This serum revealed two bands in all samples: one band of the 50 kDa DCLK-short splice variants and an 84 kDa DCLK-long splice variant (Fig. 2). In contrast to the DCLK antiserum, the CPG16 antiserum does not recognise other DCLK-short variants, because these lack the epitope to which CPG16 has been raised (Engels et al., 2004). Western blotting with the pDCLK antiserum, to determine which of the DCLK splice variants were present in their phosphorylated form, demonstrated a single immunoreactive band of 84 kDa corresponding to DCLK-long (Fig. 2).

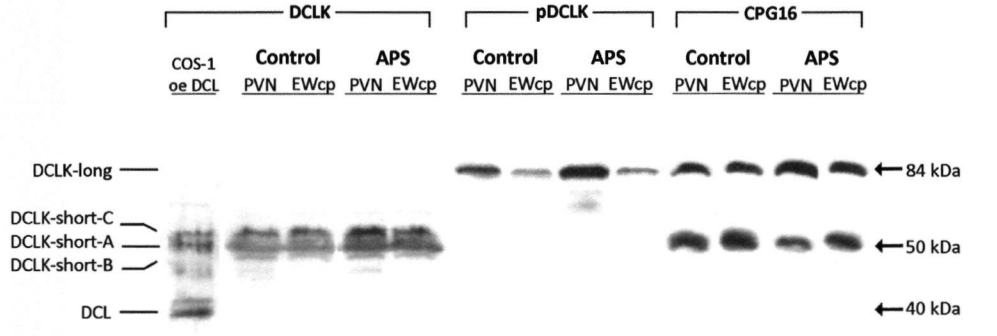

Figure 2. *Western blot analyses of long and short isoforms of doublecortin-like kinase (DCLK), phosphorylated DCLK (pDCLK) and candidate plasticity-related gene (CPG16) in the PVN and the EWcp in a control and an APS rat. An 84 kDa band corresponds to DCLK-long, bands around 50 kDa correspond to DCLK-short-A, -B and -C, and the 40 kDa band corresponds to DCL. COS-1, COS-1 cells over-expressing recombinant rat DCL.*

The (intra)cellular localisation of pDCLK was studied by immunohistochemistry. pDCLK was detected in several brain areas, including the hippocampus, dorsal raphe, lateral septum and basolateral amygdala (data not shown). The presence of pDCLK-long in neurons of the PVN and the EWcp of APS animals is shown in Fig. 3. In the PVN, both magnocellular and

parvocellular neurons were immunopositive. Double staining of pDCLK and CRF indicated co-localisation of the two factors in the parvocellular neurons. In the midbrain, DCLK staining was only present in the EWcp, and double staining demonstrated co-localisation of pDCLK with Ucn1 in EWcp neurons. In both brain areas pDCLK-immunoreactivity was present in perikarya and in proximal parts of dendrites, but was absent from the cell nucleus. The staining revealed a distinct punctuated pattern (Fig. 3).

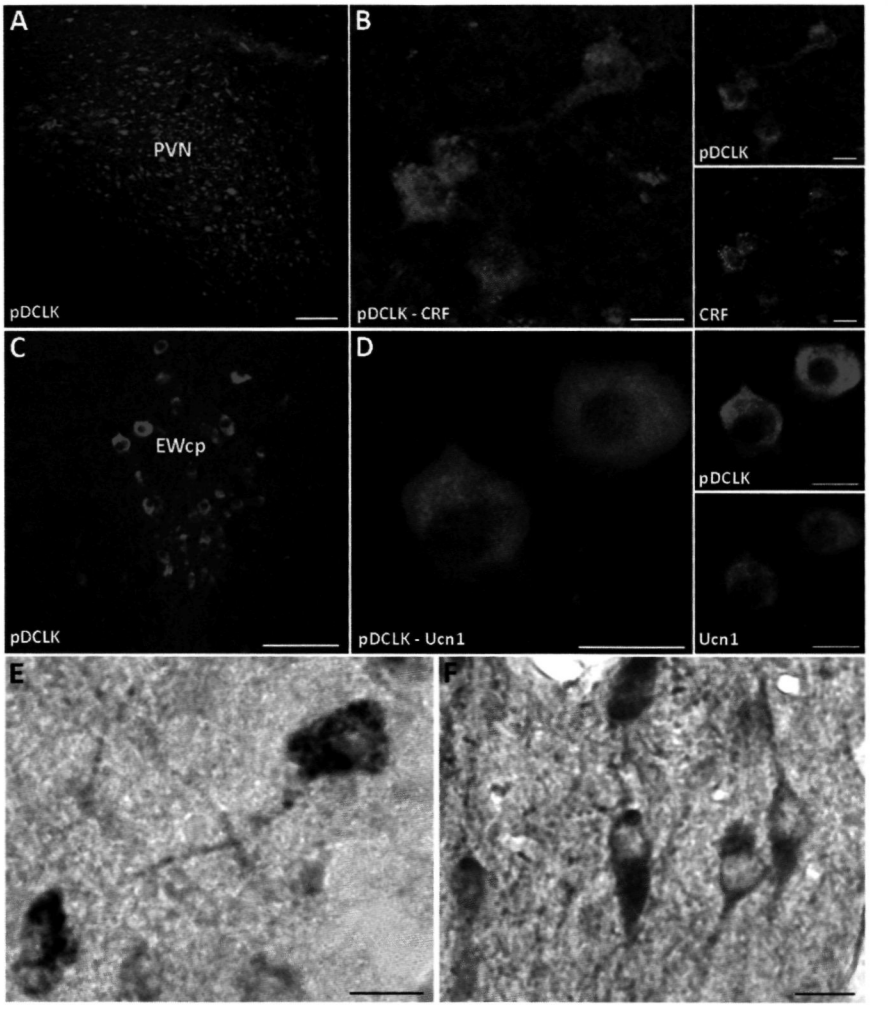

Figure 3. *Immunofluorescence labelling for pDCLK in the PVN (A) and the EWcp (C), with co-localisation of pDCLK (red) and corticotropin-releasing factor (CRF; green) in the PVN (B), and of pDCLK (red) and urocortin 1 (Ucn1; green) in the EWcp (D). DAB-immunohistochemistry showing that pDCLK in PVN (E) and EWcp neurons (F) is restricted to the perikarya and proximal dendrites. Bars = 100 μm in A, C and 25 μm in B, D-F.*

Effect of acute pain on pDCLK

Quantitative immunohistochemistry for pDCLK was performed in both parvocellular neurons of the PVN and in Ucn1-producing neurons of the EWcp, to determine their possible reaction to acute pain. Immunoreactivity to pDCLK occurred throughout the PVN in both controls and APS rats (Fig. 4). Quantification of the number of immunopositive neurons and their SSD did not reveal a significant difference in the parvocellular part (Figs. 4 C, D) nor in the magnocellular part (data not shown).

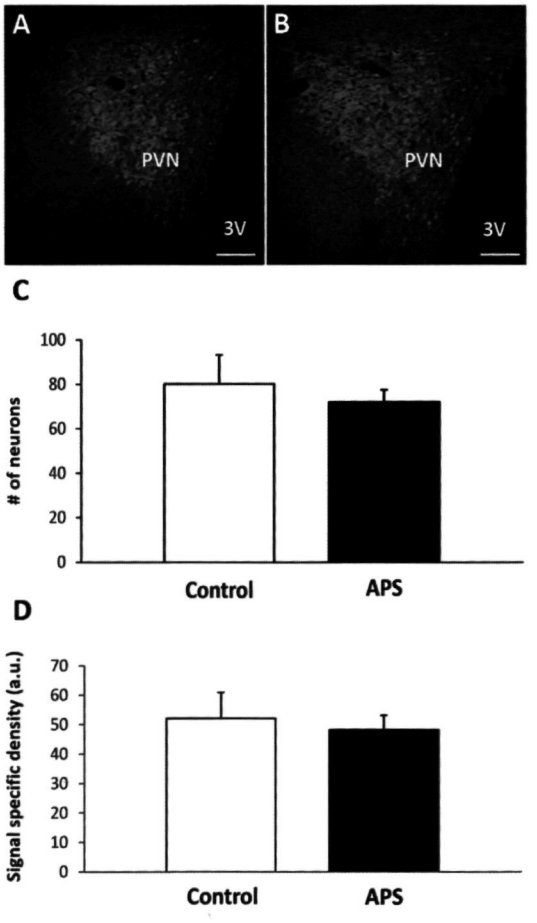

Figure 4. *Immunohistochemistry for pDCLK in the PVN of control (A) and APS rats (B). Quantification of the number of pDCLK-positive neurons (C) and the specific signal density (SSD) of immunostaining (D) in the PVN. n = 5 per group. 3V, third ventricle. Scale bars = 100 μm.*

In the EWcp staining was restricted to neurons in the centrally projecting part (Fig. 5) and the number of pDCLK-immunoreactive neurons in APS animals was 28.0% higher than in the controls ($P < 0.05$; Figs. 5 A, B). Also the SSD per neuron was clearly higher in APS rats, namely 65.3% above control value ($P < 0.05$; Figs. 5 C, D).

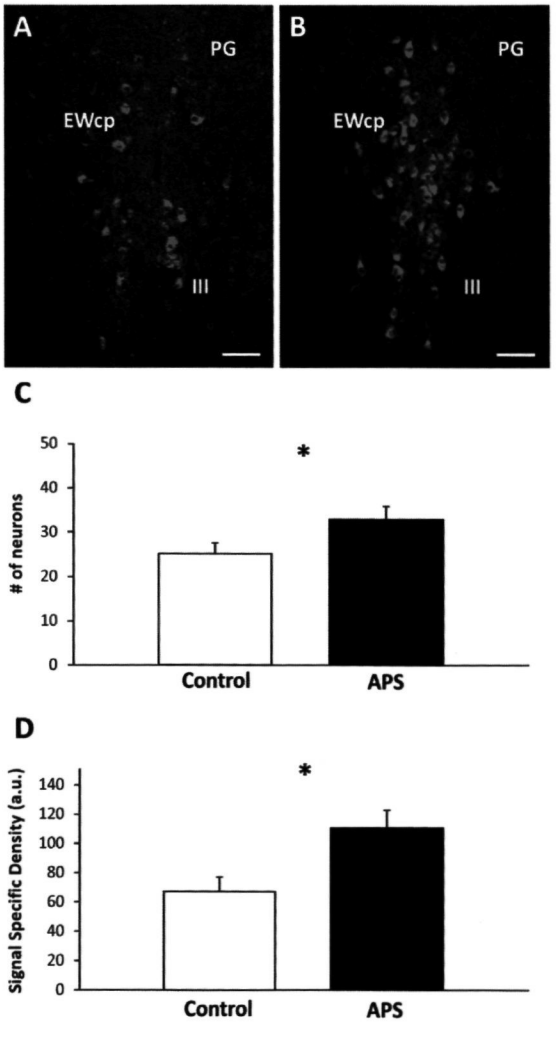

Figure 5. *Immunohistochemistry for pDCLK in the EWcp of control (A) and APS animals (B). Quantification of the number of pDCLK-positive neurons (C) and SSD of individual neurons in the EWcp (D). n = 5 per group. * P < 0.05. PG, periaqueductal grey; III, oculomotor nucleus. Scale bars = 100 μm.*

Discussion

The results from this study support our hypothesis that acute pain activates both the parvocellular neurons in the hypothalamic PVN and the Ucn1 neurons in the midbrain EWcp. However, intracellular signalling cascades recruited by acute pain were different in these stress-sensitive brain centres. We show for the first time that DCLK-short and DCLK-long, but not DCL, are present in these brain nuclei. Remarkably, in the EWcp but not in the PVN, acute pain-induced neuronal activation is accompanied by increased phosphorylation of DCLK-long at its serine residue in the PTSP-motif of the SP-rich domain. We therefore conclude that in the EWcp, DCLK-long phosphorylation of the PTSP-motif is a regulatory step involved in acute pain-induced activation of Ucn1 neurons. Below we will discuss this conclusion in detail.

Acute pain activates PVN and EWcp in a differential manner

Subcutaneous injection of formalin in the hind paw is a widely used method to study the effects of acute pain stress in small rodents. It not only induces homeostatic responses, such as an increase in blood pressure and heart rate (Culman et al., 1997), elevation of the plasma levels of catecholamines and behavioural responses like licking, shaking, lifting or biting of the paw (Abbott et al., 1995; Culman et al., 1997; McCall et al., 1996), but also activates the central stress response because the release of ACTH from the anterior lobe of the pituitary gland as well as corticosterone secretion from the adrenal cortex are increased (Kant et al., 1982; Pacak et al., 1998; Vissers et al., 2004). This activation of the HPA-axis by acute pain also appears from increased expression of cFos in the PVN (Palkovits, 2008). The midbrain EWcp responds to acute pain as well, with increases in Ucn1- and cFos-immunoreactivities (Kozicz, 2001). Our study is the first to quantify immunoreactivity against cFos in the PVN and in the EWcp after an acute pain stressor in the same experiment. We confirm here that acute pain activates both centres. However, in our study acute pain has a quantitatively differential effect on these two centres. Whereas acute pain increases the number of cFos-labelled neurons in the PVN by almost 4 times, it activates the EWcp by only 2.5 times. Possibly, this quantitative difference in recruitment of cFos induction is due to a difference in the response time of the nuclei to the acute pain stressor, because various stressors, including acute pain, are known to activate the PVN more rapidly than the EWcp (Kozicz, 2001; Viau and Sawchenko, 2002).

DCLK splice variants in the PVN and EWcp are differentially regulated

The differential time dynamics of PVN and EWcp activation (Kozicz, 2001; Viau and Sawchenko, 2002) indicate the existence in these nuclei of different intracellular mechanisms

of stressor-induced neuronal activation. Important regulators of intracellular signal transduction and major players in neuronal plasticity are the MAPKs, extracellular signal-regulated kinases (ERKs), p38, and c-Jun N-terminal kinase (Ji et al., 2009). All three MAPKs are involved in pain sensitisation after tissue and nerve injury via distinct molecular en cellular mechanisms (Ji et al., 2009). In particular, ERK activates neurons in the amygdala, which is required for inflammatory pain sensitisation, and neurons in the dorsal horn of the spinal cord by nociceptive activity (for rev. see Ji et al., 2009). However, the role of MAPKs in the EWcp and the PVN remains to be established. MAPKs can furthermore phosphorylate a variety of effector proteins, with cAMP-response element-binding protein as a major target of MAPK-signalling during neuronal plasticity (Impey et al., 1999). Since the DCLK splice variants contain a well-conserved PTSP-motif that functions as a MAPK phosphorylation site, we here investigated whether phosphorylation of this motif is involved in the intracellular signalling of acute pain in the PVN and EWcp.

DCLK splice variants are expressed during development and are involved in neurogenesis, corticogenesis and the control of neuronal migration (Deuel et al., 2006; Koizumi et al., 2006; Shu et al., 2006; Vreugdenhil et al., 2007). Some of these variants are also expressed in the adult brain (Engels et al., 2004; Omori et al., 1998; Silverman et al., 1999) but in contrast to the developing brain, little is known about their function. A first indication that DCLK splice variants play a role in the stress response came from a recent *in vitro* study showing that DCL, the splice variant lacking the kinase domain, is involved in the translocation of the activated glucocorticoid receptor to the nucleus of neuronal progenitor cells (Fitzsimons et al., 2008). In the present study we did not detect DCL in the PVN or EWcp, but showed multiple DCLK splice variants in these centres. More specifically, in parvocellular PVN neurons and in EWcp neurons pDCLK co-localised with CRF and Ucn1, respectively. Three DCLK-short isoforms and DCLK-long are present in the PVN and the EWcp. Both in control and APS animals none of the DCLK-short splice variants were detected by our phospho-specific antiserum, so that, possibly, no phosphorylation at the serine of its PTSP-motif occurs. In contrast to the DCLK-short variants, DCLK-long was present in its phosphorylated form in both control and APS rats. The fact that during conditions of acute pain DCLK-long phosphorylation is increased in the EWcp but not in the PVN, as shown by the quantitative immunohistochemistry data, suggests that the regulation of PTSP-motif phosphorylation of DCLK-long in response to acute pain specifically occurs in the EWcp. Which of the MAPKs in the EWcp is responsible for DCLK-long phosphorylation remains to be established. DCLK-long is the product of the *dclk-1* gene and the *dclk-2* gene (Edelman et al., 2005; Tuy et al., 2008), which could not be distinguished from each other by our antisera as the epitope to which these antisera have been raised is shared by both gene products. However, irrespective of whether *dclk-1* and/or *dclk-2* gene products are present in

the rat PVN and EWcp, our data show that in these two brain centres the functional protein(s) is/are differentially regulated.

By immunohistochemistry pDCLK was detected in several brain areas, but we focused on two brain areas involved in the stress response, viz. the PVN and the EWcp, which are putative brain centres for pain processing. They revealed pDCLK-staining in neuronal perikarya and proximal dendrites but not in cell nuclei. DCLK-long may be involved in axonal transport and neuronal outgrowth by interacting with microtubules via its doublecortin domain (Sapir et al., 2000; Taylor et al., 2000) and in the coordination of actin/microtubule dynamics and the accessory regulation of secretory vesicle sorting and transport (Deuel et al., 2006). A difference between the axonal projections of the PVN and EWcp is the distance over which their secretory vesicles need to be transported; parvocellular PVN neurons project mainly to the median eminence in the hypothalamus (Henry et al., 2005), which is a relative short distance compared to the distance between the EWcp and some of its main projection sites, such as the lateral septal nucleus and spinal cord (Bittencourt et al., 1999). Possibly, an increased phosphorylation of the PTSP-motif of DCLK-long warrants a more efficient axonal transport of Ucn1-containing secretory vesicles over long distances in EWcp neurons. MAPK-signalling may be involved in activity-dependent potentiation of large dense-core vesicle release (Park et al., 2006), a process mediated by calcium-dependent activation of ERK-signalling, as inhibition of ERK-signalling suppresses activity-dependent vesicle translocation. Clearly, in the absence of specific experimental data, other functions of DCLK in the neurons studied remain speculative.

Conclusions

In this study we have demonstrated the presence of DCLK-short and DCLK-long isoforms in the hypothalamic PVN and midbrain EWcp of adult rats. Acute pain-mediated up-regulation of phosphorylated DCLK-long in the EWcp but not in the PVN indicates a differential regulation of acute pain-induced DCLK-long in two stress-sensitive brain nuclei. This differential response opens new avenues for the treatment of pain. We propose DCLK as a possible novel therapeutic target for the development of specific inhibitors of MAPK pathways involved in stress and pain signalling.

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Chapter 6

The amygdala, a relay station for switching on and off pain

Role of limbic corticotropin-releasing factor in neuropathic pain

Tom P.H. Rouwette, Pascal Vanelderen, Eric W. Roubos, Tamás L. Kozicz, Kris C.P. Vissers

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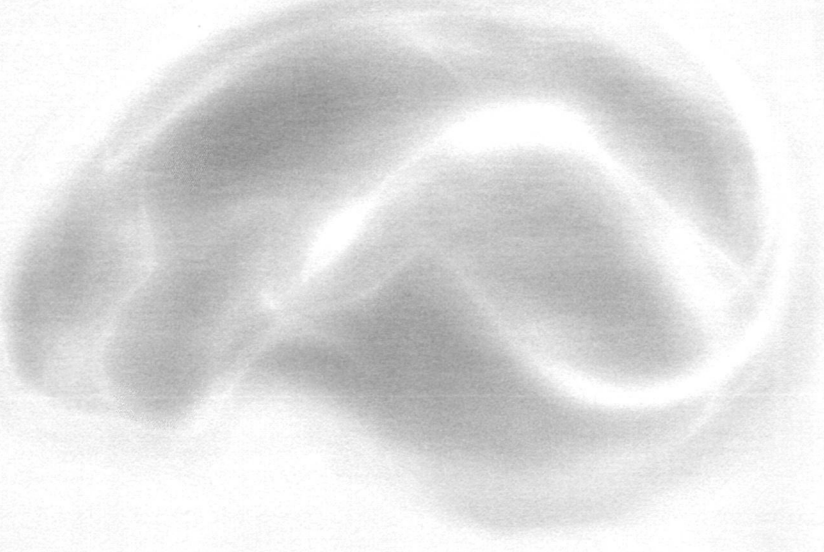

Abstract

Neuropathic pain is strongly associated with mood disorders like anxiety and depression. Corticotropin-releasing factor (CRF) plays a prominent role in these disorders as it is up-regulated in limbic structures such as the amygdala, upon experimentally-induced neuropathy. This review discusses recent literature on the role of CRF in pain processing and highlights the amygdala as a potential hot-spot in supraspinal descending pain control. Many studies have demonstrated analgesic effects of CRF following local and systemic administration, but more recently also hyperalgesic effects were shown upon endogenous amygdalar CRF increase or by blocking the CRF type 1 receptor (CRFR1). On the basis of the reviewed literature, we postulate a central mechanism for pain control in which the amygdala plays a critical role by turning chronic pain on and off. In this mechanism, upon pain stimuli, CRFR1 in the amygdala is activated by CRF to induce hyperalgesia. When the activated CRFR1 is internalised (pain initiation) it triggers the translocation of the cytoplasmic CRF type 2 receptor (CRFR2) to the plasma membrane. Here, CRFR2 can be recruited by either high (pharmacological) concentrations of CRF or by endogenous CRFR2 ligands, the urocortins, leading to analgesia (pain termination). This on-off switching of pain is completed by redistribution of the CRF receptors to their initial activity state. We furthermore propose that in neuropathic pain, this amygdalar switch is dysregulated and causes a state of permanent hyperalgesia, and present an integrative (patho)physiological model for the way disturbed CRF receptor signalling in the amygdala could initiate neuropathic pain.

Introduction

Corticotropin-releasing factor (CRF) is essential in stress adaptation, as it evokes physiological stress responses via the hypothalamo-pituitary-adrenal (HPA-) axis and produces a variety of behavioural (De Souza, 1987; Dunn and Berridge, 1990; Smagin and Dunn, 2000; Yarushkina, 2008), autonomic (Chrousos and Gold, 1992; McNally and Akil, 2002) and endocrine effects (Steckler, 2001; Vale et al., 1981). CRF exerts these effects by acting as a neurotransmitter and/or neuromodulator in the central nervous system (CNS), especially in extra-hypothalamic centres like the limbic bed nucleus of the stria terminalis (BST) and the central amygdala (CeA)(Deyama et al., 2007; Ji and Neugebauer, 2008). Chronic pain often disturbs HPA-axis functioning, and is highly co-morbid with anxiety and depression (Blackburn-Munro and Blackburn-Munro, 2001), mood disorders frequently associated with dysfunctioning of limbic CRF (Arborelius et al., 1999; Davis and Shi, 1999; Regev et al., 2011). However, only since recently, the role of CRF in pain processing has gained the strong attention it deserves (Lariviere and Melzack, 2000). Evidence is increasing that limbic CRF is involved in nociceptive sensitivity during neuropathic pain (Rouwette et al., 2012; Ulrich-Lai et al., 2006) and that *Crf* gene responsiveness is down-regulated following this pain (Bomholt et al., 2005). This review summarises recent data on brain regions involved in neuropathic pain control, with emphasis on CRF, and proposes that the amygdala is one of the key centres in pain processing, being able to turn nociception and analgesia on and off.

Neuropathic pain

Neuropathic pain arises as a direct consequence of a lesion or disease affecting the somatosensory system (Treede et al., 2008). Patients with neuropathic pain suffer from various painful symptoms, including spontaneous and provoked pain, the latter consisting of allodynia and hyperalgesia. Allodynia is pain triggered by normally innocuous stimuli, whereas hyperalgesia corresponds to an exaggerated response to a pain stimulus. Although the exact prevalence of neuropathic pain is unknown, several studies assume the occurrence of neuropathic pain in the general population to range from 1% to 8% (Bouhassira et al., 2008; Dieleman et al., 2008; Torrance et al., 2006). Common causes of neuropathic pain are posttraumatic and postsurgical nerve injury, diabetic polyneuropathy and postherpetic neuralgia (Daousi et al., 2004; Davies et al., 2006; Finnerup et al., 2001; Galil et al., 1997). Patients with neuropathic pain suffer significantly more from depression, anxiety and sleep disorders than patients without this type of pain (Freynhagen et al., 2006). Neuropathic pain is a complex disease to treat, and many patients respond differentially to common medications with unpredictable effectiveness, complicated dosing, delayed analgesic onset

and occurrence of various side-effects (Dworkin et al., 2007). To improve the treatment of neuropathic pain in patients, better insight into the underlying mechanisms of this disorder is necessary (Vissers, 2006).

Pain processing in the CNS takes place via ascending and descending pathways. First, peripheral pain sensory signals are transported up to the dorsal horn of the spinal cord and then distributed to different brain centres where processing takes place. The two main ascending pathways are the spinoparabrachial pathway, which projects to brain areas involved in mood, like the amygdala, and the spinothalamic pathway, which is particularly concerned with sensory effects (Hunt and Mantyh, 2001). The main descending pathways originate in the cortex, hypothalamus and amygdala, and relay information via the periaqueductal grey, to control autonomic and antinociceptive responses to the pain stimulus (Fields, 2004; Hunt and Mantyh, 2001). Both ascending and descending pathways involve brain areas implicated in mood control and the generation of mood disorders (*e.g.* the amygdala), indicating a clear link between persistent pain and affective disorders like anxiety and depression. This also indicates that the amygdala serves as an integrative centre of affective (via the spinoparabrachial ascending pathway) and sensory (via the descending pathway) components of pain signalling. Anxiety and depression arising as a consequence of neuropathic pain are thought to be primarily mediated by inactivation of inhibitory descending pain pathways and activation of stimulatory descending pain pathways (McWilliams et al., 2003, 2004; Ro and Chang, 2005). Therefore, therapy of neuropathic pain complaints focuses on strengthening the descending inhibitory pathways, mainly via increasing serotonin and noradrenalin concentrations in the synaptic cleft at spinal and supraspinal levels (Dharmshaktu et al., 2012). At present, pharmaceutical treatment of neuropathic pain mainly involves application of antidepressants and anti-epileptics and, to a lesser extent, opioids, whereas simple analgesics have shown little or no efficacy to treat this type of pain (Attal et al., 2006). Not only are antidepressants useful in neuropathic pain therapy for their effect on concomitant depression, they also contribute to the reinforcement of descending inhibitory pathways by affecting noradrenalin and serotonin concentrations in the synaptic cleft, both supraspinally and spinally (Dharmshaktu et al., 2012).

Antidepressants have been proven to be effective in the treatment of peripheral neuropathic pain. However, their mechanisms of action in the brain are not well-understood. Therefore, further studies exploring not only peripheral but also central mechanisms of neuropathic pain, with focus on brain centres involved in (mood) disorders arising from neuropathic pain, are needed.

Based on our recent work (Rouwette et al., 2012), we postulate a major function of limbic amygdalar CRF in the descending control of pain, and suggest the CRF system of the amygdala as a potential therapeutic hot-spot.

Corticotropin-releasing factor

CRF is a 41-amino acid peptide originally isolated from the ovine hypothalamus (Vale et al., 1981). It plays an important role in physiological processes, especially the central stress response, and is implicated in the pathogenesis of various disorders (Bale and Vale, 2004). The biological action of CRF and related peptides is mediated by the G-protein-coupled CRFR1 and CRFR2 receptors, of which CRFR1 has highest affinity for CRF (Hauger et al., 2006). CRF is strongly involved in the processing of pain signals, and is analgesic when administered either centrally or peripherally (Lariviere and Melzack, 2000). More than a decade ago, Lariviere and Melzack (2000) thoroughly reviewed the role of CRF in pain and analgesia. They provided evidence that: 1) CRF may produce analgesia at various levels of the CNS, 2) β-endorphin release is not responsible for the analgesia observed after intracranial or intravenous CRF administration, 3) CRF evokes analgesia only in the presence of inflammation, and 4) the analgesic action of CRF is specific for prolonged pain. These considerations suggest a significant role of CRF in chronic pain syndromes, and indicate the possibility that CRF and CRF-related peptides may form a new class of painkillers.

Whereas the analgesic effects of CRF have been demonstrated in many studies, focus has been largely on peripheral effects, for mainly methodological reasons (Lariviere and Melzack, 2000). However, in the past decade, the role of CRF in the CNS in pain and analgesia gained increasing attention, and it has become clear that the peptide has pleiotropic effects: it can induce analgesia but in some rodent pain models opposite, hyperalgesic effects of CRF have been shown (Bourbia et al., 2010; Lariviere et al., 2011). To substantiate the significance of forebrain CRF in pain control, we will first discuss the CNS effects of CRF and CRF (receptor) antagonists on pain behaviour. Secondly, we will look at changes in CRF-expressing brain centres upon neuropathic pain induced by peripheral lesions. Finally, we will propose the amygdala as a key centre in switching pain on and off, and discuss the implications of this idea for future research and therapy.

CRF administration

After the ground-breaking work by Lariviere and Melzack (2000), their view has been extended by several publications on the effect of CRF or CRF receptor agonists/antagonists on pain behaviour in rodents. Intracerebroventricular (i.c.v.) injection of CRF produces dose-dependent antinociceptive effects on the perception of mechanical and heat stimuli in Lewis and Fischer rats (Vit et al., 2006). In the same study, i.c.v. CRF administration was shown to cause analgesia during the interphase of pain stimulation by formalin application, but did not show an effect during the first or second phase of this test (Vit et al., 2006). Antinociceptive effects of CRF administration during the interphase of the formalin test were also

demonstrated in male and female rats (Lariviere et al., 2011), which indicates that CRF is antinociceptive by activating descending pain inhibitory pathways to the spinal cord. However, rats showed an increase in licking behaviour during the first and second phase of the test, indicating a hyperalgesic effect of CRF during these phases (Lariviere et al., 2011). Not only CRF affects pain behaviour, also CRFR1 antagonists are effective in reducing or even completely reversing thermal and mechanical hypersensitivity produced by a number of different types of pain, including ulcer pain, and inflammatory- and neuropathic-like pain (Hummel et al., 2010). More specifically, CRF in the CeA of the limbic system plays an important role in this pain signalling. It was found that intra-CeA administration of high concentrations (0.1 and 0.01 nmol) of CRF, but not of a low concentration (0.001 nmol), substantially increases the latency of the hind paw withdrawal response (HWL) to noxious thermal and mechanical stimuli, which indicates the antinociceptive action of exogenous CRF in the CeA (Cui et al., 2004). This effect was attenuated by an intra-CeA injection of CRF_{9-41}, a non-selective CRF receptor antagonist. Administering this antagonist alone decreased the HWL. The authors state that both exogenous and endogenous CRF exert an antinociceptive effect in the CeA that is mediated by CRF receptors (Cui et al., 2004). However, injection into the CeA of CRF_{6-33}, an inhibitor of CRF-binding protein (CRF-BP), which increases free, endogenous CRF, increases sensory pain, whereas CRF_{9-41} reverses the effects of CRF_{6-33} on pain sensation (Bourbia et al., 2010). Together, these studies strongly argue in favour of a role of low dose, endogenous CRF in stimulating pain sensitisation and of an antinociceptive action of high concentrations of exogenous CRF. Systemic (intraperitoneal) or intra-amygdalar administration of NBI27914, a selective CRFR1 antagonist, has an antinociceptive effect, evidenced by an increased HWL, and it augments the preference for the open arms in the elevated plus maze test, in rats where anxiety and pain-like behaviour was induced by arthritis (Ji et al., 2007). These findings were confirmed by showing that low concentrations of CRF facilitate nociceptive processing in the CeA via CRFR1 and protein kinase A activation (Ji and Neugebauer, 2008). In contrast, high concentrations of CRF have inhibitory effects via CRFR2, which is evident from the fact that they can be reversed by the selective CRFR2 antagonist astressin-2B (Ji and Neugebauer, 2008). This notion is in agreement with the idea that CRFR1, with CRF as its main ligand, is responsible for the initiation of pain responses, and CRFR2 for dampening or even reversing the CRFR1-initiated responses (de Kloet et al., 2005; Rouwette et al., 2011). Indeed, both CRFR1 and CRFR2 are present in the amygdala as well as in several subpopulations of the BST (Korosi et al., 2006; Van Pett et al., 2000). Below, these findings will be discussed in more detail.

CRF expression in the brain following neuropathic pain

Although a vast number of reports describe changes in anxiety and depressive-like behaviour following CRF or CRF antagonist injections, both systemically and brain region-specifically, only few experimental studies have been published that concern the expression of CRF mRNA and CRF peptide in the rodent brain following neuropathic pain. Ulrich-Lai et al. (2006) investigated the long-term impact of chronic pain on the HPA-axis and the limbic system in a rodent model for neuropathic pain: chronic constriction injury (CCI) of the rat sciatic nerve. They showed that CCI does not affect indices of basal or restraint stress-induced HPA-axis activity, but that it does increase CRF mRNA expression in the CeA but not in the PVN and the BST. This suggests that increased nociceptive sensitivity during chronic pain is particularly associated with changes of the limbic system but is dissociated from activation of the HPA-axis. Unchanged HPA-axis functioning is also seen following CCI, which does not affect the expression in the PVN of the immediate early gene products cFos and deltaFosB, markers for acute and long-term neuronal activity, respectively, nor does it alter the blood corticosterone titre (Bomholt et al., 2005). Also, CRF mRNA in the PVN does not differ between shams and CCI rats. In contrast, after 20 min of acute (restraint) stress, both immediate early genes in the PVN and the corticosterone titre had been significantly up-regulated. However, whereas shams displayed an increase in CRF mRNA, as often seen in acute stress paradigms, CCI rats did not show this increase (Bomholt et al., 2005). This indicates that neuropathic pain leads to a down-regulation of *Crf* gene responsiveness in the PVN, possibly repositioning the set-point of HPA-axis activation. Therefore, the authors concluded that their data do not support the hypothesis that sustained pain-like behaviour in the CCI model of neuropathic pain leads to a primary disturbance of HPA-axis functioning, but that further research is required to substantiate that conclusion (Bomholt et al., 2005). This notion has led us to investigate, in the CCI model, not only the dynamics of CRF mRNA in the PVN, CeA and BST, but also to extend these analyses to CRF peptide and the related peptide Ucn1. We showed that CCI leads to an increase in CRF mRNA in both the BST and CeA. In addition, in these rats, the CeA, but not the BST, contains more CRF peptide. In contrast, CRF mRNA and CRF peptide in the PVN are unaffected by CCI. Similarly, neurons of the Edinger-Westphal centrally projecting neuron population (EWcp; Kozicz et al., 2011a), producing the CRF family member urocortin 1 (Ucn1) and constitutively activated by various stressors including acute pain, did not show an effect of CCI on Ucn1 mRNA or Ucn1 peptide. Also, cFos and deltaFosB in the EWcp were unaffected by CCI (Rouwette et al., 2012). These results indicate that neuropathic pain does not act via the HPA-axis or the EWcp, but involves CRF in the limbic system, which is in clear contrast to acute and chronic stressors primarily acting on the PVN and the EWcp (Rouwette et al., 2011, 2012).

Tumor necrosis factor-alfa

There are several experimental animal models for neuropathic pain, such as CCI (Bennett and Xie, 1988; Kim et al., 1997; Kontinen et al., 2003; Xie et al., 1995), partial sciatic nerve ligation (Kim et al., 1997; Kim and Chung, 1992; Seltzer et al., 1990), spinal nerve transection (Liu et al., 2000), sciatic nerve transection (Wall et al., 1979), and spared nerve injury (Decosterd and Woolf, 2000), and whereas these different models involve various degrees of nerve damage, they all share the presence of post-injury inflammation, with mast cell degranulation and the recruitment of macrophages and polymorphonuclear neutrofils (Daemen et al., 1998; Olsson, 1967; Perry et al., 1987). It is evident that this inflammatory microenvironment is of crucial importance for the development of neuropathic pain, because the CCI model reveals thermal hyperalgesia even though the ligatures are placed loosely around the sciatic nerve without actually mechanically damaging it (Frisen et al., 1993; Maves et al., 1993; Sommer et al., 1993). Furthermore, suppression of the inflammatory response attenuates thermal hyperalgesia, whereas increasing this response strengthens pain hypersensitivity (Clatworthy et al., 1995). This notion led to on-going research on immune and pro-inflammatory mediators in neuropathic pain, including the involvement of eicosanoids, bradykinins, serotonin, ATP/ADP, neurotrophins, cytokines, chemokines and reactive oxygen species, all of which contribute to the development of neuropathic pain (Leung and Cahill, 2010). These mediators do not only act at the peripheral site of injury but are also active in the CNS. This appears from the fact that in arthritic mice 24 hours after neutralisation of tumor necrosis factor-alfa (TNF-α) by intravenous infusion of a monoclonal antiserum to TNF-α, nociceptive activity of the limbic system was blocked, whereas at the same time clinical and laboratory markers of inflammation in the affected limb remained unaffected (Hess et al., 2011). These authors furthermore found that arthritic mice over-expressing TNF-α reveal altered pain behaviour and that their brain activity upon nociceptive stimulation is much higher than in wild-type mice. After immunoneutralisation of TNF-α these changes were rapidly reversed. These results suggest that TNF-α is involved in the generation of nociceptive brain activity, at least in the context of arthritis, and that neutralisation of TNF-α affects this changed activity long before it induces anti-inflammatory phenomena in the joints. Furthermore, cytokines like interleukin-1β, interleukin-6 and interferon-β are up-regulated in the forebrain upon peripheral inflammation induced by polyinosinic-polycytidylic acid (Konat et al., 2009). Similarly, intraperitoneal lipopolysaccharide administration in the amygdala induces not only TNF-α but also interleukin-1β and interleukin-6. Interestingly, these changes in amygdaloid activity are accompanied by an increase in anxiety (Engler et al., 2011). These data show that peripheral inflammation can increase various types of cytokine in forebrain nuclei including the amygdala, to induce behavioural changes.

A model

CRF can act both stimulatory and inhibitory on pain sensation; low CRF concentrations in the amygdala have a nociceptive effect via activation of CRFR1, whereas high, pharmacological concentrations show analgesic effects by recruiting CRFR2 in the amygdala, as stated above. Activation of CRFR1 in the amygdala could occur via CRF released from the BST, a nucleus that shows up-regulation of CRF mRNA following peripheral neuropathy (Rouwette et al., 2012) and heavily projects to the amygdala (Dong et al., 2001). The parabrachial nucleus (PBN) in the pons shows a high presence of CRF-containing neurons and links the CRF system in the amygdala to the spinoparabrachial pain pathway and implicates CRF as a transmitter of nociceptive information to the amygdala (Ji et al., 2007; Merchenthaler et al., 1982). However, CRF has a very low affinity for the CRFR2, and is not an endogenous ligand for this receptor. On the other hand, CRFR2 binds the endogenous ligands Ucn1, Ucn2 and Ucn3 with high affinity and high specificity (Bale and Vale, 2004). This fact raises the possibility that urocortins released in the amygdala could play a role in CRFR2-mediated analgesia. Indeed, several brain areas containing Ucn1-, Ucn2- and Ucn3-expressing neurons heavily project to the amygdala. The EWcp, which is the brain's richest source of Ucn1 and implicated in pain and stress responses (Bale and Vale, 2004; Kozicz, 2001, 2007; Kozicz et al., 2011b; Rouwette et al., 2011), seems to innervate the amygdala (da Costa Gomez and Behbehani, 1995; Li et al., 1990). However, the lack of Ucn1 terminals in the amygdala and our recent data showing that Ucn1 in the EWcp does not differ between shams and CCI rats do not support a role of Ucn1-EWcp in chronic neuropathic pain (Rouwette et al., 2012). This raises the question if other pain-sensitive and Ucn-producing centres might control the amygdala. The locus coeruleus (LC) in the pons, expressing Ucn2 and classically considered as a source of pain inhibition, also strongly projects to the amygdala (Millan, 2002; Tsatsanis et al., 2007). To date, however, no Ucn2-immunoreactive axon terminals have been demonstrated in the amygdala. In contrast, dense networks of Ucn3-immunoreactive axons innervate this nucleus (Lewis et al., 2001), strongly suggesting that Ucn3 might be the endogenous ligand responsible for CRFR2-mediated inhibitory effects on pain modulation in the amygdala. Ucn3-expressing neurons occur in the rostral perifornical area of the hypothalamus (PFH) and in the posterior BST (Lewis et al., 2001; Li et al., 2002; Wittmann et al., 2009a), brain areas sending efferents to the amygdala (Wittmann et al., 2009b). Furthermore, the PFH is indeed involved in stress-induced analgesia in rats (Gerashchenko et al., 2011). The above presented pieces of neuroanatomical evidence clearly provide the morphological framework for distinct actions of CRF-CRFR1 (hyperalgesia) and Ucns-CRFR2 (analgesia) in the amygdala.

On the basis of integrating the morphological and pharmacological data reviewed above, we put forward the following novel concept of pain processing in the amygdala. In the

initial response to pain, CRF would be released from the dorsolateral BST or PBN to the amygdala, and activates CRFR1 in amygdalar neurons. Initial activation of CRFR1 produces strong hyperalgesia that is necessary to protect the organism against further tissue damage. Simultaneously, activation of CRFR1 results in transport of CRFR2 towards the plasma membrane where it will bind CRFR2 ligands such as the urocortins released from the posterior BST, PFH and/or LC. This second phase of CRF receptor activation produces an analgesic effect important for the termination of pain. This concept implicates that balanced and proper sequential recruitment of CRF receptors in the amygdala is crucial for successful management of the pain response. Consequently, imbalance in this process or, for instance, failed activation of CRFR2 could result in an enhanced and chronic pain response such as seen during neuropathic pain. This proposed mechanism is explained in more detail in Figure 1. Such a CRF receptor-mediated response is not without precedent; a similar involvement of CRF receptors has been suggested to control the initial and later adaptive phase of the stress response in the serotonergic dorsal raphe system (Kozicz, 2010; Waselus et al., 2009).

Meanwhile, the question arises how this process would derail. Possibly, excessive release of cytokines during neuropathic pain would contribute to this disturbance, as patients suffering from complex regional pain syndrome experience elevated interleukin levels in the cerebrospinal fluid, and patients with failed back surgery show an increased TNF-α titre in the CSF (P. Vanelderen, pers. comm.). In particular, a number of studies have demonstrated the involvement of cytokines in CRF-signalling in the amygdala. More specifically, IL-2 stimulates *in vitro* amygdalar CRF in a dose-dependent manner (Raber et al., 1995), whereas IL-6 increases CRF mRNA in a time- and dose-dependent manner in dissociated amygdalar cultures and elevates both secretion and intracellular content of CRF peptide in this nucleus (Kasckow et al., 1997). Also, microinjection of TNF-α into the CeA facilitated anxiety-like behaviour and adding SSR125543, a CRFR1 antagonist, prior to the repeated TNF-α administration partly reduced this behaviour (Knapp et al., 2011). These findings provide evidence for involvement of cytokine action on CRF receptors in the neuropathic pain response, either directly on the amygdala or indirectly via CRF receptors in the BST.

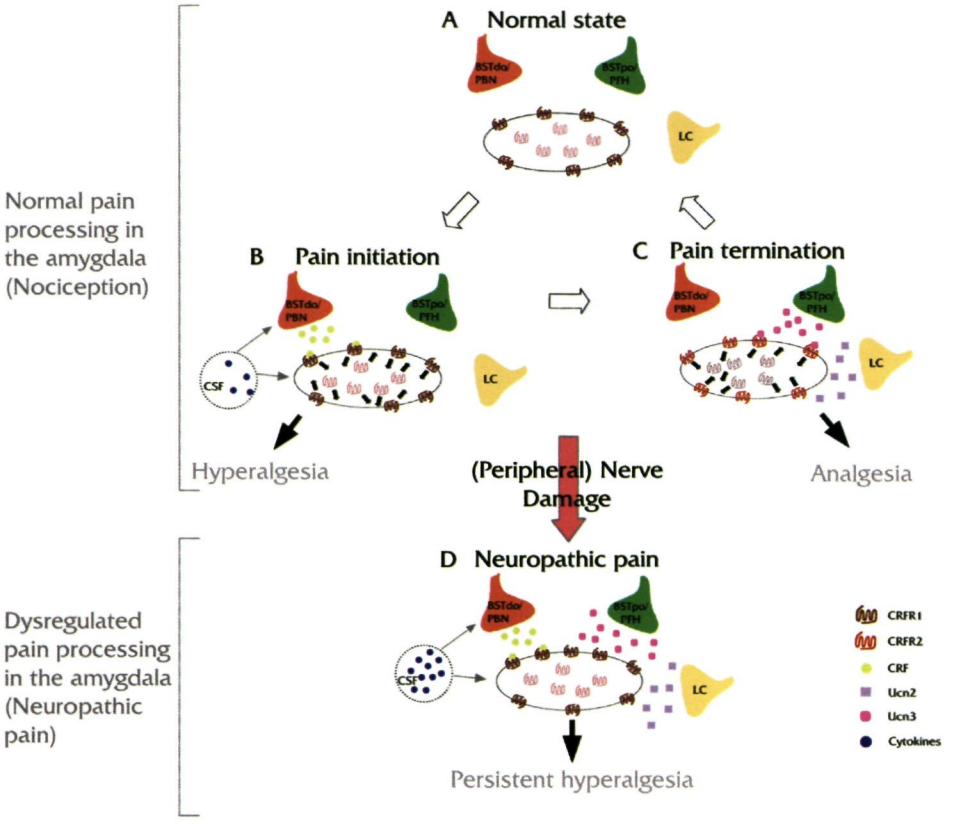

Figure 1. *Proposed mechanism for pain processing in the amygdala. In the normal state, corticotropin-releasing factor receptor 1 (CRFR1) is located on the plasma membrane of amygdalar neurons and CRFR2 in the cytoplasm (A). Upon painful stimuli, CRF is released from the dorsolateral bed nucleus of the stria terminalis (BSTdo) or parabrachial nucleus (PBN) and activates CRFR1, leading to hyperalgesia (B). Then, CRFR1 internalises and CRFR2 is recruited to the plasma membrane. Pain termination occurs when urocortin 3 (Ucn3), released from the posterior BST (BSTpo) or perifornical hypothalamus (PFH), or Ucn2 from the locus coeruleus (LC), binds to CRFR2. Binding of urocortins to CRFR2 results in analgesia and triggers CRFR2 to internalise and CRFR1 to be transported again to the plasma membrane (C), returning the amygdala to its normal state (A). In neuropathic pain, this amygdalar on-off switching is disturbed, so that pain initiation does not lead to redistribution of CRF receptors. As a consequence, urocortins cannot bind the still cytoplasmic CRFR2 and pain continues. This amygdalar dysfunction is triggered by an excessive release of cytokines from the cerebrospinal fluid (CSF) under chronic pain conditions (D).*

Implications of the model

The possible added value of our hypothesis is that it does not only help to explain the pathogenesis of neuropathic pain, but also reveals why neuropathic pain is so often associated with mood disorders. As stated before, imbalance and/or dysfunction of CRF receptors in the amygdala has been implicated in mood disorders such as anxiety and depression (Argoff, 2007; Blackburn-Munro and Blackburn-Munro, 2001; Hunt and Mantyh, 2001; Manas et al., 2011; Sherbourne et al., 2009) and with anxiety and depressive-like behaviour in rodents (Hu et al., 2009; Jesse et al., 2010; Roeska et al., 2008, 2009). CRF plays a prominent role in these disorders, because over-expression of this neuropeptide in the mouse CeA and dorsolateral BST affects anxiety and depressive-like behaviour, respectively (Regev et al., 2011), and peripheral mononeuropathy evokes an increase in CRF in the rat CeA en BST (Rouwette et al., 2012; Ulrich-Lai et al., 2006). Although there is a large body of evidence that CRF secreted from PVN neurons plays a key role in human mood disorders (Blackburn-Munro and Blackburn-Munro, 2001), this link cannot be fully established using animal models of neuropathic pain. Furthermore, there is growing evidence that neither the PVN of the hypothalamus nor the EWcp is involved in the induction of neuropathic pain in rats (Bomholt et al., 2005; Rouwette et al., 2012; Ulrich-Lai et al., 2006). These findings led us to postulate that HPA-axis dysfunctioning is not necessary for the development of anxiety and depressive-like behaviour in animals with neuropathic pain induced by constriction of the sciatic nerve. Although there are several studies reporting changes in HPA-axis activity and concomitant prevalence of mood disorders following chronic pain, we assume that the major centres involved in neuropathic pain and co-morbid depression and anxiety are located in the limbic system with, especially in the amygdala, important roles for CRF, CRF-related peptides and their receptors. (Other) limbic centres may be triggered by anti-inflammatory factors, and undergo and maintain a changed phenotype even when the initial source of inflammation has disappeared. Furthermore, imbalance between the action of CRF receptors and their ligands in the limbic system may explain the strong pain sensitivity observed in both neuropathic rodents and humans. Therefore, it is likely that targeting specifically CRF receptors in the amygdala would be an important therapeutic target in the treatment of neuropathic pain and co-morbid mood disorders.

To date, several CRF antagonists have entered clinical trials to establish an effect on depression, anxiety and pain (Binneman et al., 2008; Coric et al., 2010; Zorrilla and Koob, 2004). Although a phase IIa trial showed reduction in anxious and depressive symptoms in patients, the development of this drug had to be discontinued because of the occurrence of elevated liver enzyme levels (Zorrilla and Koob, 2010). Currently, several other CRF antagonists are being tested, which will hopefully further underline the potential for

therapeutic use of (antagonists of) CRF and its related peptides like the urocortins, to combat depression, anxiety and chronic neuropathic pain.

Future perspectives

To validate our model of the amygdala as a relay station in turning the pain response on and off, some essential questions have to be answered, for example as to the precise distribution of the CRF receptors within the amygdala. Understanding the mechanism of pain processing in the amygdala requires detailed knowledge of the distribution of CRF receptors in this nucleus. The notion that under basal conditions CRFR2 is mainly located in the cytoplasm, and pain-induced activation of CRFR1 results in the translocation of CRFR2 from the cytoplasm to the plasma membrane, needs to be validated using ultrastructural approaches. Also, the question should be answered whether or not both CRF receptor types are co-localised within the same neuron. In this respect, two major populations of neurons in the amygdala, viz. CRF- and met-enkephalin-positive neurons, are of particular interest. It is intriguing to assume that CRF activates CRFR1 in CRF neurons and plays a role in pain initiation concomitant with hyperalgesia, whereas urocortins might act on CRFR2 to recruit the met-enkephalin-positive neuron population mediating pain termination concomitant with analgesia.

The PBN as well as the dorsolateral subdivision of the BST are candidate pain-sensitive brain areas sending strong CRF-positive efferents to the amygdala, thereby possibly activating CRFR1 to initiate the pain response. However, the exact origin of urocortins possibly involved in the amygdalar switch should be established, with the PFH, posterior BST and the LC as likely candidates as the source of origin of CRF and urocortinergic fibre terminals in amygdala.

In order to have proper pain initiation and termination, it is likely that upon painful stimuli, first CRF is released in the amygdala. Urocortins, being able to cause analgesia and to terminate the pain response, would be released at a later time point. It is therefore critical to establish such a temporal pattern for the release of CRF and urocortins within the amygdala. In addition, it should be tested if in neuropathic pain, this temporal pattern of CRF/urocortins release is derailed or does not happen at all.

In animal models, with viral vectors, optogenetic tools and specific CRFR agonists/antagonists the level of expression and activation of CRF receptors can be manipulated and consequently linked to pain-related behavioural indices. In this way, the role of the (dysregulated) amygdalar CRF/Ucns system in the (patho)physiology of the pain response may be further elucidated.

Complex regional pain syndrome and failed back surgery lead to elevated cytokine concentrations in the cerebrospinal fluid (P. Vanelderen, pers. comm.) and cytokines cause

an up-regulation of amygdalar CRF that can be counteracted by administration of CRFR1 antagonists (Kasckow et al., 1997; Knapp et al., 2011; Raber et al., 1995). This shows that cytokines play a role in CRF-mediated pain signalling in the amygdala. It needs to be studied if chronically and strongly elevated cytokines in the amygdala are causative in the dysregulation of balanced CRF receptor activation during pain.

As strong individual differences exist in vulnerability to develop neuropathic pain, the possibility that single nucleotide polymorphisms (SNPs) in CRFR1, CRFR2 and cytokine receptors underlie these individual differences in susceptibility to developing chronic neuropathic pain should be assessed. In that way it can be elucidated if genetic factors contribute to increased vulnerability to neuropathic pain.

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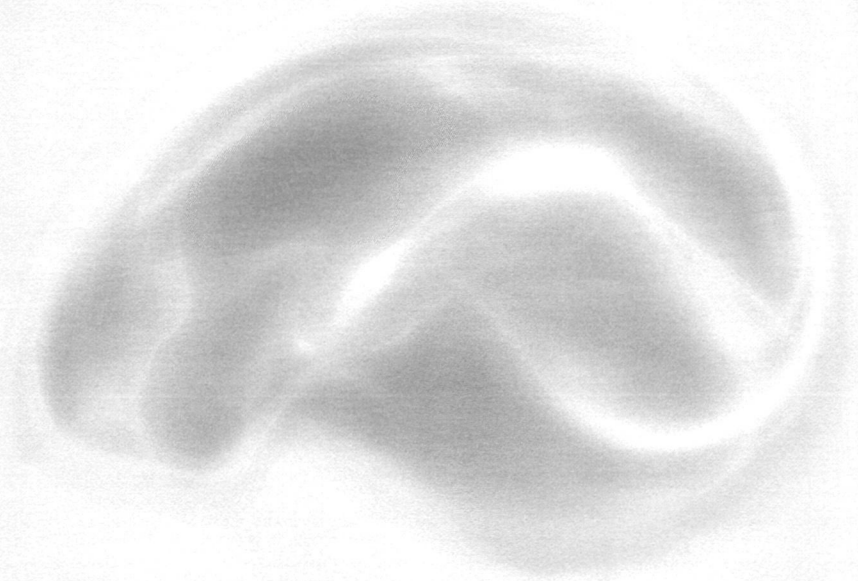
Chapter 7

General Discussion



Testing the hypotheses

Patients who suffer from chronic neuropathic pain (CNP), for example as a consequence of surgical intervention, have a high chance of obtaining mood disorders like anxiety and depression (Argoff, 2007; Freynhagen et al., 2006; Hunt and Mantyh, 2001; Manas et al., 2011). The aetiology of these pain-related mood alterations is not well known, but there is evidence that dysregulation of the brain's stress response system, with corticotropin-releasing factor (CRF) as a main messenger, is involved. The aim of this thesis research was to increase the insight into the mechanisms underlying the pathogenesis of CNP, by testing the following hypotheses:

1. The Sprague-Dawley (SD) chronic constriction injury (CCI) rat is a suitable animal model to study effects of peripheral mononeuropathy on pain sensitivity, anxiety and depressive-like behaviour as seen in humans and, therefore, to test novel drugs for their anti-neuropathic effects.

2. CCI affects the functioning of brain centres involved in the regulation of the stress response and of mood, in particular the hypothalamo-pituitary-adrenal (HPA-) stress axis and CRF-producing forebrain areas.

3. CNP-associated sensory and mood disorders are not (only) caused by the later chronic but (also) by the initial, acute phase of neuropathic pain.

In this General Discussion, it will be considered if and to what degree the results of the experiments described in the previous chapters contribute to supporting the above hypotheses. First, the suitability will be discussed of the SD CCI rat model for testing CNP-associated changes in pain sensation and anxiety and depressive-like behaviours, as well as its potential to test novel drugs to treat these neuropathy-associated symptoms.

Suitability of the SD CCI rat for CNP research and drug development

A reliable animal model for studying the neuronal basis of human neuropathic pain should have high face validity, meaning that upon neuropathy induced by CCI it should reveal similar symptoms of neuropathic pain and associated behaviours as shown by neuropathic patients, such as hyperalgesia and allodynia, and anxiety and depression. In this thesis research we have used the SD CCI rat as a model to increase insight into the brain mechanisms underlying CNP. The SD rat is an albino rat widely used in medical research because of its calmness and ease of (proper) handling, which gives it a clear advantage in the ability to handle compared to *e.g.* the more vicious Wistar rats. CCI is an inflammatory neuropathic model suitable for the study of pain and, in contrast to other nerve ligation paradigms, demonstrating a very high correlation between the experimental model and clinically relevant underlying mechanisms (Kontinen and Meert, 2003). Indeed, SD CCI rats

reveal clear allodynia and hyperalgesia (Bomholt et al., 2005; Ulrich-Lai et al., 2006), but at the start of this research it was not clear whether they also exhibit anxiety and depressive-like behaviour as revealed by neuropathic pain patients. Therefore, to determine to what extent our SD CCI rats can be used to study the various neuronal and behavioural aspects of neuropathic pain, we have tested the occurrence of these behaviours and the possible influences on these behaviours of drugs used in the clinic to treat neuropathic pain-associated symptoms (**Chapter 2**). It is shown that upon CCI, our rats develop allodynia to a cold stimulus and hyperalgesia to mechanical stimuli. Moreover, we demonstrate that these symptoms last till up to six weeks after surgery, which does not only correspond with data obtained from studies on both SD and Wistar rats with CCI (Bennett and Xie, 1988; Roeska et al., 2008; Ulrich-Lai et al., 2006) but also with the prolonged occurrence (up to over 50 months) of these symptoms in human neuropathic patients (Doth et al., 2010). These results confirm the suitability of our SD CCI rat to study the underlying brain mechanisms of increased pain sensitivity associated with human neuropathic pain. Moreover, of the anti-neuropathic drugs tested, minocycline appeared to be able to reduce thermal allodynia on the cold plate, indicating the potential of this drug to partially reverse pain sensation caused by CCI, and, by extrapolation, the suitability of the SD CCI rat to test (some types of) novel drugs for their possible antinociceptive effectiveness.

In contrast to increased nociceptive sensitivity we found no CCI-induced effects on parameters for anxiety and depressive-like behaviours, and likewise, we did not observe any effect on these behaviours by anti-neuropathic drug administration (**Chapter 2**).

This would suggest that the SD CCI rat does not reveal mood disorders associated with human neuropathy and that effects of CCI found on these rats should be (cor)related to increased pain sensitivity rather than to behavioural disorders. However, this conclusion should be drawn with care, because at the time of our behavioural studies, building construction activities were going in our animal facility (T.P.H. Rouwette, unpubl. obs.). Therefore, the possibility cannot be excluded that chronic 'construction stress' had increased anxiety and depressive-like behaviour (*cf.* Schipper et al., 2011) in control, non-CCI-operated rats, obscuring possible effects of CCI- and drug treatment on these behaviours. Also, a recent neuropathic pain study with mice shows that anxiety and depressive-like behaviour is only seen 1 and 2 months, respectively, after induction of neuropathy (Yalcin et al., 2011), which could indicate that at the time of our behavioural testing, anxiety and depressive-like behaviour had not developed yet.

Taken the above data together, we conclude that the SD CCI rat has a high face validity to study especially the underlying mechanisms of human neuropathy-related allodynia and hyperalgesia, whereas the model's face validity with respect to human

neuropathy-associated anxiety and depression may be low, a possibility, however, that deserves further study.

Using SD CCI rats, the neural mechanisms possibly underlying neuropathy have been studied with particular attention to the important neurochemical messenger corticotropin-releasing factor (CRF). Emphasis was on the possible relations between acute and chronic neuropathic pain and the activities of the paraventricular nucleus of the hypothalamus (PVN), the bed nucleus of the stria terminalis (BST), the central amygdala (CeA) and the midbrain Edinger-Westphal centrally projecting neuron population (EWcp), and the roles of these brain areas in the initial and adaptive phases of the pain response, as will be discussed below.

Chronic neuropathic pain and acute pain differentially affect brain centres

There are many indications for an involvement of CRF and its peptide family member urocortin 1 (Ucn1) in the response to neuropathic pain. Not only is CNP associated with depression and anxiety (Freynhagen et al., 2006), disorders often associated with dysregulated CRF and Ucn1 systems (Arborelius et al., 1999; Davis and Shi, 1999; Kozicz et al., 2008, 2011; Regev et al., 2011), but it is also considered a chronic stressor activating the HPA-axis (Blackburn-Munro and Blackburn-Munro, 2001), of which CRF is the dominant neurochemical regulator (Tsigos and Chrousos, 2002) and which activity depends on Ucn1 as well (Kozicz et al., 2011). Therefore, we have tested the hypothesis that CNP affects brain centres involved in the stress response and producing CRF or Ucn1. Since CNP can be divided into a chronic and an acute phase (Gray, 2008), we have studied the effects of both chronic and acute pain on the activities of CRF/Ucn1 brain centres. Chronic effects were induced by long-term CCI, whereas for acute effects another rat paradigm was used, namely formalin injection into the hind paw, a well validated procedure to induce acute pain stress (Dubuisson and Dennis, 1977; Tjølsen et al., 1992; Vissers et al., 2004).

In rat, chronic variable mild stress increases CRF mRNA in the PVN and consequent corticosterone release from the adrenals (Sterrenburg et al., 2012), and up-regulates Ucn1 mRNA and peptide in the EWcp (Derks, 2010; Korosi et al., 2005; van Wijk, 2011). Furthermore, chronic stress is associated with increased gene expression as revealed by increased neuronal cFos and deltaFosB, markers for early and prolonged gene activation, respectively, in both the PVN and EWcp (Nunez et al., 2010; Xu et al., 2010). However, we show that in SD rats, CCI does not change cFos- or deltaFosB-immunoreactivities in the PVN (see also Bomholt et al., 2005) or in the EWcp (**Chapter 3**), nor does it influence plasma ACTH and corticosterone titres (Bomholt et al., 2005; Ulrich-Lai et al., 2006; Vissers et al., 2003). This indicates that CCI does not act as a regular chronic stressor. This conclusion is further underpinned by our finding that CCI does not change the amounts of CRF and CRF

mRNA in the PVN nor of Ucn1 and Ucn1 mRNA in the EWcp (**Chapter 3**). Therefore, CNP does not seem to be associated with sustained activation of the HPA-axis and the EWcp, at least not in its chronic phase, but rather with activity changes in other brain areas. The BST and CeA are likely candidates to mediate neuropathy-induced changes in mood, because we found that in CCI rats CRF mRNA is up-regulated in both nuclei and, moreover, CRF is increased in the CeA (**Chapter 3**). This idea is in line with the observation in mice mutants that dysregulation of the limbic CRF system by continuous over-expression of CRF affects anxiety and depressive-like behaviour (Regev et al., 2011). In our study (**Chapter 3**) we could correlate CCI-induced changes in the BST and CeA with increased pain sensitivity (increased allodynia and hyperalgesia), but we did not find an effect of CCI on anxiety or depressive-like behaviour (**Chapter 2**). Whether this latter absence of effect is due to a strain property, SD rats being less vulnerable to chronic neuropathic changes in their behaviour, or that chronic construction stress has masked mood responses, or that anxiety and depressive-like behaviour had not yet developed, remains to be investigated. In any case, it is interesting to note that the CeA and BST do not only play roles in anxiety and depressive-like behaviour but also in pain sensitivity control (Deyama et al., 2007; Hunt and Mantyh, 2001). So, our studies strongly suggest that the effects of CNP on nociception may be exerted via these forebrain areas.

In view of our hypothesis that CNP-associated sensory and mood disorders are not (only) caused by the later chronic but (also) by the initial, acute phase of neuropathic pain, in **Chapters 4** and **5** we focused on the possible role of acute pain in the induction of CNP. Theoretically, it would have been ideal to study this in our CCI rat in the acute phase of CNP, shortly after CCI-surgery. However, CCI-operated rats need several hours to recover from the anaesthesia applied for CCI-surgery, making it impossible to interpret the acute effects of CCI, *i.e.* within the first hours after CCI, without confounding anaesthesia effects. Therefore, we have used the acute pain stress (APS) model, where acute pain is induced by a formalin injection into the sole of the left hind paw. Our results reveal that, in contrast to chronic pain, APS rapidly activates gene expression in all four brain areas studied (as is clear from the increased neuronal cFos contents) and from changed dynamics of CRF in the PVN and of Ucn1 in the EWcp (**Chapter 4**). Interestingly, the responses of these brain areas are temporally different, the EWcp reacting later than the PVN. These results indicate that CRF in the PVN may be a major player in the initiation phase of the response to an acute pain stressor, whereas Ucn1 in the EWcp may rather be involved in the adaptive phase of this response.

Towards the intracellular level of pain signalling: a role of DCLK in Ucn1 signalling

In our search for the mechanisms underlying CNP, we followed an integrative approach, including not only the study of behavioural and cellular aspects of CNP but also the investigation of molecular components of the signalling processes concerned. In the present thesis research, we made a first step by looking at the intracellular level, focusing on a rather recently discovered messenger, doublecortin-like kinase (DCLK), a mitogen-activated protein kinase that is an important player in intracellular signalling pathways and plays a role in the induction and maintenance of neuronal plasticity during pain modulation (Ji et al., 2009). DCLK-short, a splice variant of the *dclk* gene, has been linked to the generation of anxiety behaviour, because mice over-expressing a truncated form of DCLK-short spend less time on the open arms of the elevated plus maze (Schenk et al., 2010).

As we had found that APS exerts clear effects on both PVN and EWcp functioning, (**Chapter 4**) we subsequently explored the possibility that these responses would depend on DCLK action, via a DCLK-mediated signalling pathway. Western blotting studies reveal that two splice variants of the *dclk* gene are present in both centres, viz. the DCLK-short and DCLK-long forms (**Chapter 5**). Phosphorylation and dephosphorylation of these proteins regulate their functions (Schaar et al., 2004), and we demonstrate that in both nuclei only DCLK-long is phosphorylated at its PTSP-motif. Two hours after acute pain initiation, phosphorylated DCLK-long is up-regulated in the EWcp but not in the PVN. These data indicate that these centres have different DCLK-associated intracellular signalling mechanisms to process pain stimuli. However, it is also possible that their mechanisms are the same but act at different speeds. In any case, this study indicates that DCLK is an important messenger involved in pain signalling in the EWcp. It remains to be tested if CCI leads to long-term changes in DCLK expression, possibly in the amygdala, as this would help answer the question if neuropathy-associated anxiety and depressive-like behaviours would depend on DCLK-mediated signalling as well.

The amygdalar pain switch

In view of the dominant response of CRF neurons in the limbic system to neuropathy-associated stressors as shown in **Chapters 3** and **4**, we have performed an extensive literature search focusing on recent data on the role of CRF in the limbic system in nociception (**Chapter 6**). This study showed that CRF not only exerts analgesic effects when administered locally or systemically in supra-physiological concentrations, but also employs analgesic effects when endogenous amygdalar CRF is increased and/or by blocking the CRF type 1 receptor (CRFR1; Bourbia et al., 2010; Cui et al., 2004; Ji et al., 2007; Ji and Neugebauer, 2008). This leads to the idea that in normal, healthy individuals, in response to peripheral

noxious stimulation, CRF is released from the BST or the parabrachial nucleus (PBN), to bind to CRFR1 in the amygdala and switch on pain sensation (algesia). The activated CRFR1 is then internalised and triggers the translocation of the CRF type 2 receptor (CRFR2) from the cytoplasm towards the plasma membrane, where it becomes activated by the specific CRFR2 ligands, the urocortins 2 and 3 released from the BST, perifornical hypothalamus (PFH) and locus coeruleus (LC; Lewis et al., 2001; Millan, 2002; Tsatsanis et al., 2007; Wittmann et al., 2009). The activated CRFR2 then switches pain sensation off (analgesia). Finally, CRFR1 returns from the cytoplasm towards the plasma membrane and CRFR2 from the plasma membrane to the cytoplasm, restoring the situation from before the noxious stimulation (see Fig. 1, **Chapter 6**).

This amygdalar on-off switch might play a crucial role in the start and termination of peripherally induced pain sensation in the normal individual.

The mechanism of neuropathic pain – a model

We hypothesise that dysregulation of the amygdalar on-off switch is a major component of the generation of CNP. Such dysregulation might be the result of the action of cytokines that change the dynamics of CRF mRNA and CRF peptide in the amygdala (Kasckow et al., 1997; Raber et al., 1995). Cytokines, produced at sites of inflammation, might be released from the inflamed site of sciatic nerve constriction as applied in CCI rats (P. Vanelderen, pers. comm.; Engler et al., 2011). On the basis of this idea and the data collected in this thesis research, we have put forward a model that describes the generation of neuropathic pain in two consecutive phases (Fig. 1A). According to this model, CCI-surgery evokes a pain signal that travels via the ascending pathways to the brain where it activates the brain's stress adaptation system, stimulating pDCLK-mediated Ucn1 release from the EWcp and CRF, ACTH and corticosteroid release from the HPA-axis, to maintain homeostasis of body and mind. This process can be considered as the acute phase of neuropathy. In this phase, CRF release is also triggered from the BST or PBN to switch on pain sensation by the amygdala. In addition to their role in stress adaptation, urocortins released from the BST, PFH and/or LC will act on the amygdala to turn pain sensation off. However, in certain conditions their analgesic action fails. It is hypothesised that cytokines released from the inflamed CCI-surgery site may disable turning off pain in the amygdala, and as a consequence pain sensation persists, representing the second phase of neuropathic pain, which is often accompanied by anxiety and depressive-like behaviours. Whether these behaviours are a primary result of amygdalar dysregulation or, as depicted in our model, result secondarily from the persistent release of cytokines during CNP remains to be determined.

Although it is an intriguing assumption that increased cytokine levels are responsible for dysregulation of a postulated amygdalar switch and the development of CNP and

associated behavioural responses, there are several other pain syndromes with the same pathophysiology but in the absence of inflammation or damage to the neural system. The common denominator in these pain disorders is central sensitisation, which manifests as pain hypersensitivity, and extensive experimental medicine and clinical investigation over the past decades revealed it to be an important component in many pain patients. This research also showed that a considerable amount of progress had been made in elucidating cellular and molecular mechanisms involved, and although much remains still to be clarified (Woolf, 2011), in CCI, inflammatory factors (cytokines) are likely contributors to central sensitisation underlying chronic pain development.

According to our model, the two phases of neuropathic pain involve different brain centres, the acute phase depending on the HPA-axis and the EWcp, exerting the rapid adaptation response, and the chronic phase depending on limbic brain regions, evoking mood changes such as anxiety and depression. It is important to note that the second phase does not involve activation of the HPA-axis, as the corticosterone titre is probably elevated by CCI only during the acute phase, and has dropped to pre-surgery value in the chronic neuropathy phase (Fig. 1B).

Perspectives for fundamental and clinical research

CNP is a disorder affecting health, society and economy, and leading to behavioural disturbances like anxiety and depression, disorders linked to a dysregulation of the stress system. Although a lot of factors involved in the pain signalling are known, and there are several medications to relieve the pain or treat its symptoms, though with concomitant side effects, the causative neuronal mechanisms underlying CNP are not known. This thesis research provides evidence for a role of the brain's stress adaptation system, including the involvement of CRF and Ucn1, in pain processing and shows brain centres influenced by acute pain and by CCI. To further substantiate the model presented above, the following basic and clinical studies may be helpful.

First of all, repetition of our CCI studies with Wistar rats may show the general validity of our model and that CCI leads to an up-regulation of CRF in the BST and CeA and increased anxiety and/or depressive-like behaviour. Chronic application of clinically used medications, in a dose-dependent manner, will reveal the potential of these drugs to attenuate or completely inhibit this behaviour, possibly via reversion of CCI-induced changes in limbic CRF.

As a first step in unravelling the molecular signalling pathways underlying pain responses of the brain, we obtained evidence for an early involvement of DCLK phosphorylation in the response of the EWcp to acute pain stress (**Chapter 5**). Since we showed that Ucn1 in the EWcp and CRF in the PVN play a major role in the brain's response

to acute pain, where CRF dynamics appear to be faster than Ucn1 dynamics (**Chapter 4**), we here propose that DCLK-long phosphorylation might already have been up-regulated and de-regulated again within the first two hours. This could be tested by a similar experiment where DCLK-immunoreactivity is measured at different time points after pain initiation. Also, it would be of interest to see if DCLK is also involved in the response of the nuclei studied to CCI that leads to CNP. If so, pharmacological interference with the DCLK pathways might be a basis for development of drugs that prevent or treat CNP.

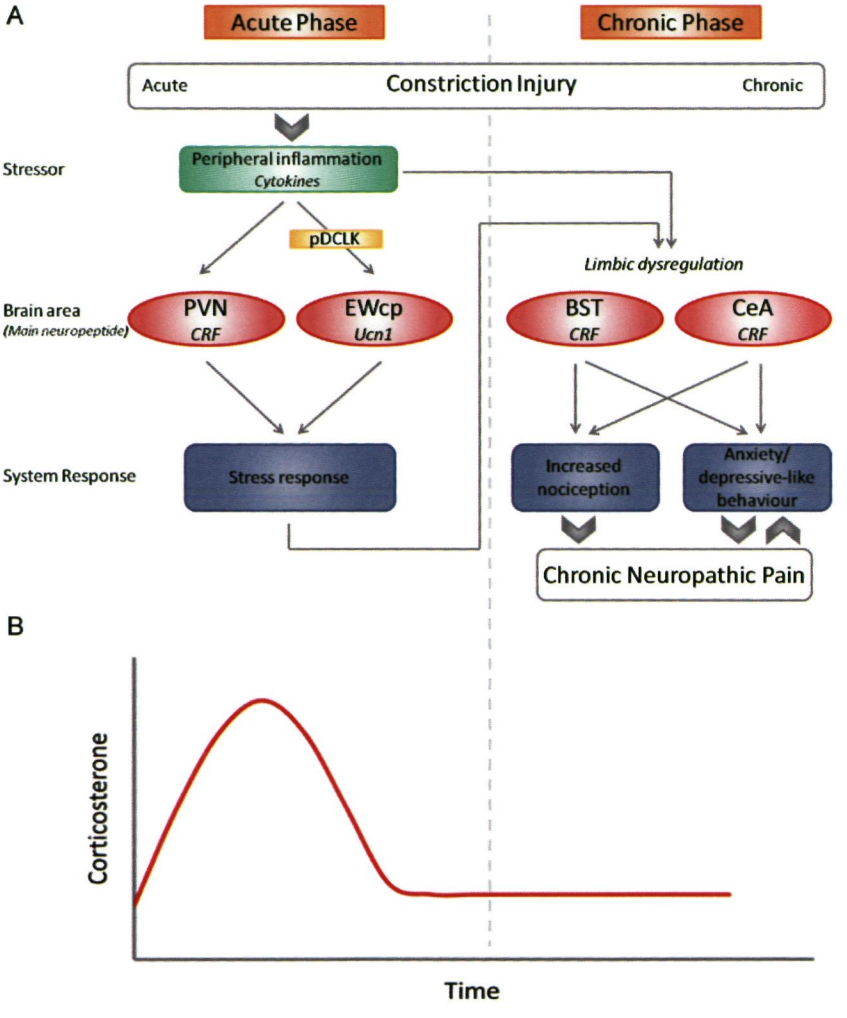


Figure 1. *Proposed model for the two-step generation of neuropathic pain and the involvement of components of the CRF stress system. BST, bed nucleus of the stria terminalis; CeA, central amygdala; CRF, corticotropin-releasing factor; EWcp, Edinger-Westphal centrally projecting neuron population; pDCLK, phosphorylated doublecortin-like kinase; PVN, paraventricular nucleus; Ucn1, urocortin 1.*

As we demonstrated a differential involvement of CRF and Ucn1 in acute and chronic neuropathic pain in the rat, it is a challenge to test if the same mechanism exists in human neuropathy. Studies on *post-mortem* brains from patients suffering from chronic neuropathic pain and associated mood disorders might confirm this idea.

Finally, development of highly specific CRF receptor agonists and antagonists could help to dissect the acute from the chronic neuropathic pain phase. This might provide the basis for the development of specific drugs that treat neuropathy in each phase, and may help prevent patients in the acute phase to develop chronic neuropathy that is accompanied by strong pain sensitivity, anxiety and depressive-like behaviour.

Final considerations

The main aim of this thesis research was to elucidate if and how chronic constriction injury leads to a disturbance in the brain's stress system and affects the regulation of the CRF peptide family in the HPA-axis, limbic system and EWcp, causing mood disorders like anxiety and depression. We have shown that our pain stressor activates the stress system in the acute phase, and that the later, chronic phase of neuropathy occurs in the absence of the key symptoms of chronic stress but clearly reveals thermal allodynia and mechanical hyperalgesia. On the basis of our experimental and theoretical research, we postulate a mechanism where dysregulation of amygdalar CRF signalling (the on-off pain switch) is the basis for the transition from acute to chronic neuropathic pain. Our results, put together in an integrative model, extend the knowledge on pain signalling following peripheral neuropathy, broadening the insight into the way CRF-containing brain areas may collaborate in healthy and neuropathic patients in response to peripherally-induced neuropathy. It is conceivable that these results will contribute to the identification of novel and target-specific therapeutics that can prevent or treat the chronic neuropathy.

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Summary

Chronic neuropathic pain (CNP) is a disorder affecting tens of millions of people worldwide, causing behavioural disorders like anxiety and depression. Its treatment is largely symptom-based, due to a lack of knowledge of the underlying neuronal mechanism(s). The research described in this thesis provides evidence for the involvement of the brain's stress adaptation system in pain processing, using rat models to study nociception, behaviour and brain activities.

Chapter 1 provides the background information for this thesis research, including an overview of the basic neuronal correlates and clinical features of CNP. Furthermore, an analysis is made of the known and unknown aspects of pain processing in the central and peripheral nervous systems, with particular attention to the (possible) roles of supraspinal brain centres that are involved in the stress response and express the stress neuropeptide corticotropin-releasing factor (CRF) or its family member urocortin 1 (Ucn1). Finally, hypotheses are presented for the ways these centres contribute to the pathogenesis of CNP.

In **Chapter 2** Sprague-Dawley (SD) rats with chronic constriction injury (CCI) of the sciatic nerve have been tested for the development of increased nociception (thermal allodynia and mechanical hyperalgesia) and the induction of anxiety and depressive-like behaviour. The study shows that the SD CCI rat is a suitable model for investigating CNP-associated allodynia and hyperalgesia that occur in humans and, to some degree, for testing the effectiveness of novel drugs to alleviate these disturbances. However, regarding human CNP-associated anxiety and depression, the face validity of this rat model seems to be low.

Chapter 3 is concerned with the neuronal correlates of CNP. Using the SD CCI rat, research focuses on neuropathy-induced changes in supraspinal brain centres involved in the stress response. With quantitative *in situ* hybridisation and immunohistochemistry techniques to assess CRF production (neuronal CRF mRNA contents) and CRF storage and secretion (numerical density of CRF neurons and their CRF peptide contents), it is shown that three weeks after CCI, CRF mRNA is up-regulated in the bed nucleus of the stria terminalis (BST) and the central amygdala (CeA), and CRF peptide contents are increased in the CeA. On the other hand, CRF in the paraventricular nucleus of the hypothalamus (PVN) does not appear to be affected, and the midbrain Edinger-Westphal centrally projecting neuron population (EWcp) does not show altered general gene activation (indicated by neuronal contents of the immediate early gene product cFos) or changed Ucn1 mRNA and Ucn1 peptide contents. These data indicate that neuropathic pain is not primarily associated with the hypothalamo-pituitary-adrenal (HPA)-axis or the EWcp, which play main roles in the organism's response to acute and chronic (pain) stressors, but rather with the CRF-producing brain centres in the limbic forebrain, viz. the BST and CeA.

As we hypothesised that chronic neuropathic pain may actually start with an acute phase induced by a dominant stressor, in **Chapters 4** and **5** the responses of the CRF- or Ucn1-producing brain centres to acute pain stress have been investigated. For that purpose, Wistar rats were injected into the left hind paw with formalin. The results indicate that the PVN, BST, CeA and EWcp all respond to the acute pain stressor with induction of immediate early genes (cFos mRNA and cFos protein). CRF and Ucn1 dynamics, however, reveal brain centre-specific responses to acute pain stress, as to kind and timing of the response. In fact, they strongly suggest a major role of CRF in the PVN in the initiation phase of the acute pain response, whereas Ucn1 in the EWcp may act especially in the later, termination phase of this response (**Chapter 4**). These different responses of the PVN and EWcp in pain processing have been studied at the intracellular level in **Chapter 5**. Here, it is shown that doublecortin-like kinase (DCLK), which is assumed to be a main player in intra-neuronal signalling, is present in the CRF neurons of the PVN and in the Ucn1 neurons of the EWcp. Phosphorylation of the DCLK-long protein is increased by acute pain stress in the EWcp, whereas this phosphorylation in the PVN remains unaffected. This indicates a differential regulation to acute pain by DCLK-long between the stress-responsive PVN and EWcp.

In view of the dominant response of CRF neurons in the limbic system to neuropathy-associated stressors, in **Chapter 6** a literature search has been presented, focusing on recent data on the role of limbic forebrain CRF in nociception. Based on the results of this study, it is postulated that CRF and its receptors in the amygdala play a critical role in the transition from acute to chronic pain sensation. Upon painful stimuli, CRF is released from the BST and/or parabrachial nucleus (PBN) to activate CRFR1 in the amygdala that turns on pain sensation. Subsequently, the amygdalar neurons switch CRFR1 for CRFR2 receptors that turn pain sensation off upon binding to urocortins released from the BST, the perifornical hypothalamus and the locus coeruleus.

By combining this latter notion with the conclusions arrived at in the previous chapters, a model for the pathogenesis of neuropathic pain has been proposed in the General Discussion (**Chapter 7**). In this model CCI acts as an acute stressor that activates the PVN and EWcp to start the stress response, but also induces CRF release from the BST or PBN, which turns on the amygdalar pain switch. In contrast to healthy controls, in neuropathy the switch cannot be turned off by urocortins anymore as it is disabled by cytokine release from the inflamed site of CCI-surgery. As a result, permanent chronic pain is generated together with mood disturbances like anxiety and depressive-like behaviour.

Finally, in the General Discussion a series of experiments have been suggested that may further support this model, providing more insight into the extracellular and intracellular pathways underlying neuropathic pain-induced disorders of body and mind, and in this way hopefully improving the development of novel drugs to treat this major disease.

Samenvatting

Wereldwijd lijden tientallen miljoenen mensen aan chronische neuropathische pijn (CNP), die angststoornissen en depressie tot gevolg kan hebben. De behandeling van CNP is vooral gericht op het bestrijden van de symptomen, hetgeen te wijten is aan het gebrek aan kennis omtrent de cellulaire mechanismen die aan deze pijn ten grondslag liggen. In het onderzoek dat in dit proefschrift staat beschreven, wordt op deze mechanismen ingegaan. Het laat zien dat het stress-adaptatiesysteem in de hersenen een belangrijke rol speelt bij de verwerking van pijnprikkels. Met behulp van twee rattenmodellen is de relatie tussen pijngevoeligheid, gedrag en hersenactiviteit onderzocht.

Hoofdstuk 1 schetst de achtergrond van het onderzoek, waarbij een overzicht wordt gegeven van de neurale componenten die een rol spelen bij CNP en van de daarbij optredende klinische verschijnselen. Voorts wordt een analyse gegeven van de bekende en onbekende aspecten van pijnverwerking in het centrale en het perifere zenuwstelsel. Hierbij wordt speciale aandacht besteed aan de (mogelijke) rol van hersengebieden die betrokken zijn bij de stressrespons en die het neuropeptide 'corticotropin-releasing factor' (CRF) dan wel het aan CRF gerelateerde neuropeptide urocortine 1 (Ucn1) tot expressie brengen. Tenslotte worden de hypothesen voorgesteld ten aanzien van de wijzen waarop deze hersengebieden bijdragen aan het ontstaan en de ontwikkeling van CNP.

In **Hoofdstuk 2** wordt beschreven hoe het chronisch afbinden van de *nervus ischiadicus* van Sprague-Dawley (SD)-ratten leidt tot verhoogde pijngevoeligheid ('chronic constriction injury'; CCI). CCI is gekenmerkt door belangrijke symptomen van CNP bij de mens, namelijk thermische allodynie en mechanische hyperalgesie. Tevens is onderzocht of en in welke mate CCI leidt tot het ontstaan van angststoornissen en depressief gedrag. Het blijkt dat de SD CCI-rat zeer geschikt is om allodynie en hyperalgesie te onderzoeken, en om medicijnen te testen op hun vermogen om deze symptomen te onderdrukken. Dit rattenmodel lijkt echter minder geschikt voor studies aan angststoornissen en depressie.

Hoofdstuk 3 richt zich op de neurale componenten van CNP, en wel op de effecten van CCI in de SD-rat op hersencentra die betrokken zijn bij de stressrespons. Met kwantitatieve *in situ* hybridisatie en immunohistochemische technieken is gekeken naar de productie van CRF (bepaling van CRF mRNA) en de opslag en secretie van CRF (telling van CRF-positieve neuronen en meting van hun CRF-gehalte). De resultaten laten enerzijds zien dat drie weken na zenuwafbinding het CRF mRNA-gehalte verhoogd is in de bed nucleus van de stria terminalis (BST) en in de centrale amygdala (CeA), waarbij de laatste kern tevens een verhoging vertoont van CRF-peptide. Anderzijds blijkt CCI geen effect uit te oefenen op het CRF-gehalte van de paraventriculaire hypothalame kern (PVN). Ook de 'Edinger-Westphal

centrally projecting neuron population' (EWcp) in de middenhersenen laat geen verandering zien in algemene genexpressie (zoals gemeten aan het 'immediate early gene' cFos), noch een verandering in de gehalten van Ucn1 mRNA en Ucn1-peptide. Deze data wijzen er op dat CNP niet direct geassocieerd is met de hypothalamus-hypofyse-bijnier-as of met de EWcp, die allebei een belangrijke rol spelen bij adaptatie aan acute en chronische (pijn) stressoren, maar veeleer met limbische hersengebieden die CRF produceren, zoals de BST en CeA.

Eén van onze te testen hypotheses was dat CNP begint met een acute fase die geïnduceerd wordt door een dominante pijnprikkel. Daarom is in de **Hoofdstukken 4** en **5** gekeken of een acute pijnstressor effect heeft op de activiteit van CRF- en Ucn1-producerende hersengebieden. Hiertoe zijn Wistar-ratten in de linker achterpoot geïnjecteerd met formaline. De resultaten wijzen uit dat deze acute pijnstressor leidt tot een snelle inductie van cFos mRNA en cFos-eiwit in zowel de PVN, BST en CeA als in de EWcp. Ook treden dynamische veranderingen op in de gehalten van CRF en Ucn1, maar deze veranderingen verschillen per hersengebied, zowel qua richting als qua timing van de respons. Dit experiment geeft aan dat CRF in de PVN een belangrijke rol speelt in de initiatiefase van de acute pijnrespons, terwijl Ucn1 in de EWcp vooral betrokken is bij de eindfase van deze respons (**Hoofdstuk 4**). In **Hoofdstuk 5** wordt daarom dieper ingegaan op de intracellulaire aspecten van pijnverwerking in de PVN en EWcp. Hieruit komt naar voren dat het 'doublecortin-like kinase' (DCLK) een belangrijke rol speelt bij intraneuronale communicatie in de CRF-neuronen van de PVN en in de Ucn1-neuronen van de EWcp. In de laatste kern vindt als reactie op pijn een verhoging plaats van de fosforylatie van het DCLK-long-eiwit, een proces dat niet optreedt in de PVN. Blijkbaar verschillen de stressgevoelige PVN en EWcp in de wijze waarop acute pijn DCLK-long reguleert.

Omdat CRF in het limbisch systeem een belangrijke rol lijkt te spelen in CNP, is een literatuuronderzoek verricht naar de rol van CRF in de BST en de CeA bij pijnverwerking (**Hoofdstuk 6**), en op basis van de conclusies van dit onderzoek is vervolgens de stelling geponeerd dat CRF en CRF-receptoren in de amygdala cruciaal zijn voor de overgang van acute naar chronische pijn. De pijnprikkel maakt CRF vrij in de BST en/of in de parabrachiale kern (PBN) dat vervolgens wordt getransporteerd naar de amygdala om daar de CRF-receptor type 1 te activeren, hetgeen de pijnsensatie opwekt. Vervolgens worden de type 1 receptoren in de amygdala verruild voor type 2 CRF-receptoren die urocortines binden die afgegeven zijn door de BST, de perifornicale hypothalamus en de locus coeruleus. Hierdoor schakelt de amygdala de pijn weer uit.

Door dit mechanisme van het aan- en uitschakelen van pijn te koppelen aan de conclusies die getrokken zijn uit de experimentele studies, is in de Algemene Discussie (**Hoofdstuk 7**) een model opgesteld voor de wijze waarop CNP zich ontwikkelt. Hierin werkt CCI als een acute pijnprikkel die de PVN en EWcp activeert en zo de stressrespons start. Er

komt echter ook CRF vrij vanuit de BST en/of de PBN, waardoor de amygdala de pijnsensatie aanschakelt. In tegenstelling tot de situatie bij gezonde dieren, kan deze switch bij neuropathie niet uitgezet worden door urocortines, omdat hun werking geblokkeerd wordt door cytokines die vrijkomen als gevolg van de door het afbindingsproces ontstane ontsteking in de achterpoot. Hierdoor wordt de pijn chronisch, hetgeen leidt tot angststoornissen en depressie.

Tenslotte worden in de Algemene Discussie experimenten voorgesteld die bovenstaand model verder kunnen onderbouwen. Ook kunnen ze nader inzicht verschaffen in de intra- en extracellulaire mechanismen die ten grondslag liggen aan lichamelijke en geestelijke stoornissen die het gevolg zijn van CNP, en kunnen ze bijdragen aan de ontwikkeling van nieuwe medicijnen om deze stoornissen te bestrijden.

Dankwoord

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en Eva thuis was het altijd erg gezellig. Met heel veel goede herinneringen zal ik hieraan terugdenken. Ik moet je helaas wel teleurstellen: de cover van mijn proefschrift is niet fluorescerend roze geworden.

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Curriculum Vitae

Tom Rouwette werd geboren op 10 december 1981 te Venray. Hij groeide op in Oostrum (Lb) en voltooide het Gymnasium aan het Raaylandcollege Venray in 2000. Datzelfde jaar begon hij met de studie Algemene Natuurwetenschappen aan de (toenmalige) Katholieke Universiteit Nijmegen. Tijdens de doctoraalfase heeft hij tijdens twee stages onderzoek verricht, waarbij het multidisciplinaire karakter van zijn studie duidelijk naar voren kwam. Zijn eerste stage liep Tom bij de afdelingen Cellulaire Dierfysiologie en Organische Chemie van dezelfde universiteit, inmiddels omgedoopt tot Radboud Universiteit Nijmegen, waar hij een antagonist voor een van de CRF-receptoren synthetiseerde en de werking hiervan onderzocht in *Xenopus laevis*. In Toms tweede stage ontwikkelde hij op de afdeling Biomoleculaire Chemie een tumorcellijn die een bepaald protease tot overexpressie brengt. De werking van dit protease in de betreffende cellijn werd onderzocht op het Overall Lab in Vancouver, Canada, met behulp van geavanceerde technieken waar dat laboratorium in gespecialiseerd is. In maart 2007 behaalde hij zijn diploma om vervolgens in diezelfde maand te beginnen met zijn promotie-onderzoek bij de afdelingen Anaesthesiologie aan het Universitair Medisch Centrum St Radboud en Cellulaire Dierfysiologie aan de Radboud Universiteit Nijmegen. De resultaten van dit onderzoek staan in dit proefschrift beschreven en zijn gepresenteerd op diverse nationale en internationale wetenschappelijke bijeenkomsten.

Donders Graduate School for Cognitive Neuroscience Series

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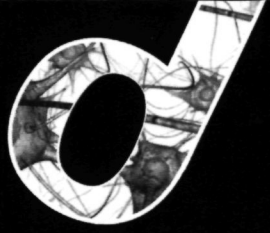

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