Relationship between pain and anxiety in rats

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Kerstin Röska

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SUMMARY

Clinically, it is well known that neuropathic pain often induces comorbid symptoms such as anxiety. In turn, also anxiety has been associated with a heightened experience of pain. Although, the link between pain and anxiety is well recognized in humans, the neurobiological basis of this relationship remains unclear. Therefore, the aim of the current study was to investigate the influence of neuropathic pain on anxiety and vice versa in rats by assessing not only pain-related behaviour but also by discovering possible key substrates which are responsible for the interrelation of pain and anxiety.

In rats with a chronic constriction of the sciatic nerve (CCI model) anxiety-like behaviour was observed. Since anxiety behaviour could be completely abolished after the treatment of the pure analgesic drugs gabapentin and morphine, we concluded that anxiety was caused by the strong persistent pain. Furthermore, we found that the neuropeptides oxytocin and vasopressin were upregulated in the amygdala of CCI rats, and the intra-amygdala treatment of an oxytocin antagonist but not the vasopressin antagonist could reduce anxiety-like behaviour in these animals, while no effect on mechanical hypersensitivity was observed. These data indicate that oxytocin is implicated in the underlying neuronal processes of pain-induced anxiety and helps to elucidate the pathophysiological mechanisms of neuropathic pain.

To assess the influence of trait anxiety on pain sensation in rats, we determined mechanical hypersensitivity after sciatic nerve lesion (CCI) in animals selectively bred for high anxiety or low anxiety behaviour. The paw withdrawal thresholds were significantly decreased in high anxiety animals in comparison to low anxiety animals 2 and 3 weeks after surgery. In a second model state anxiety was induced by the sub-chronic injection of the anxiogenic drug pentylentetrazol in naive rats. Pain response to mechanical stimuli was increased after pharmacologically-induced anxiety. These results provided evidence for the influence of both trait and state anxiety on pain sensation.

The studies contribute to the elucidation of the relationship between pain and anxiety. We investigated that the neuropathic pain model displays sensory as well as emotional factors of peripheral neuropathy. Changes in expression levels of

neuropeptides in the central nervous system due to neuropathic pain may contribute to the pathophysiology of neuropathic pain and its related symptoms in animals which might also be relevant for human scenarios. The results of the current study also confirm that anxiety plays an important role in the perception of pain.

A better understanding of pain behaviour in animals might improve the preclinical profiling of analgesic drugs during development. The study highlights the potential use of the rat model as a new preclinical tool to further investigate the link between pain and anxiety by determining not only the sensory reflexes after painful stimuli but also the more complex pain-related behaviour such as anxiety.

INTRODUCTION

Pain- a sensory and emotional experience

Pain is a sensory perception which is defined by the International Association for the Study of Pain (IASP) for clinical and scientific purposes as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage” (143). The most important physiological function of this subjective sensory system is warning the body to avoid tissue damage and protect from painful and serious impairment.

Pain can be divided into two different modalities. While nociceptive pain occurs after peripheral somatic or visceral tissue damage, neuropathic pain, which is the main topic of this thesis, is defined as “pain, initiated or caused by a lesion or disease in the somatosensory system” (214). During neuropathic pain state the original warning function is lost, and the pain experience becomes manifested in chronic pain without tissue damage.

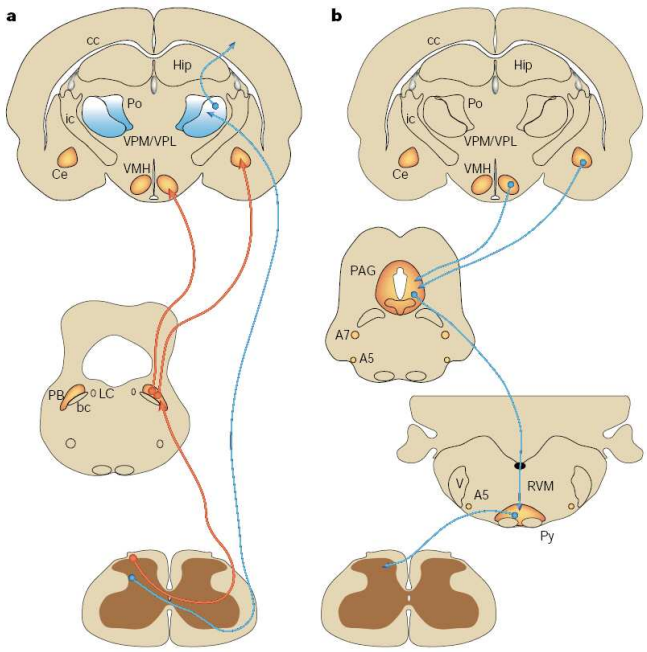
A pain survey - performed in 16 European countries - showed that 19 % of European population are suffering from chronic pain (29). In Germany about 14 million people complain about strong and long-lasting pain, which often affects daily life activities, quality of life and their effectiveness at work. Common types of chronic pain include back pain, headaches, arthritis, cancer pain, and neuropathic pain, which results from nerve injuries. Regardless of the type of chronic pain, the physical and emotional effects can be devastating. Patients often report about associated comorbidities such as depression, anxiety and sleep disturbances. Despite our understanding of nociceptive processing and of plastic changes after persistent noxious input has immensely increased within the last decades, neuropathic pain is still a big clinical scientific challenge. There is an unmet need to increase knowledge in the underlying mechanisms and investigation of new targets to improve the therapeutic options for chronic pain patients.

Neuroanatomy of pain

Noxious impulses from primary afferent fibres (Aβ-, Aδ- and C- fibres) in the periphery are conducted through the dorsal root ganglia (DRG) into the dorsal horn of the spinal cord. The majority of nociceptive Aδ and C-fibres stretch out in the lamina

I – II, the superficial layer and only a few perform synapses in the deeper regions. Pain is thought of having a sensory ("discriminative") and affective ("unpleasantness") component which is transmitted via two different ascending pathways to higher brain areas in the CNS (Figure 1). Sensory information is directly mediated via the spinothalamic pathway to thalamic structures and from there to cortical areas (95) such as primary and secondary somatosensory cerebral cortical areas (SI and SII). The neurons of this pathway originate from layer I and layer V - VI and contain predominantly wide dynamic range (WDR) neurons. This pathway is processing the different modalities and stages of pain and is therefore critically involved in the discriminative nature of pain (6). Serial interconnections to cortico-limbic structures also allocate the spinothalamic pathway and contribute to pain-related affect (65; 86).

Figure 1. The main ascending and descending pain pathways.



a: Two primary ascending pathways, conduct nociceptive information from the superficial dorsal horn to higher brain areas. The spinoparabrachial pathway (red) is responsible to distribute the affective component of pain, while the spinothalamic circuitry (blue) conducts the discriminative and sensory aspects of pain.

b: The descending pathway, originating from the amygdala and hypothalamus control nociceptive information via the periaqueductal grey (PAG) and terminate in lower brain stem regions. A, adrenergic nucleus; bc, brachium conjunctivum; cc, corpus callosum; Ce, central nucleus of the amygdala; Hip, hippocampus; ic, internal capsule; LC, locus coeruleus; PB, parabrachial area; Po, posterior group of thalamic nuclei; Py, pyramidal tract; RVM, rostroventral medulla; V, ventricle; VMH, ventral medial nucleus of the hypothalamus; VPL, ventral posteriolateral nucleus of the thalamus; VPM; ventral posteriomedial nucleus of the thalamus. Figure taken from Hunt *et al*. 2001 (95).

The spinoparabrachial pathway is the second ascending circuit, which consists of nociceptive specific neurons (NS neurons) and conducts more the affective-motivational component of pain. It derives mainly from lamina I neurons of the dorsal horn and terminates within the parabrachial area and periaquaeductal grey, which in

turn project on brain areas such as the ventral medial nucleus of the hypothalamus and the amygdala. These pathways are thought to be responsible for the development of pain-related secondary symptoms such as anxiety and depression.

The painful sensation however is not only dependent on the transmission via the ascending pathways and central modulation. The descending pathways are another very important system to control and to influence nociceptive information. They are predominantly noradrenergic and serotonergic and can be modulated by stimulation of opiate receptors. Descending influences are both facilitatory and inhibitory in nature. Several studies provide evidence that the neurotransmitter serotonin contributes to the pro-nociceptive facilitatory pain system and plays a role in the development and maintenance of hyperalgesia. For instance a depletion of spinal 5-HT reduces pain-related behaviour after nerve injury (175; 209).

The inhibitory system in the spinal cord plays a crucial role to control and attenuate the sensitivity of descending pain neurons. Norepinephrine (NE) from the brain stem initiate inhibitory descending processes via activation of α_2-adrenoceptors (145). Moreover, a diminished expression of μ-opioid receptors has been observed after nerve injury, which results in a decreased inhibitory transmission (109; 174). Therapies that intensify mechanism of descending inhibition or diminish descending facilitation are furthermore an important target for research and novel analgesic drug development.

Mechanisms and pathophysiology of pain

Physiological pain arises as a consequence of activation of primary nociceptive afferents due to damaging stimuli from peripheral tissues such as cutaneous, muscular, joint tissue and visceral structures. Pain is named neuropathic when the nervous system itself is damaged. A nerve injury initiates a range of peripheral and central nervous system processes that contributes to persistent pain and abnormal sensations and finally leads to a state of hyperexcitability in primary afferent nociceptors (46). The peripheral sensitised nociceptors in turn innervate central neurons, which undergo dramatic functional changes including hyperexcitability termed central sensitisation. This process leads to the common neuropathic pain symptoms such as allodynia, spontaneous pain and hyperalgesia. One of the following processes can be responsible for these symptoms:

1. increased primary afferent nociceptor firing (peripheral process)
2. decreased inhibition of neuronal activity in central structures (spinal process)
3. altered central processing (central sensitisation), leading to an amplification of sensory input (central process)

Increased peripheral excitability can be caused by an unusual distribution of ion channels as well as abnormal responses to endogenous pain producing substances such as cytokines. Evidences for instance suggest that the cytokine interleukin-1 and tumor necrosis factor-α both may be involved in the generation and maintenance of hyperalgesia (201; 202). Furthermore, an injured nerve may develop chemosensitivity to various substances, including bradykinin, histamine, serotonin and many others, which are released from adjacent non-neuronal cells or the afferent fibres itself (125; 232). An accumulation of Na^+-channels has been detected on the surface of injured dorsal root ganglion axons, which contribute to the elevated excitability and spontaneous activity common for neuropathic pain (118). Beside the peripheral processes of neuropathic pain, there are indications for sensitisation processes in the spinal cord in particular in the dorsal horn. Enhanced synaptic transmission emerges by wind-up phenomena or long-term potentiation (LTP) in the dorsal horn following a peripheral nerve injury (128). Voltage-gated calcium channels, which are localized at the pre-synaptic endings of primary afferent neurons, lead to a stronger neurotransmitter release following painful stimulation resulting in a sensitisation process of the projection neurons in the lamina I of the spinal cord (186). Recent studies found out that neuropathic pain and hyperalgesia may be due to a dysfunction of endogenous inhibitory systems. The spinal pain transmission system is under inhibitory control from brainstem centres such as the periaqueductal grey and the locus coeruleus. A decrease of descending inhibition as well as an increase of descending facilitation of dorsal horn neuronal transmission (167; 168) due to a reduced efficacy of the endogenous opioid system contribute to elevated pain sensation following a peripheral nerve lesion. Finally, changes in supra-spinal centres triggered by noxious stimulation have been detected by imaging studies. Seifert and Maihöfner found that pain stimuli recruit a complex cortical network, including nociceptive, motor and cognitive areas (191). It is furthermore reported that prolonged pain is accompanied by enhanced responsiveness of neurons in the central amygdala, due to altered membrane properties (154). This overview of plastic cellular changes triggered by nociception is not complete, however it demonstrates

the complexity of neuropathic pain. Some processes can become irreversible over time and contribute to nociception without a centre of injury or nerve damage. The rapid gain of knowledge about abnormal signalling and neuronal substrates involved in the generation of neuropathic pain provide potential targets for the development of new analgesic drugs.

Signs and symptoms

Pain can be divided in two main classifications. (1) Nociceptive pain is characterized by the activation of nociceptors mainly caused by tissue damage. A subtype of the nociceptive pain is the inflammatory pain, which is mediated by several pro-inflammatory mediators. (2) Neuropathic pain occurs after a lesion of the somatosensory system (214). Since the current project primarily investigates neuropathic pain, nociceptive pain is less considered.

A variety of diseases of the nervous system such as spinal cord injury, diabetes, herpes zoster infection, human immunodeficiency virus (HIV) infection or cancer are associated with neuropathic pain. Pain intensity can be rated with one of the validated verbal, numerical or visual analogue scales. Patients often describe their pain as “burning” or “stabbing” and experience numbness, tingling or needles and pins´ sensations. It is quite difficult to translate pain complaints and sensory findings into specific pathophysiologic mechanisms: Different mechanisms often lead to the same symptoms in pain patients. On the other hand patients with similar pain disorders exhibit completely different symptoms.

A simple peripheral nerve injury causes a wide range of molecular and cellular processes and alterations in the nerve system, which leads to a state of hyperexcitability or to ectopic nerve activity. These processes evoke common positive symptoms such as paraesthesia, spontaneous pain, allodynia and hyperalgesia (13). However, neuropathic pain also comprises negative sensory as well as negative motor symptoms such as inappropriate response to painful stimuli, hypotonia and decreased muscle strength. The particular profile of positive and negative symptoms often corresponds to the specific insult to the nervous system. Beside the more physical indications of pain, there are also several symptoms concerning the psychological aspects such as, anxiety, depression, disability and decrease of daily life activities (156).

Treatment of neuropathic pain

A successful treatment not only includes the relief of pain but also aims to improve the activities of daily life. Effective treatment usually combines non-pharmacological methods with pharmacological medication (60). The most important function of an analgesic drug is reduction of neuronal hyperexcitability, either peripherally or centrally. The efficacy of an analgesic drug is determined by the number needed to be treated (NNT). This value demonstrates the number of patients needed to be treated with a certain drug to obtain at least 50 % pain relief. Table 1 shows an overview about the available drugs and their efficacy in diabetic neuropathy, post herpetic neuralgia, peripheral nerve injury (trauma/compression) or trigeminal neuralgia. As first-line medications for neuropathic pain gabapentin, tricyclic antidepressants, opioids, sodium channel blockers and tramadol are discussed. But also drug classes such as seizure medicine, local anaesthetics, antiarrhythmics and anticonvulsants were considered as therapeutic options, since their proposed mechanism are implicated in pain relief (12; 67). The antiarrhythmic drug mexiletine as well as the anticonvulsant lamotrigine and carbamazepine exert their analgesic properties via ion-channel-blocking activity and silence spontaneous and evoked nerve activity. Selective serotonin reuptake inhibitors act through their specific inhibition of presynaptic reuptake of serotonin. The 5-HT and norepinephrine reuptake inhibitor duloxetine is not only used to treat depression or anxiety disorders but also shows efficacy in neuropathic pain (208).

Despite the increasing number of clinical trials and treatment options for neuropathic pain, adverse effects and low efficacy in neuropathic pain syndromes remain a major clinical challenge due to an inadequate understanding of the mechanisms involved in the development and maintenance of neuropathic pain. Therefore, the development of more effective and tolerable analgesic drugs is essential for neuropathic pain treatment.

Medication	NNT for Efficacy/Adverse Effects			
	Diabetic Neuropathy	Postherpetic Neuralgia	Peripheral Nerve injury	Trigeminal Neuralgia
Gabapentin	3.7/1.8	3.2/3.4	—	—
Lidocaine	—	5	—	—
Oxycodone	—	2.5/ND	—	—
Tramadol	3.4/ND	—	—	—
Amitriptyline	2.0/9.7	2.3/6.2	2.5/ND	—
TCA (all types)	2.4/4.9	2.3/6	2.5/ND	
Desipramine	3.4/20	1.9/4.8	—	—
SSRIs (all types)	6.7/ND	—	—	—
Duloxetine	5.7/ND	—	—	—
Paroxetine	2.9/ND	—	—	—
Citalopram	7.7/ND	—	—	—
Carbamazepine	3.3/1.9	—	—	2.6/3.4
Lamotrigine	—	—	—	2.1/ND
Mexiletine	10.0/6.3	—	—	—
Baclofen	—	—	—	1.4/ND

Table 1. Common treatment options for neuropathic pain: Numbers needed to treat to obtain one patient with more than 50% pain relief (NNT) (60; 197). TCA: Tricyclic antidepressants

Animal models for neuropathic pain

Neuropathic pain is caused by nerve injury and includes conditions such as trigeminal neuralgia, postherpetic neuralgia and painful diabetic neuropathy. There are several animal models described, which resemble neuropathic pain state as depicted in Table 2. In some models a direct nerve ligation leads to hypersensitivity In other models, the injection of e.g. a virus or streptozotocine, a toxic agent for insulin producing cells, induce peripheral neuropathy and are used to resemble postherpetic neuralgia or painful diabetic neuropathy in rats. In the current study the chronic constriction injury model (CCI) according to Bennett *et al*. (20) and the partial sciatic nerve ligation model (PNL) according to Seltzer *et al*. (192) were selected to

imitate peripheral neuropathic pain. In the CCI model of Bennett *et al*., four catgut ligatures are loosely tied around the sciatic nerve. This injury leads to intraneural oedema, a focal ischemia and an axonal degeneration. Animals display a pronounced chemical and heat-evoked hyperalgesia, as well as cold and mechanical allodynia and symptoms of spontaneous pain such as flinching and licking for a period of more than 2 month. In the Seltzer model one third to half of the sciatic nerve is tightly ligated with a single ligature. This injury induces mechanical allodynia, heat-evoked hyperalgesia and ongoing pain, which are present for up to 7 months. Both models were chosen in the present studies, since they have been used and characterized in many studies and a stable mechanical and heat hypersensitivity – all sensations reported by neuropathic pain patients - could always be observed (58). The CCI model seems to be a more severe model than the PNL model since a stronger spontaneous pain reflected in a more frequent lifting of the injured paw, could be detected (58).

Animal model	Resembled indication	Surgery/ injection	Symptoms	Reference
Complete sciatic transection (CST)	nerve transection	sciatic nerve transection	peripheral ectopic firing, selfmutilation, spontaneous pain	(223)
Chronic constriction injury (CCI)	neuroma, peripheral neuropathy	four chromic cat cut ligatures around sciatic nerve	allodynia, hyperalgesia, spontaneous pain	(20)
Partial nerve ligation (PNL)	neuroma, peripheral neuropathy	1/2-1/3 ligation of sciatic nerve	mechanical allodynia, heat evoked hyperalgesia, spontaneous pain	(192)
Tibial and sural transcetion model (TST)	peripheral neuropathy (sympathetical independent)	injury of sural and tibial nerves	mechanical allodynia, cold allodynia spontaneous pain	(83)
Spinal nerve ligation (SNL)	peripheral neuropathy	L5 and L6 spinal nerve ligation	mechanical and heat-evoked hyperalgesia, spontaneous pain	(38)
Diabetic neuropathy model	diabetic-associated neuropathy	streptozotocine injection; death of pancreatic cells	mechanical hyperalgesia	(151)
HIV-associated pain model	HIV-induced peripheral neuropathy	application of HIV 1-gp120 in rat sciatic nerve	mechanical hypersensitivity, anxiety	(226)
Herpes Zoster associated pain model	postherpetic neuralgia	varicella zoster virus injection in footpad	mechanical hypersensitivity, anxiety	(86)
Cancer pain model	cancer-associated pain	Inoculation of squamous carcinoma cells	Spontaneous pain, heat hyperalgesia, mechanical allodynia	(9)
Chemotherapy model	chemotherapy-associated peripheral neuropathy	injection of paclitaxel or vincristine	mechanical and cold allodynia/hyperalgesia	(64)

Table 2. Common animal models for neuropathic pain

Assessment of pain in animals

Assessing the level of pain in animals is a key element in pre-clinical pain research. In contrast to humans who can describe a particular pain sensation very specifically, noxious behaviour in animals can only be estimated by observing their reactions. In general in animals the most reliable signs of pain are physical ones. However, it has to be considered that every species exhibits individual pain behaviour. Brief, sharp pains are mainly related with motor responses (withdrawal), escape or attack behaviour. However, it has to be noted, that also passive motor responses, such as immobility or freezing behaviour might indicate a pain experience in animals.

An animal model for nociception should possess the following characteristics: Sensitivity, specificity, validity, reliability and reproducibility. Current pain assessment techniques are mainly limited to estimating animal responses elicited by stimuli to the affected area using either:

1) Thermal (paw immersion test, hot plate test, radiant heat test, acetone test),
2) Tactile (von Frey filaments), or
3) Mechanical (paw pressure) stimuli

To determine mechanical hypersensitivity, an abnormal sensation to mechanical stimuli, we selected the “electro von Frey-test” This test is described as a method with small inter-animal variability and can be used to ascertain whether the nerve lesion has been successful (58).

Evoked pain tests are important in the assessment of painful states and the sensory component of pain. They are reflexive in nature, and only comprise information about the simple pain responses related to the applied modality. However, they do not resemble the complete aspects of perceptual pain experience. As already mentioned chronic pain patients also complain about psychological symptoms such as anxiety, sleep disturbances and reduced quality of life. In clinical trials with chronic pain patients sensory aberrations, spontaneous pain, several comorbidities such as anxiety or depression are considered (140). However, in animals, paw withdrawal threshold and reflexive behaviour are mainly used to determine painful states of animals, while the more complex behavioural aspects of pain are often disregarded.

Therefore, one goal for future pre-clinical pain research is to develop animal models were also emotional aspects of pain, such as anxiety are determined (181).

Anxiety – a particular form of stress

Stress is a very common emotional state which is defined as a “non-specific response of the body to any demand” (80). The exposure to a stressor induces several coordinated responses composed of alterations in behaviour, autonomic function and the secretion of multiple hormones. Some of the physiological changes associated with stress include increased attention on the perceived threat, heightened cerebral perfusion, enhanced cardiovascular output and modulation of immune function.

Stressful stimuli can be grouped in three categories (217):

(a) Psychological stress, based on a learned response to the threat of an impending adverse condition (fear, anxiety)
(b) Stress that consists of a physical stimulus and has a strong psychological component (pain, foot shock)
(c) Stressful stimuli which challenge cardiovascular homeostasis (heat exposure, exercise, haemorrhage)

Stressors are the major elements of a number of anxiety disorders, which are prevalent psychiatric disorders in western countries with an incidence of about 10% of the population. Anxiety disorders are often a debilitating chronic condition and are accompanied by emotional symptoms such as decreased concentration, irritability, restlessness and by physical symptoms such as headache, sweating and hypertension. There are seven different types of anxiety disorders, each with its own distinct symptom profile: Panic disorder (PD), agoraphobia, post-traumatic stress disorder (PTSD), generalized anxiety disorder (GAD), social anxiety disorder, obsessive-compulsive disorder (OCD) and special phobias.

Anxiety itself may be interpreted as an emotional anticipation of an aversive situation, difficult to predict and to control, which is likely to occur. Fear is not seen as a basal state, but a complex response elicited during danger to facilitate appropriate defensive behaviours to reduce danger or injury. In both animals and humans,

anxiety and fear are characterized not only by harm avoidance behaviour but also by a bias favouring negative associations of a given scenario (121).

Neuroanatomy of anxiety

The neurocircuitry of anxiety and fear has been well studied and it turns out that several brain areas such as hypothalamus, septohippocampal system, amygdala, cingulate and prefrontal cortices, hindbrain regions, parabrachial nucleus, cuneiform nucleus and dorsal raphe nucleus are involved in conducting anxious information (74; 77; 96; 170). Anxious stimuli are transmitted via external and visceral sensory pathways to the thalamus, which function as relay station for amygdala, sensory cortex and cingulate gyrus (4; 112; 164). Information reaches the basolateral amygdala via two parallel neural circuits.

1. A rapid subcortical path (short loop, unprocessed information).
2. A slower regulatory cortical pathway (long loop, processed information) encompassing the primary somatosensory cortices, insula and anterior cingulate cortex (126).

Anxiety disorders may be conceived as an imbalance in the control of the long loop over the short loop. Imaging studies proved a participation of the amygdala in anxiety disorders, since an increased amygdala reactivity was observed in patients across a range of disorders (187; 189). These findings show that the amygdala has a pivotal role in the transmission of fear- and anxiety-inducing information (126; 217). The neuronal connections between the amygdala and higher subcoritcal regions enable the individual to initiate adaptive behaviours to threat. The amygdala is controlled and regulated by medial prefrontal regions, including orbifrontal cortex and subgenual anterior cingulate cortex (ACC) (178; 179). The efferent pathways of anxiety-fear circuits include several brain regions such as amygdala, locus coeruleus, hypothalamus, periaqueductal grey and striatum, which activate autonomic, neuroendocrine and skeletal-motor responses.

Pathophysiology of anxiety disorders

Pharmacological studies, genetical approaches, as well as clinical and basic research have contributed to the elucidation of underlying mechanisms of anxiety. Although the pathophysiology of anxiety disorders is still not completely understood,

several neuronal substrates of the central nervous system seem to be implicated in the biology of anxiety. Stress responses include the activation of the hypothalamic-pituitary-adrenocortical axis, which results in an increased release of catecholamine from the sympathetic nervous system as well as a release of corticotrophin releasing hormone (CRH), vasopressin and oxytocin (see below) from the hypothalamus (37; 71; 217). CRH has a major role in modulation stress and anxiety and coordinates the endocrine, autonomic, behavioural and immune responses to stress by the release of ACTH and cortisone (53; 217).

The neurotransmitter and neurohormone neuropeptide Y (NPY) may play an important role in anxiety, since intracerebroventricular injection of NPY produces potent anxiolytic effects in an operant rodent model for anxiety (30; 31). Cholecystokinin (CCK) is a neuropeptide found in high density in the central nervous system and mediates its anxiogenic action via the CCK-B receptor. In PTSD patients, anxiety was induced by the receptor agonist CCK-4 which supports the hypothesis that this neuropeptide is critically involved in the pathophysiology of panic attacks (28).

There are also several classical neurotransmitters which are involved in anxiogenic and stress situations. For instance after a stressful stimulus gabaergic interneurons contribute to the inhibitory influence upon noradrenergic (locus coeruleus), serotonergic (raphe nuclei) and dopaminergic cell clusters, and cause reduced neurotransmitter release (146; 229). The locus coeruleus is the major noradrenergic cell body which is activated during fear and anxiety states (205) and may be of particular significance to the induction of panic attacks (206). Dopamine release is activated after uncontrollable stress in the medial prefrontal cortex, thereby indicating a contribution in anxious states (219). Serotonergic neurons originating in raphe nuclei provide a massive input to corticolimbic structures such as amygdala, hypothalamus and hippocampus and are activated by anxiogenic stimuli (150). GABA agonists such as diazepam or propofol result in a hyperpolarization of centrally located neurons by opening the Cl^- channels, which elicits an anxiolytic action (91; 111).

These data showed that the neurocircuitry of anxiety is very complex and that several neuronal substrates are involved. Apart from conventional neurotransmitters, such as

monoamines, GABA and glutamate, neuropeptides such as vasopressin and oxytocin play an important role in the modulation of anxious states. Their role in anxiety and pain is additionally described in the chapter “Link between pain and anxiety”.

Treatment of anxiety

Over the past decade, there has been substantial progress in our understanding of anxiety disorders. This also leads to an increase of effective treatment for the variety of anxiety disorders. Treatment can be grouped broadly into psychosocial and psychopharmacological approaches. Today selective serotonin reuptake inhibitors (SSRI) such as fluvoxamine, fluoxetine and paroxetine are considered first-line treatments for OCD, social phobia and panic disorder, GAD and PTSD. However, the frequent occurrence of symptoms such as sweating and tremor has to be considered. Furthermore, benzodiazepine such as diazepam, clonazepam and alprazolam are a proper treatment option for anxiety disorders but several adverse events such as sedation, depression, tolerance and withdrawal difficulties have to be taken into account (182; 190). Estimates suggest that approximately 70 % of GAD patients will respond to a therapy with benzodiazepines (75). Anticonvulsants such as gabapentin and pregabalin provide an alternative therapeutic option for social phobia not only due to their significant effects, but also since they are tolerated very well and have a safety profile (159). The tricyclic antidepressant (TCA), such as imipramine and clomipramine offer a second line treatment for several anxiety disorders. Potential advantages of TCAs include their ability to treat symptoms of both anxiety and depression and the absence of abuse and physical dependence effects. However, the utility of TCAs is limited due to their cardio toxic potential and their adverse events such as weight gain, hypotension, oedema, and constipation. It is obvious that a variety of medication are available for the treatment of anxiety disorders. Due to these different affected mechanisms by the treatment it is very important to adjust each patient individually to an optimal therapy.

Models to detect anxiety-related behaviour

In order to characterize anxious states in animals, behavioural tests for the evaluation of potential anxiolytic and anxiogenic properties are available:

- Geller-Seifert conflict test
- Vogel conflict test
- Social interaction test
- Light dark exploration test
- Elevated plus maze test
- Elevated zero maze
- Open field test

Most animal models imply an element of “conflict”, whereby the animal is driven from the natural exploratory desirable behaviour and the fear or avoidance behaviour of a potentially aversive stimulus or dangerous situation. Since the determination of concrete parameters reflecting “anxiety” such as arterial pressure or hyperthermia are limited, emotive components of anxiety such as avoidance, escape and freezing are useful indications for this behaviour. In the thesis the elevated plus maze was selected for measuring anxiety-like behaviour in rats, since it is presently the most widely used model for drug discovery (49). This model is based on the naturalistic conflict between the tendency of rodents to explore a novel environment and the aversive properties of a bright open arm and height. This test is a widely used paradigm, which is unconditioned, relatively simple and ethologically relevant (93; 224). The apparatus consists of a plus-maze elevated from the floor with two open and two closed opposite platforms. The longer the rats spent time in the closed and protected arms the more anxious they are. Anxiolytic drugs specifically increase the number of entries into the open arms and the time spent in open arms. Anxiogenic as well as anxiolytic effects are detectable.

Link between pain and anxiety

In the previous pages a detailed and independent description was given about neuropathic pain and anxiety. In the following chapter an overview about the proposed link between neuropathic pain and anxiety will be described. In fact the

reciprocal relation between anxiety and pain perception has been element of latest clinical and pre-clinic research and was assessed from different perspectives:

First it is acknowledged that chronic pain can have a considerable impact on daily life activities and is associated with psychiatric disorders such as anxiety. It was for instance shown that chronic neuropathic pain impairs the quality of life, reflected in increased anxiety, depression, loss of mobility and independence (18). It is known that patients with persistent pain have a 30 – 54 % higher incidence for the comorbidity anxiety and anxiety disorders have been associated with a large number of somatic complaints, including chronic pain (207). It is possible that the enhancement of comorbidities might in turn lead to increased neuropathic conditions, following an even more intense pain experience (180).

Only a few studies have been conducted to investigate anxiety-like behaviour in animal models of neuropathic pain. Anxiety-like behaviour was observed in a model for HIV-related neuropathic pain (225) and in a rat model of varicella zoster virus-associated pain (86). These findings underscore the influence of neuropathic pain on the affective component of pain and provide evidence that pain-associated symptoms such as anxiety can also be determined pre-clinically in rodents. This is of particular interest since in clinical trails sensory as well as emotional symptoms and comorbidities such as anxiety and depression are assessed, while such endpoint measures are mainly disregarded in preclinical research. It would be an improvement of animal models used in drug discovery to also consider such affective components of chronic pain.

There are clear evidences for the influence of pain experience on anxiety. However, it is also well documented that anxiety has recently emerged as an important vulnerability factor in pain experience. Anxiety can modulate pain thresholds, decrease tolerance to pain and increase patient self-reported pain ratings (43; 115). Results from clinical and preclinical studies demonstrated that anxiety sensitivity or anxiety-like behaviour influences pain experience (11; 107; 108; 177; 211). These clinical and pre-clinical observations provide evidence that pain sensation is correlated with anxiety in animals as well as in humans. However, it is still not clear why such a relationship exists and how anxiety can result in different pain

experiences. Therefore, the current study investigates the interrelationship between pain and anxiety in rats.

Amygdala modulates emotion and pain

Lines of evidence suggest that the amygdala plays an important role in processing anxious and fearful stimuli as well as in modulating pain information (*quod vide* chapter “Neuroanatomy of anxiety”). Through its composition of several anatomically and functionally distinct nuclei the forebrain structure is a key area in processing and integrating sensory information from the periphery (Figure 3). Sensory input from the lateral amygdala and the basolateral amygdala to the central amygdala are part of the fear and anxiety-related circuitry (126; 171). Through extensions of the spinohypothalamic and spinothalamic pain pathways, the central “nociceptive” amygdala receives pain-related information from the thalamus, the anterior cingulate cortex and the insular cortex (144; 195; 203). The amygdala plays a dual role in nociceptive processes and is involved in both pain enhancement and pain reduction. Early analysis of pain mechanisms in the amygdala by using electrophysiological, anatomical and behavioural techniques raised the idea that sensory input modulates the amygdala reflected in a sensitization of neurons and synaptic plasticity which contribute to the activation of cognitive affective functions via cortical circuitry.

The central amygdala builds connections with forebrain areas, hypothalamus and brainstem, to regulate emotional behaviour (47; 126). Further efferent pathways reach the periaqueductal grey (PAG), parabrachial nucleus (PB), reticular formation, solitary tract and nucleus and ventrolateral medulla (126; 172). Insights in the pharmacological and biochemical mechanisms of the amygdala may provide a novel target area for the development of drugs relieving pain and its emotional affective symptoms. Two of the possible neuronal substrates are described in the following chapters.

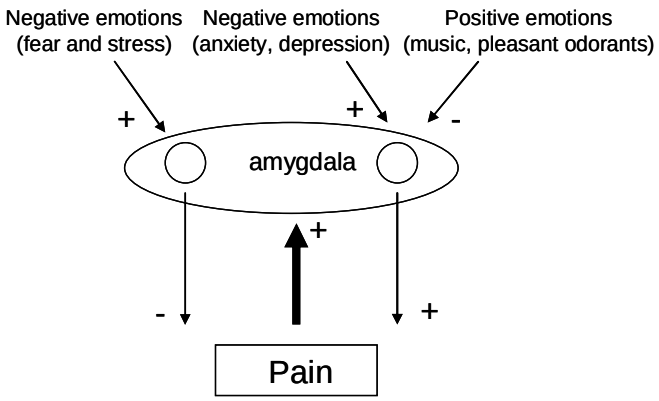


Figure 2. Hypothetical model of interaction between emotions and pain through the amygdala

The amygdala is linked to both facilitatory and inhibitory pathways to modulate pain. Fear and stress lead to hypoalgesia while anxiety and depression lead to hyperalgesia via amygdala outputs: Amygdala output either activates descending inhibitory or facilitatory pathways. Positive emotions inhibit amygdala-mediated pain facilitation. Pain in turn enhances amygdala activity (155).

Vasopressin

Vasopressin is a nine-amino acid neuropeptide that is synthesized in different hypothalamic nuclei, including the supraoptic nucleus, stria terminalis, paraventriuclar nucleus and suprachiasmatic nucleus (54; 148; 215). Vasopressin is generated from a precursor peptide, which consists of three domains: arginine vasopressin, neurophysin II and glycoprotein. After posttranslational modifications the functional active nonapeptide vasopressin is set free (188). The effect of vasopressin are mediated by V_{1a}, V_{1b} and V_2 receptors. While V_{1a} and V_{1b} stimulate phospholipase C via G_q , V_2 subtype activates adenylatcyclase via G_s protein (82; 130). These three receptors for vasopressin have unique tissue distributions. Since V_2 receptors are only found in peripheral tissue such as the lung or the kidney, it plays a minor role for the modulation of anxiety and pain. In contrast V_{1a} receptors are mainly expressed in the central nervous system e.g. in the cortex as well as the septum, the hippocampus and the amygdala (34; 82). V_{1b} receptors have also been identified in the amygdala, cingulate cortex, frontal cortex, nucleus accumbens and hippocampus as well as in locus coeruleus (82; 129; 216). The neuropeptide regulates several body functions such as plasma volume, osmolarity, blood pressure, brain development and modulation of behaviour or memory. Lines of evidence suggest a role of vasopressin in anxiety-like behaviour. A central vasopressin synthesis in the amygdala upon exposure to stress and the release of arginine vasopressin within the septum after the forced swimming test is described (2; 62; 222). Furthermore, anxiolytic properties of mixed V_{1a}/V_{1b} receptor antagonists have been documented (127). On the other hand there are multiple studies supporting the anti-nociceptive effect of vasopressin

after intrathecal (213) intracerebroventricular (21) or systemic (212) application. These findings indicate that vasopressin plays a key role in modulating and processing anxiety as well as pain-related information in the amygdala.

Oxytocin

The nonapeptide oxytocin is synthesized in specific nuclei of the hypothalamus, the paraventricular nucleus (PVN) and the supraoptic nucleus (SON). The magnocellular neurons of the PVN send their projections to the neurohypophysis where the peptide is released in the blood circulation (169), while parvocellular neurons of the PVN mainly project to extrahypothalamic brain areas such as amygdala, substantia nigra, hippocampus, substantia gelatinosa of the spinal cord and brainstem (33; 69; 122). Oxytocin is packaged in large, dense-core vesicles, where it is bound to neurophysin I, to build the larger precursor protein molecule from which oxytocin is derived by enzymatic cleavage. Exocytosis of the neuropeptide is regulated by electrical activity of the hypothalamus. After activation of the oxytocin receptor, which is coupled to G_q protein, effects are mediated via activation of phospholipase C.

The neurophypophyseal peptide is well known for its role in parturition and lactation, learning and memory, maternal behaviour, sexual behaviour and thermoregulation. It has also been demonstrated to be involved in anti-nociception. Oxytocinergic terminals have been identified in pain modulation areas of the brain and oxytocin-containing neurons project to the spinal cord (147; 200), which is implicated in the descending pain pathways. Heat withdrawal latencies and mechanical pain thresholds were increased after injection of oxytocin into the nucleus accumbens and after intrathecal injection, respectively (78; 149; 244). It is also well described that noxious stimuli lead to a release of oxytocin from the pituitary via noradrenergic transmission from the medulla (158). Additionally, there is a wide range of literature which implies a role of oxytocin in anxiety-like behaviour. For instance oxytocin secretion correlates with the level of anxiety (17) and oxytocin deficient mice were found to display an anxiogenic profile (135). However, it has been reported that oxytocin also exhibits anxiolytic properties after central administration to rodents (14; 204). Intriguingly, it appears that oxytocin may fulfil opposite anxiolytic and anxiogenic roles. Taken together, these studies clearly show that oxytocin and vasopressin are implicated in nociceptive transmission as well as in anxiety states. Although the neuropeptides oxytocin and vasopressin are reported to be involved in

anxiety as well as in pain processes it is not known if they are also important key modulators in the affective component of pain. Therefore, in the current study (*quod vide* chapter IIIChapter III) their mRNA levels in animals with pain-induced anxiety were determined, and the effect of an antagonist on mechanical hypersensitivity and pain-induced anxiety was measured.

Aim of the study:

Neuropathic pain, a disease due to a lesion or disease of the somatosensory system, is often associated with several disabling comorbidities such as anxiety or depression. In turn, anxiety is also widely reported to influence pain sensation. Anxiety may exacerbate pain and in turn pain increases the associated symptoms, thereby aggravating the disease and complicating the treatment of neuropathic pain. The influence of affective illness and neuropathic pain and vice versa is well recognised in humans yet there is little known about this phenomenon in animals. Rodent models have successfully been used to assess emotional disorders and several animal models are available to identify neuropathic pain behaviour in rats. However, the majority of such studies rely on measures of evoked withdrawal responses to identify hypersensitivity but reveal hardly any information regarding emotional or subjective aspects of pain. This is one of the drawbacks of preclinical pain research, since in human clinical trails not only evoked pain but also spontaneous pain and emotional and subjective aspects of pain are commonly assessed. In the present thesis this problem was approached, by measuring sensory components of pain as well as anxiety-like behaviour in a rat model of neuropathic pain. The hind paw withdrawal test “electro von Frey” was combined with the anxiety paradigm “elevated plus maze” to assess both, sensory aspects as well as pain-related symptoms of neuropathic pain in rats.

Since it is hypothesized that anxiety can either induce or increase pain sensation in humans, it is important for improving therapeutical approaches, to fully understand the link between pain and anxiety in the preclinical models by assessing not only behavioural tasks but also discover responsible cellular changes contributing to the pathophysiology of neuropathic pain and associated anxiety. Additional investigations are required to identify the key substrates responsible for pain and anxiety.

To completely identify the relationship between anxiety and pain it was essential to examine whether pre-existing anxiety also influences pain response in rats, as it was observed in anxiety disorder patients.

In the present study the reciprocal relationship between anxiety and pain in rats is assessed. By using behavioural, pharmacological and molecular biological techniques the following questions were addressed:

1. Does neuropathic pain induce anxiety-like behaviour in a model of mononeuropathy? Can we reverse the pain-induced anxiety-like behaviour by treating the rats with the anxiolytic drug midazolam and the analgesic drugs morphine and gabapentin? (chapter I)
2. Which effects exhibit other analgesic drugs on pain-induced anxiety-like behaviour? (chapter II)
3. Are there any changes of mRNA level of the neuropeptides oxytocin or vasopressin due to neuropathic pain in the amygdala of rats with sciatic nerve lesion? Do the high levels of both neuropeptides affect mechanical hypersensitivity or pain-induced anxiety in CCI lesioned rats? (chapter III)
4. Can pain sensitivity be influenced by anxiety?
    a. Can we affect pain sensitivity by using a model for trait anxiety? (chapter IV)
    b. Can we affect pain sensitivity by using a model for state anxiety? (chapter V)

Answers to these questions can clarify the link between anxiety and pain. A better understanding of pain behaviour in animals might be an advantage to improve the characterization of analgesic drugs during development. Cellular changes (e.g. expression levels) due to neuropathic pain can give insights into the pathophysiology of neuropathic pain and its related symptoms in animals. The anxiety-induced hypersensitivity model would represent a new preclinical tool to further investigate the link between pain and anxiety and may be relevant to detect new therapeutic targets and strategies for several pain and anxiety-associated disorders.

MATERIALS AND METHODS

Animal care

Male Wistar rats (CrlGlxBrlHan:WI, Charles River), weighing 210 - 230 g at delivery day, were housed in groups of 4 - 5 per cage. In the microinjection experiment (experiment 2) animals were separated into two rats per cage in order to avoid mutual injury. Animal rooms were adjusted to normal light cycle (6.00 a.m. – 18.00 p.m.) and temperature-controlled environment (23 ± 1 ℃). Food and water were available *ad libitum*. Animals were habituated in the animal room for at least 7-10 days after delivery before starting surgery or experiments. Behavioural tests were performed during the inactive phase (9.00 – 14.00 h). Housing, handling and testing of the animals were conducted according to the *Guidelines on Ethical Standards for investigation of Experimental Pain in Animals* (250). The experiments were approved by the local committee for animal care and use.

Low anxiety and high anxiety behaviour rats

Experiments were performed with male Wistar rats (350 – 450 g) selectively bred for either low or high anxiety-related behaviour. The LAB and HAB rats used in this study were bred in the animal facilities of the University of Regensburg (Germany). A detailed description of the breeding procedure is given in the quoted references (120; 127). Animal care and keeping of animals was similar to our conditions as described previously.

Surgeries

Chronic constriction injury (CCI) and partial nerve injury (PNL) of the sciatic nerve

Rats were anaesthetised with isoflurane (2% in 100% O2; Forene®, Abbott GmbH & Co. KG, Wiesbaden, Germany). All surgical procedures were carried out under sterile conditions and were performed by the same person. Animals were inspected every day and tested 21-28 days after surgery. Chronic constriction injury was performed according to the method of Bennett (20). The left common sciatic nerve was exposed at the middle of the thigh by blunt dissection through *biceps femoris*. Proximal to the sciatic trifurcation, about 7 mm of nerve was freed of adhering tissue and 4 ligatures (4/0 USP, Resocat® chrom. Resorba, Germany) were tied loosely around it with

about 1 mm spacing. Thus, the total length of affected nerve was 4-5 mm. The ligatures were loosely tied in order to minimize nerve constriction. After neuropathic surgery, the skin was closed by double knots with the use of vicryl thread (3/0 USP, Ethicon, Johnson & Johnson, Belgium).

PNL surgery was applied according to the method of Seltzer (192). The rat's left sciatic nerve was exposed and one-third of the nerve diameter was ligated by Vicryl (6/0, Ethico GmbH, Norderstedt, Germany). After the surgery, the skin was closed by double knots with the use of Vicryl thread (3/0 USP, Ethicon, Johnson & Johnson, Belgium).

Sham controls were performed for each surgery by exposing the nerves without inducing any lesion or ligation.

Microinjection in amygdala (insertion of cannula)

The rats were anesthetized by intraperitoneal barbiturate Narcoren and fixed in the stereotaxic instrument. Stainless steel guide cannulas (26 gauge) were lowered bilaterally into the amygdala based on coordinates provided by the atlas of Paxinos and Watson (161) (anterior/posterior to bregma -2.6 mm ; lateral to bregam ± 4.3 mm, ventral to bregma 7.4 mm) and was fixed to the skull by dental cement (PermaCem, DMG, Germany). On the day of experiment an internal cannula (26 gauges) were directly inserted into the guide cannula. The cannulas were connected, via polyethylene tubing, to syringes mounted on an infusion pump (Precidor, Infors AG Basel, Switzerland). 1 µl of oxytocin antagonist (OAT), vasopressin antagonist or saline were injected into the amygdala during 1 min.

Histological verification of the injection site

At the end of the experiment rats received a microinjection of methylenblue to stain the location of the cannula. Immediately afterwards rats were sacrificed by an overdose of the barbiturate Narcoren. Brains were isolated placed in tissue-tek (Sakura, the Nederlands) and then immediately shock frozen in liquid nitrogen. Coronal sections (20 µm) were taken and the injection sites of the cannulas were verified. All animals with dislocated injection site were excluded from analysis. The illustration in the appendix shows an example of the injection site in the amygdala and a schema of the affected area (Figure 17).

Behavioural tests

Measurement of mechanical hypersensitivity

Mechanical hypersensitivity was measured by using an electronic von Frey algometer (Somedic, Hörby, Sweden). The animals were placed in a plexiglas cage (16 x 24 x 14 cm) with a grid bottom and adapted for at least 15 minutes. Mechanical stimuli were generated by touching the plantar region of the left and right hind paw of the rat with a continuous increasing pressure (5 g/sec). For the paw withdrawal threshold the mean of three independent measurements was calculated. The values of the paw withdrawal thresholds were automatically recorded and stored. The observer was blinded to pharmacological treatments.

Motility test – activity box

The spontaneous locomotor activity and the total number of rearings were assessed in a dark environment with an automatic measurement system (Coulbourn Instruments, USA). The rats were placed in a transparent box (40 x 40 x 40 cm) equipped with two series of photocells located 3 and 13 cm above the floor. Lateral movement of the animals was detected with a set of photocells at the bottom while rearing was measured with the upper set. The total distance travelled and the total number of rearings were measured over a time period of 30 minutes by generating a data set every 5 minutes. A decrease in spontaneous motility in a dark field could reflect motor dysfunction or sedation.

Anxiety test – elevated plus maze

The elevated plus-maze test was used to assess the anxiety-like behaviour state. The rats were adapted in their home cages in the experimental room for about 24 hours. The animal was placed in the middle of the EPM and the behaviour was recorded by a video camera for 10 minutes. After each test, the maze was carefully cleaned with 70 % ethanol and tissue paper. The apparatus comprised two open arms (100 lux) and two closed arms (45 x 40 x 10 cm; 20 lux) which were connected by a common central area (10 x 10 cm). The maze was constructed from black PVC synthetic material and elevated to a height of 80 cm above floor level. The elevated plus-maze was surrounded by an opaque curtain to avoid disturbances by the observer. The experiments were carried out in a room with low (100 lux) illumination conditions. The complete experiment was directly recorded on DVD. The following

parameters were scored during the trial by a custom-made program: Total number of entries into open arms and the time spent in open arms. The observer was blinded to surgical and pharmacological treatments. Anxiety test at university of Regensburg was performed under slightly different conditions:

High or low anxiety-like behaviour rats were tested on the EPM at the age of 8 weeks to characterize their behaviour. The EPM apparatus made of dark grey plastics had a raised edge (0.5 cm) on the open arms, which provided additional grip for the rats. The test session lasted 5 minutes. Behaviour was measured by means of a video camera mounted above the platform and scored by a trained observer pressing pre-set keys on a computer (Plus-maze version 2.0; Ernst Fricke). An open/closed-arm entry was defined as both fore-paws of the rat being on the respective arm of the elevated plus-maze.

Pharmacologically induced anxiety

Anxiety was induced by oral treatment of pentylentetrazol (40 mg/kg) once (for acute experiment) or three times daily over a period of 9 days (for subchronic treatment).

Quantitative real-time polymerase chain reaction (Taqman RT- PCR)

Expression of Oxytocin and Vasopressin was detected in neuropathic animals sham and naive rats, three weeks after operation (n = 3 - 4). After performing the EPM task and determining mechanical hypersensitivity, animals were sacrificed by an overdose of the barbiturate Narcoren. The experiment was done twice with a group of 3 - 4 animals. Ipsilateral and contralateral amygdala and hypothalamus were dissected from each rat and incubated over night in mRNA stabilizing solution at 4℃ (RNAlater, Ambion, Applied Biosystems, USA). The next day tissues were homogenized with 1 ml of Trizol (Invitrogen, Canada) per 100 mg tissue and isolation of mRNA was performed (Qiagen RNeasy Kit, Venlo, The Netherlands) according to the manufacturer’s instruction. RNA was resuspended in RNAse free water and quantity of mRNA was calculated by A 260 measurement. RNA quality was assessed by electrophoresis. cDNA synthesis was performed using the High Capacity cDNA Archive Kit (Applied Biosystems) on pooled samples. Quantitative real-time PCR was carried out using the 7500 FAST real-time PCR system (Applied Biosystems). Primer mixes were purchased from Applied Biosystems. Relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and

expresses it relative to the levels of an internal control RNA and analysed to the housekeeper 18S RNA.

Drugs and anaesthetics

The $GABA_A$ receptor modulator midazolam (Dormicum®) was obtained from LA Roche AG (Basel, Switzerland), the opioid-receptor agonist morphine from Merck KGaA (Darmstadt, Germany) and gabapentin from Chempacific (Baltimore, USA; Ca^{2+}-channel modulator). The $5HT_{1A}$ receptor agonist 8-OH-DPAT, the µ-opioid receptor agonist tramadol, which also affects the 5HT system, and the sodium channel blocker mexiletin were purchased from Sigma Aldrich (USA), lacosamide (sodium channel modulator and collapsin-response mediator protein 2 modulator) and the peptidergic B_1- antagonist R-715 from Tetranov Biopharm (China). The synthetic B_1- antagonist SSR240612 was produced in house. All compounds were dissolved in saline (0.9 % NaCl) except the B_1-antagonist SSR240612 and the anti-epileptic drug lacosamide, which were dissolved in 90% Natrosol 0.5%, 10% Tween 80 0.1%. If not mentioned drugs were administered intraperitoneally. Oxytocin antagonist (d(CH2)51,Tyr(Me)2,Thr4,Orn8,Tyr-NH29)-Vasotocin (dissolved in Saline) was purchased from Bachem and vasopressin antagonist Conivaptan (Vaprisol) from Komtur. Isoflurane (Forene®) was purchased from Abbott.

Dosages were chosen in a range which does not impair motility, as indicated in the literature and assessed in the activity box in our laboratory (Table 4).

Statistics and design of experiments

The experiments were performed in a randomized and blind manner between 9 a.m. and 15 p.m. Data are given as mean ± SEM. An adjusted p-value less than 0.05 indicates a significant difference. The statistical evaluation was prepared by using the “Graph pad prism”, Version 5.

Chapter I: The assessment of activity, pain and anxiety-like behaviour in sham-, PNL- and CCI-operated animals was done by analyzing the absolute values using one-way analysis of variance (ANOVA). Pairwise comparisons were carried out by *t-tests* and values were adjusted to Bonferroni Holm (Figure 4). To analyse the analgesic properties of the utilised drugs midazolam, morphine and gabapentin, (Figure 5) a paired *t-test* (Wilcoxon signed rank test) between the thresholds of the

injured paw before and after treatment was performed. The experiments, in which drug effects were investigated in the elevated plus maze, were analysed performing a parametric two-way ANOVA test, including the factors surgery (sham and CCI), treatment (midazolam, morphine and gabapentin) and interaction, followed by Bonferroni-Holm pairwise *post hoc* analysis, to compare the effects within all groups (Figure 6). Graphs of pharmacological studies using the EPM test are presented with data normalised to the mean value of the sham (vehicle) group.

Chapter II: To compare the drug related effects on locomotion between the control group and the treatment group, an unpaired *t-test* was performed between the vehicle and the treatment group. The analgesic effect of a drug was analysed by using a paired *t-test* (Wilcoxon signed rank test) between the thresholds of the injured paw before and after drug administration. Two-way ANOVA, including the factors surgery (sham and CCI), treatment (analgesic drug) and interaction, followed by Bonferroni-Holms pairwise *post hoc test*, were used to determine the effect of the compound on pain-induced anxiety in the EPM (Table 4).

Chapter III: The assessment of mechanical hypersensitivity of oxytocin and vasopressin was done by analyzing the absolute values using analysis of variance (ANOVA) including the factors time (0, 10, 30 60 minutes after treatment), operation (sham and CCI surgery) and treatment (vehicle and vasopressin antagonist; Figure 8e; Figure 9e). The determination of pain-induced anxiety was performed by using analysis of variance (ANOVA) including the factors operation (sham and CCI surgery) and treatment (vehicle and vasopressin antagonist). Pairwise comparisons were carried out by *t-tests* and values were adjusted to Bonferroni Holm (Figure 8c-d and Figure 9c-d). Independent groups were used to investigate the effect of vasopressin and oxytocin antagonists on anxiety and pain behaviour, respectively. First pain behaviour was investigated to find the time of maximal effect. In each experiment two sham groups (vehicle and drug) and two CCI groups (vehicle and drug) were used.

Chapter IV: To investigate the influence of anxiety on pain behaviour over a period 8 weeks an analysis of variance (ANOVA two-way) for repeated measurements was performed including the factors experimental group (HAB and LAB) and time point (day 0, 7, 14, 21, 36 and 57 post-injury). Adjustment of p-values for multiple testing was performed by the method of Bonferroni-Holm for each pair wise comparison over

all time points (Figure 10). To analyse statistical differences in paw withdrawal thresholds between the injured and the non-injured paw in LAB and HAB rats, and to analyse the effect of gabapentin on mechanical hypersensitivity in HAB and LAB rats, paired *t-tests* were performed separately for the measurements before and after treatment (Figure 11). For analysing pain-induced anxiety-like behaviour in LAB rats with chronic constriction injury the same statistical procedure was applied (Figure 12). Animals were tested on the elevated plus maze before performing the experiments to characterize LAB rats and HAB rats at the age of 8 weeks (EPM 1). Rats were subjected to CCI at the age of about 12 weeks. 36 days after surgery LAB rats were tested a second time on the EPM to prove whether the chronic injury influences their behaviour in this task (EPM 2). Mechanical hypersensitivity was assessed one day before surgery and at day 7, 14, 21, 36 and 57 post-surgery. Paw withdrawal threshold (PWT) of the lesioned paw were compared with the healthy contralateral side. Pain experiments were conducted with 15 lesioned HAB and 15 lesioned LAB rats. For measuring pain-induced anxiety (EPM 2), lesioned LAB rats were compared with 12 naive LAB rats.

Chapter V: The effect of acute treatment of PTZ on mechanical hypersensitivity and on anxiety-like behaviour (acute) was analysed with an unpaired *t-test* (Figure 13). A data analysis of variance (two way ANOVA) was applied to test the effect of chronic PTZ treatment over time, including the factors time (day 1, 4, 7, 9, 14) and treatment (PTZ and vehicle; Figure 14). To calculate the effect of gabapentin (Figure 15) or midazolam (Figure 16) on PTZ-induced anxiety and mechanical hypersensitivity, the two way ANOVA with the factors (chronic treatment of PTZ or vehicle) and acute treatment (drug or vehicle) was used. Adjustment of p-values for multiple testing was performed by the method of Bonferroni-Holm for each pairwise comparison.

Dropouts:

Chapter I and II: Animals that did not develop an enhanced response to mechanical stimuli following nerve injury or showed self mutilation at the paw were excluded from the study for measuring anxiety or pain behaviour. Lack of behavioural hyperalgesia is defined as a decrease of PWT less than 20% of the average value of the control group. Chapter III: All animals where the tip of the inserted cannula was dislocated from the amygdala, were excluded from the analysis. Furthermore, 3 sham and 3 CCI injured animals, which lost the cannula pedestal, were also excluded from the

behavioural studies. Chapter IV: All animals were used during the whole test session. In Chapter V one animal was excluded due to incorrect application.

CHAPTER I

Anxiety-like behaviour in animals with mononeuropathy is reduced by the analgesic drugs morphine and gabapentin

It is reported that a considerable number of patients suffer from depression or anxiety disorders as a consequence of chronic pain (156). Although the presence of anxiety symptoms in neuropathic pain patients was frequently reported, only a few studies have been conducted in animals to investigate the emotional or subjective aspects of pain. Recently, a few groups began to address this issue by measuring related symptoms of neuropathic pain in preclinical studies. It has been shown that neuropathic pain is associated with anxiety-like behaviour in a model for varicella zoster virus-associated pain (86), partial nerve ligation (228) and HIV-associated pain (226). These data indicate that such behavioural paradigms are useful for the assessment of pain-related symptoms in rodent models and might help to discover potential pharmacological analgesic targets.

The aim of our first study was to assess anxiety-like behaviour in neuropathic pain models. To corroborate our hypothesis that pain relief also causes a reduction of pain-induced anxiety we determined exploratory behaviour on the elevated plus maze (EPM) test. Entries and time spent in open arms were taken as the readout, and this behaviour was examined in two different animal models of neuropathy: the chronic constriction injury (CCI) model and the partial nerve ligation (PNL) model. Moreover, the effects of the anxiolytic drug midazolam, which is devoid of anti-nociceptive effects, and the anti-nociceptive drugs morphine and gabapentin were assessed in this test. The analgesic efficacy and effects on locomotion of the utilised drugs were also tested in CCI- and sham-operated animals, respectively. The study may help us to better understand the relationship between pain and anxiety and to assess the effects of new analgesic drugs on both behavioural aspects.

The following study is already published in “Pain”: Roeska K, Doods H, Arndt K, Treede RD, Ceci A. Anxiety-like behaviour in rats with mononeuropathy is reduced by the analgesic drugs morphine and gabapentin. Pain 2008; 2008 Oct 15;139(2):349-57.

Pain induces anxiety in rats with sciatic nerve injury

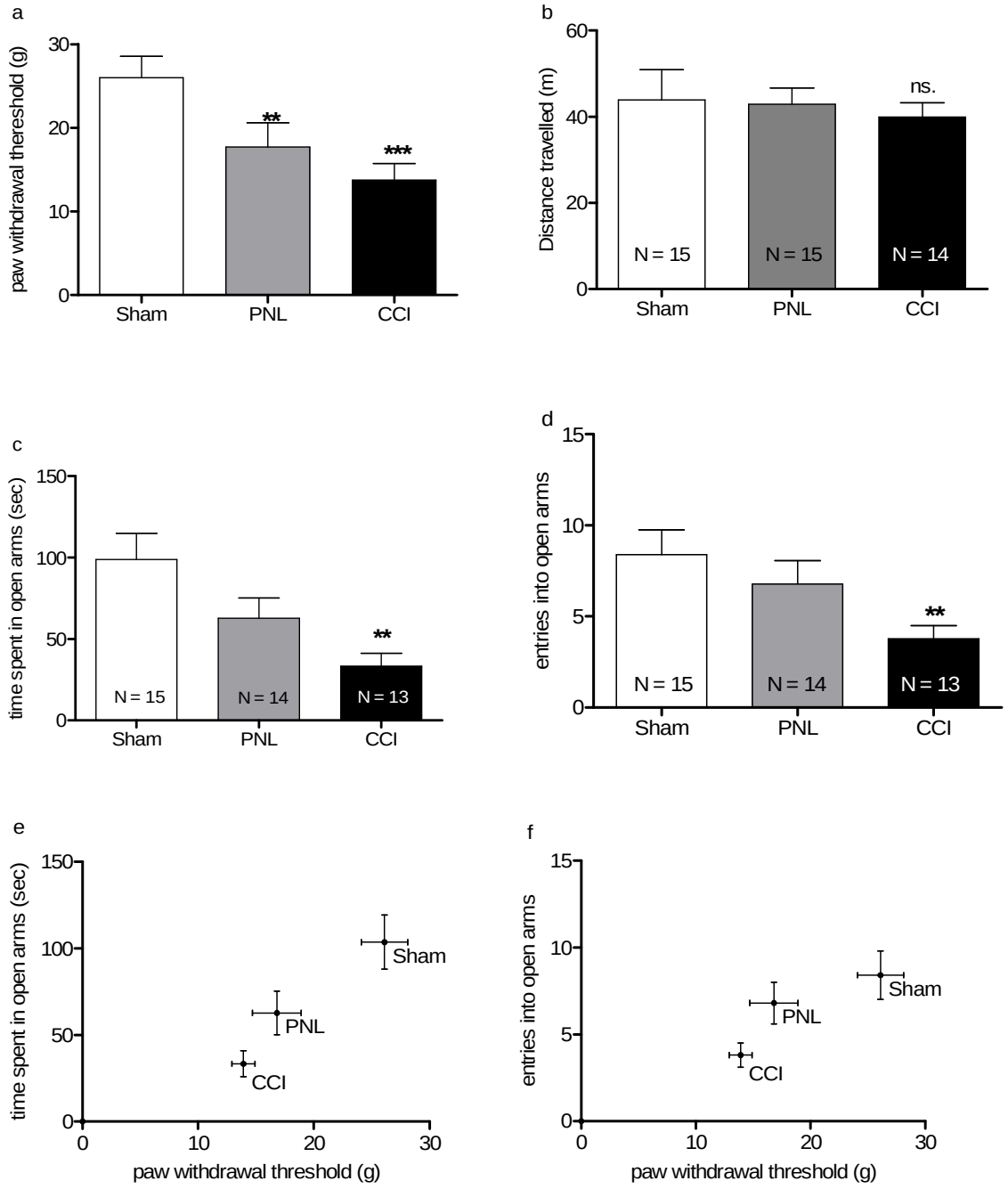


Figure 4. Assessment of mechanical hypersensitivity, locomotion and anxiety-like behaviour of two models for neuropathic pain

Mechanical hypersensitivity was measured in animals with neuropathic pain (PNL and CCI model) and sham operated animals (a). Both models exhibit a significant pain response. This is even more pronounced in rats with chronic constriction injury than in rats with partial nerve ligation. Locomotion in all animal groups is not influenced by the injury (b). Anxiety-like behaviour was determined in sham-, PNL- and CCI-operated rats by determining entries and time spent in open arms (c-d). The dot blots (e-f) demonstrate the positive relationship between the level of mechanical hypersensitivity and the level of anxiety-like behaviour (entries into open arms and time spent in open arms). Data are

expressed as means ± SEM (N = 13-15). *T-test* adjusted to Bonferroni–Holm, *P < 0.05, **P < 0.01 compared with sham-operated animals.

The animals were tested three weeks after sciatic lesion, and significant hypersensitivity to mechanical stimulation was observed in the injured paw of PNL- (N = 15) and CCI- (N = 14) operated rats in comparison to the sham group (N = 15; Figure 4a). The paw-withdrawal thresholds (PWT) significantly decreased from 26 ± 2 g in sham to 18 ± 2 g in PNL (P < 0.01) and 14 ± 1 g in CCI animals (P < 0.001). In order to eliminate the possibility that the surgery affects the motility of the animals, spontaneous locomotion was measured (Figure 4b). The distances travelled during the observation time of 30 minutes were 38 ± 2 m in CCI (N = 14), 42 ± 2 m in PNL (N = 15) and 44 ± 2 m in the sham group (N = 15). The total number of rearings was 143 ± 6 in CCI, 145 ± 7 in PNL and 142 ± 10 in the sham group (data not shown). There was no significant difference in motility between all three groups, and no impairment of movement could be observed in the nerve-injured rats in comparison to the sham group. The animals showed common health and normal behaviour. Signs of disability and distress were absent and development of body weight was regular and identical in all groups.

Having shown that lesioned animals are not impaired in locomotion, the anxiety-like behaviour was determined using the elevated plus maze task. ANOVA one-way comparison revealed significant differences in the time spent in the open arms and the total number of entries into the open arms (Figure 4c-d). A pairwise *t-test* comparison indicated a significant effect between sham (N = 15) and CCI animals (N = 13) reflected in a decreased time spent in open arms in sham animals from 99 ± 15.8 s to 33.4 ± 7.5 sec in CCI injured rats (P < 0.01). Also the second parameter entries in open arms is significantly diminished from 8 ± 1 in sham operated animals to 4 ± 1 in CCI operated animals (P < 0.05;). A *t-test* comparison between sham and PNL animals (N = 14) indicated no significant effects on the time spent in open arms (sham 103 ± 15.8 s; PNL 62.7 ± 12.6 s; P = 0.10) nor for the number of entries into the open arms (sham 8.4 ± 1.4; PNL 6.8 ± 1.3; P = 0.3). These results indicate that CCI-operated animals exhibit anxiety-like behaviour in comparison to the sham group, while in PNL animals only a trend towards significance could be observed.

The degree of hypersensitivity is associated with the degree of anxiety-like behaviour across the two models and sham animals. As depicted in Figure 4e the CCI animals

exhibited the lowest mean value of time spent in the open arms as well as the lowest mean value of paw-withdrawal thresholds to punctuate mechanical stimulation. The same relationship was observed between mechanical hypersensitivity and total number of entries into the open arms (Figure 4f). Since significant differences were only observed in the CCI animals, drug effects were determined exclusively in this model.

Effect of midazolam, morphine and gabapentin on mechanical hypersensitivity

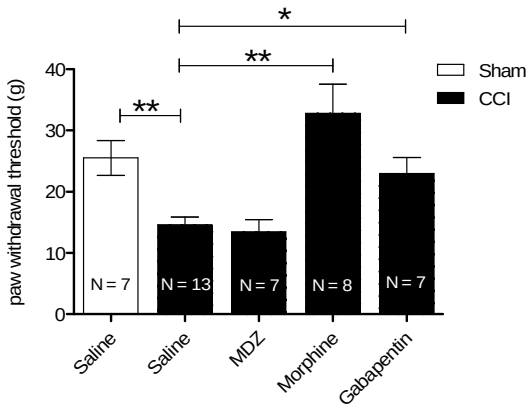


Figure 5. Effect of midazolam, morphine and gabapentin on paw-withdrawal threshold following chronic constriction injury (CCI) of the sciatic nerve.

Rats with chronic constriction injury displayed a pronounced mechanical hypersensitivity on the injured paw after applying mechanical stimulus. The anxiolytic drug midazolam (0.5 mg/kg; i.p.; N = 7) had no effect on the decreased threshold. Morphine (3 mg/kg; i.p.; N = 8) and Gabapentin (30 mg/kg; i.p.; N = 7) reduced the pain responses 1 h after treatment. Data are expressed as mean ± SEM. *T-test* adjusted to Bonferroni–Holm: **P < 0.01.

In the next stage the effect of the anxiolytic drug midazolam and the analgesic drugs morphine and gabapentin on mechanical hypersensitivity was assessed to confirm their analgesic effect (Figure 5). A one-way ANOVA comparison revealed a highly significant difference between the groups ($P < 0.001$). Paw-withdrawal thresholds were significantly lower in sham-operated animals (25.5 ± 2 g) in comparison to vehicle treated injured rats (15 ± 1 g; $P < 0.01$). This result demonstrates the development of behavioural signs of mechanical hyperalgesia three weeks post-surgery in sciatic nerve lesioned rats. The administration of the anxiolytic drug midazolam (0.5 mg/kg; i.p.) did not show anti-nociceptive effects on the paw-withdrawal threshold ($P = 0.77$). In contrast to midazolam, morphine (3 mg/kg; i.p.) significantly reversed mechanical hypersensitivity from 15 ± 1 g in saline-treated CCI animals to 33 ± 3 g in CCI animals post-administration ($P < 0.01$); this value was slightly above that in sham animals ($P = 0.22$). Gabapentin (30 mg/kg; i.p.) also had an anti-nociceptive effect and significantly increased the PWT in the injured leg to 23 ± 3 g ($P < 0.05$). These results illustrate that midazolam does not affect pain thresholds in CCI-injured animals, while morphine and gabapentin are

able to reverse mechanical hypersensitivity in the doses of 3 and 30 mg/kg, respectively.

Midazolam, morphine and gabapentin reduce pain-induced anxiety

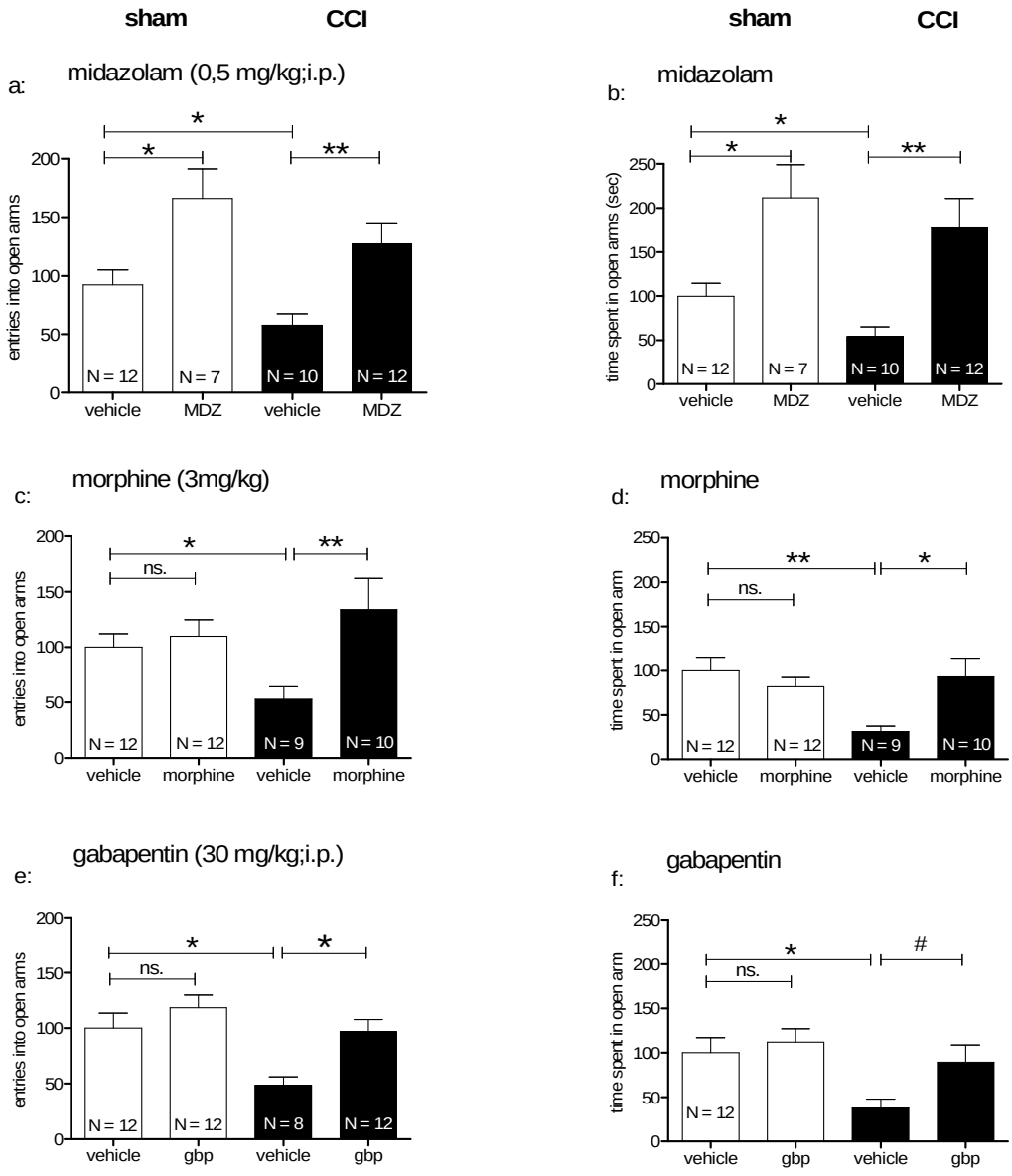


Figure 6. Effect of midazolam, morphine and gabapentin in the elevated plus maze task

Midazolam (0.5 mg/kg) exhibited an anxiolytic effect in sham- and CCI- operated animals (a and b; N = 7–12). 3 mg/kg morphine (c and d; N = 9–12) and 30 mg/kg gabapentin (e and f; N = 8–12) revealed an anxiolytic effect in the CCI-operated animals, whereas no effect in the sham group could be observed. Pain-induced anxiety can be reversed by a pure analgesic drug. Number of entries after drug treatment (a, c and e) and time spent in open arms (b, d and f) are displayed. Data are normalized to the mean value of the sham (vehicle) group and expressed as means ± SEM. *t-test* adjusted to Bonferroni–Holm indicates statistically significant differences: #P = 0.078, *P < 0.05, **P < 0.01.

After investigating the effects of the drugs in mechanical hypersensitivity, their ability to affect anxiety-like behaviour in sham-operated and in CCI-lesioned animals was determined (Figure 6). Midazolam showed a significant anxiolytic effect in sham and CCI animals when tested in the EPM paradigm (Figure 6a-b). A two-way ANOVA comparison revealed statistically significant main effects in entries into the open arms for treatment ($P < 0.001$) and for surgery ($P < 0.05$), but not for interaction. Likewise, two-way ANOVA for time spent in open arms showed significant effects of treatment ($P < 0.001$) and trend towards significance for surgery ($P = 0.099$) but not for interaction. A pairwise *t-test* revealed a statistically significant increase in number of entries into the open arms in the sham group from 5 ± 1 to 9 ± 1 ($P < 0.05$) as well as in CCI animals from 3 ± 0.5 to 7 ± 1 ($P < 0.01$). Midazolam also significantly increased time spent in open arms in sham animals from 83 ± 11 to 160 ± 28 s ($P < 0.0$) and in CCI animals from 35 ± 8.5 to 134 ± 26 s ($P < 0.01$). These results confirm the anxiolytic effect of midazolam in neuropathic and control animals.

After demonstrating that neuropathic pain causes anxiety-like behaviour which is reversible by midazolam, we investigated the ability of analgesic drugs to reduce pain-induced anxiety (Figure 6c-d). Morphine attenuated the pain-induced anxiety-like behaviour in the CCI animals without having an effect on this parameter in the sham group. In these experiments two-way ANOVA revealed a significant main effect in entries into the open arms for the treatment ($P < 0.05$) and a significant interaction ($P < 0.05$) but no significance for surgery ($P = 0.346$). Similar results were seen for the time spent in open arms. A *post-hoc* test yielded statistically significant increase for number of entries into the open arms in CCI animals treated with vehicle from $3. \pm 0.4$ to 8 ± 1 ($P < 0.01$) and for the time spent in open arms in animals from 32 ± 5.6 to 95 ± 20 s ($P < 0.05$). The morphine treatment did not influence behaviour in the sham groups. These findings clearly demonstrate that anxiety-like behaviour can be diminished as a consequence of pain relief by the analgesic drug morphine.

Similar results were obtained with gabapentin (Figure 6e-f). Two-way ANOVA revealed statistically significant main effects in entries into the open arms for the treatment ($P < 0.01$) and for surgery ($P < 0.01$). The interaction was not significant (Table 3). Similar effects could be observed in time spent in open arms. Figure 6e and f demonstrate that the effect of gabapentin in EPM behaviour is more pronounced in CCI than in sham-operated animals. *Post-hoc* results showed that

gabapentin significantly increased entries into the open arms from 5 ± 1 to 7.5 ± 1 ($P < 0.05$) in the lesioned rats without affecting this parameter in the sham group. Time spent in open arms was increased in CCI-operated animals from 54 ± 13 to 115 ± 25 s but missed significance ($P = 0.078$), while no significant difference could be observed in the sham group after treatment. These results show that gabapentin can completely reverse pain-induced anxiety-like behaviour in rats with sciatic nerve-lesion.

Drug		Entries in open arms	Time spent in open arms
Midazolam	treatment	$F_{1,37} = 20.74$; $P < 0.001$	$F_{1,37} = 24.15$, $P < 0.001$
	surgery	$F_{1,37} = 7.43$; $P < 0.05$	$F_{1,37} = 2.86$; $P = 0.099$
	interaction	$F_{1,37} = 0.01$; $P = 0.908$	$F_{1,37} = 0.06$; $P = 0.814$
Morphine	treatment	$F_{1,39} = 5.08$; $P < 0.05$	$F_{1,39} = 2.18$; $P = 0.148$
	surgery	$F_{1,39} = 0.91$; $P = 0.346$	$F_{1,39} = 3.84$; $P = 0.057$
	interaction	$F_{1,39} = 6.28$; $P < 0.05$	$F_{1,39} = 7.3$; $P < 0.05$
Gabapentin	treatment	$F_{1,40} = 7.73$; $P < 0.01$	$F_{1,39} = 3.25$; $P = 0.079$
	surgery	$F_{1,40} = 9.30$; $P < 0.01$	$F_{1,39} = 6.03$; $P = 0.019$
	interaction	$F_{1,40} = 1.58$; $P = 0.217$	$F_{1,39} = 1.75$; $P = 0.193$

Table 3. Effect of midazolam, morphine and gabapentin on pain-induced anxiety. P values and F values of two-way ANOVA test.

Discussion

The current study demonstrates that rats with neuropathic pain caused by sciatic nerve lesion develop anxiety-like behaviour reflected in a reduced exploration time of the open arms in the elevated plus maze. It is hypothesized that this particular behaviour is a consequence of persistent pain present in these animals. This is based on the observation that the analgesic drug morphine (3 mg/kg; i.p.) as well as gabapentin (30 mg/kg; i.p.) reverses the anxiety-like behaviour in neuropathic animals without having an effect on anxiety in sham-operated rats. In contrast, the anxiolytic drug midazolam (0.5 mg/kg; i.p.) reduced anxiety-like behaviour in all study groups, including the sham animals, without displaying analgesic properties.

Behavioural signs of anxiety in different neuropathic pain models

In order to investigate the relationship between neuropathic pain and anxiety, the first goal was to select a suitable model reflecting the highest level of anxiety-like behaviour. The CCI and PNL models according to Bennett and Seltzer, respectively, were selected due to their wide use in assessing neuropathic pain (20; 58; 192). A more pronounced punctuate hyperalgesia in CCI-operated animals compared to PNL-operated animals was observed. Moreover, it was shown, that CCI-operated animals also exhibit a higher level of anxiety-like behaviour, which was most likely due to the CCI lesion resulting in a more severe and intense pain sensation compared to PNL. A similar correlation was reported between anxiety-like behaviour in the open field paradigm and the degree of mechanical hypersensitivity in the varicella zoster virus model (86). Anxiogenic behaviour was furthermore described in a model for HIV-induced neuropathy (225), in a model for arthritis and after partial sciatic nerve lesion (153) as well as in rats with diabetic polyneuropathy (176). These data indicate the possibility to measure pain associated comorbidities not only in humans but also in animals. On the other hand, there are some studies where no anxiety-like behaviour was observed in the spinal nerve ligation model (87; 110). It would be appealing to assume that the development of anxiety is related to the degree of ongoing pain sensations in these models. However, parameters such as strain differences, time point of measurement and differences of surgical procedures might also explain some of the variance across studies. The finding that animals with neuropathic pain also exhibit anxiety-like behaviour is in agreement with data from

pain patients. In several clinical studies neuropathic pain-associated symptoms such as anxiety and depression are described (8; 25; 99; 153; 156). In fact, only few studies investigated associated symptoms, such as anxiety, in animals with neuropathic pain. In pre-clinical studies, anti-nociceptive effects of analgesic drugs are mostly determined by measuring changes in pain thresholds after evoking mechanical, chemical or thermal stimuli. Measurements of associated symptoms are often disregarded in animals with neuropathic pain. This is one of the drawbacks of basic pain research, since the broad spectrum of pain symptoms is only partially considered. In clinical trials the subjective assessment of pain-associated symptoms, such as ‘‘quality of life-parameters” and emotional states are included (8; 137; 138; 156). The development of novel animal models which also address domains measured in human clinical trials, such as anxiety, would narrow the gap between pre-clinical and clinical drug testing.

Pharmacological modulation of anxiety-like behaviour

The present study investigated anxiety-like behaviour in animals with neuropathic pain. An additional intriguing question was, which drugs were able to affect this behaviour. Therefore, in the second part of the study the effect of midazolam, morphine and gabapentin on anxiety-like behaviour was assessed. Since the EPM test is based on locomotion of the animals, it was important to rule out sedative drug effects. The dose which exhibited reduction of hyperalgesia (morphine and gabapentin) and no impairment of locomotion was then used in the EPM test. In the case of the anxiolytic drug midazolam the dose which had no sedative effects in the motility test was selected. The results showed that anxiety-like behaviour was diminished by midazolam in sham as well as in CCI animals without affecting nociception. Similar observations were made by Narita and colleagues (153) using etizolam in the plus maze test. These results further support the interpretation that reduced exploratory behaviour of mononeuropathic rats in the EPM is due to anxiety. Although we observed anxiety-like behaviour in nerve-ligated animals three weeks after surgery, an acute treatment with an anxiolytic drug can diminish anxiety-like behaviour.

Additionally, it was investigated whether pain-relief also results in an attenuation of anxiety-like behaviour. Morphine (3 mg/kg) attenuated mechanical hypersensitivity

without reducing motor activity, thus demonstrating the acute analgesic effect of morphine (251). Intriguingly, morphine at the dose of 3 mg/kg led to a reduction of anxiety-like behaviour in CCI animals without showing an anxiolytic effect in control animals. These data demonstrate that anxiety, as an affective component of nociception, has been attenuated by anti-nociceptive treatment. Other authors reported that morphine may have anxiolytic properties on its own for 6 mg/kg i.p. (104; 248), or may even be anxiogenic (160). These controversial data show that the activity of morphine is strongly dependent on the model used, the route of administration and the injected dosage.

Having demonstrated that the opioidergic analgesic drug morphine is able to reverse anxiety-like behaviour as a consequence of pain reduction, the effect of the anticonvulsant drug gabapentin in rats with neuropathic pain was investigated. The study was able to confirm the anti-nociceptive effect of gabapentin (Figure 5). Interestingly, the analgesic dose of 30 mg/kg could increase the time spent and the entries in open arms in lesioned animals, while no effect in the sham animals was observed. These data demonstrate that gabapentin reverses pain-induced anxiety due to its analgesic properties. In contrast to the current data, it is claimed that gabapentin may have anxiolytic properties in rats (199). De-Paris and colleagues showed an increase of time spent and entries into the open arms with 10, 30 and 100 mg/kg of gabapentin. Their experiment was performed under dim red-light conditions on a low platform (51). In the present settings the animals are confronted with more distressing conditions like bright light and increased height of the apparatus. The anxiety level in the experimental setting of this current study might be higher than under the conditions of de-Paris´ experiment and as such gabapentin dosage might be too low to demonstrate a significant anxiolytic effect. Based on these results with gabapentin, it is assumed that the drug is in fact able to reverse anxiety-like behaviour in CCI animals as a consequence of pain relief.

It is still an open question which exact pathways are responsible for the mediation of pain-induced anxiety and how analgesic drugs interact with these mechanisms. In this study only two analgesic drugs, which showed efficacy in sensory and affective pain processing, were assessed to establish the model. It will be very interesting to analyse, whether other analgesic drugs, e.g. with different modes of action have the same efficacy in reversing anxiety-like behaviour via their analgesic efficacy.

Assay sensitivity

The elevated plus maze test as employed in this study was sensitive to both reduced exploratory behaviour (e.g. in CCI lesioned animals) and increased exploratory behaviour (in sham-operated animals treated with midazolam). Thus, the absence of anxiolytic efficacy of morphine and gabapentin in sham-operated animals is not due to a ceiling effect. Differences in anxiety-like behaviour between two neuropathic pain models could be shown (PNL and CCI). Other groups determined anxiety-like behaviour in animals using the open field (OF) model. Both EPM and OF use the conflict between the natural tendency of rodents to explore new environments and their reluctance to venture into brightly lit areas (36; 173). Though in both models a reduction in exploratory behaviour is interpreted as anxiety, there are differences in the experimental settings. The OF involves forced confrontation of an animal with a large open space, whereas the EPM animals are free to explore the open or covered arms. Lighting conditions can be varied in both models, but in EPM in addition the height may be varied to adjust the level of ‘‘anxiety” created.

The main result of these experiments was that morphine and gabapentin are able to reduce mechanical hypersensitivity as well as anxiety-like behaviour in rats with neuropathic pain. We could demonstrate that it is possible to differentiate between anti-nociceptive and anxiolytic effects. Since in the clinical situation both aspects are of importance, this study highlights the potential use of the rat model and the behavioural paradigm in the validation of novel analgesic drugs for treatment of neuropathic pain.

CHAPTER II

Effect of several drugs on pain-induced anxiety-like behaviour

In the previous chapter we confirmed our hypothesis that rats subjected to neuropathic pain also generate the associated symptom anxiety. We furthermore investigated that the pure analgesic drugs gabapentin and morphine reduce pain-induced anxiety in rats with neuropathic pain. It now remains to be proved whether the attenuation of mechanical hypersensitivity following also a reduction of anxiety is a general feature of analgesic drugs or specific for morphine and gabapentin. Therefore, in the current experiment multiple analgesic drugs from different substance classes were assessed in this model (Table 4).

Since the elevated plus maze is dependent on locomotion of the animal, the effect of each compound on motility and ambulation was first determined in naive animals. The parameter distance travelled was used to represent locomotor impairments. Afterwards, the analgesic property was determined in rats with chronic constriction injury (CCI) and a pronounced pain relief was set as a prerequisite. When both conditions were fulfilled, the drug was examined in the elevated plus maze paradigm to investigate the effect on anxiety-like behaviour, while the parameter “time in open arms” was taken as an endpoint measurement. If not mentioned otherwise, compounds were administered intraperitoneally. For the anxiety and pain test the same animals were used and the tests were performed at intervals of 3 – 5 days.

Neuroanatomical and physiological studies have long implicated serotonin in the central nervous system control of pain (e.g. descending inhibition) (16). Although the 5-HT_{1A} receptor subtype has not traditionally been considered as a molecular target for pain therapy, it is reported that 5-HT_{1A} ligands are involved in nociceptive processes. Colpaert and colleagues found for instance that 5-HT_{1A} agonists exhibit pro- as well as anti-nociceptive properties, dependent on their efficacy at the receptor (40). On the other hand the serotonin system is shown to be implicated in the anxiety processes. We tested the highly selective and potent 5-HT_{1A} receptor agonist 8-OH-DPAT in our model which was reported to exert anxiolytic effects (57).

Bradykinin receptors are localized in nociceptive pathways, which support the role for bradykinin in pain mediation. It has already been shown that a B_1-antagonist reduces hyperalgesia in both acute and chronic pain models (23; 73). Therefore we assessed the B_1-antagonist R-715 and SSR240612 in the CCI model.

Tramadol is a central acting analgesic, used for the treatment of moderate to severe pain. It is thought that the compound exerts its anti-nociceptive effect through its action on the μ-opioid receptor as well as its inhibition of the reuptake of monoamines, such as norepinephrine (NE) and serotonin (5-HT) (196). Tramadol is not only described to relief pain but also reported to have efficacy in obsessive compulsive disorder (72). Therefore it is interesting to investigate the effect of the drug in the pain as well as the anxiety paradigm.

The sodium channel blocker mexiletine is used as an antiarrhythmic agent but is effective in the relief of chronic pain. Pharmacologically it is related to lidocaine, decreases excitability and diminishes pain transmission by blocking the influx of Na^+ ions during an action potential. Pain is modulated by a subset of voltage-gated sodium channels, including Nav1.3, which is expressed in dorsal root ganglia neurons, Nav1.7, mainly localised on sensory and sympathetic neurons as well as Nav1.8 and Nav1.9, which are expressed selectively in peripheral neurons. We were interested in potential effects of this drug on mechanical hyperalgesia as well as pain-related anxiety through its sodium channel blocking properties.

Lacosamide was developed as an anticonvulsant drug but also showed positive preliminary phase III results in reducing pain scores in patients with painful diabetic neuropathy (www.pharmaceutical-business-review.com). The drug demonstrated antiepileptic effectiveness in different rodent seizure models and anti-nociceptive properties in experimental animal models of neuropathic pain. Lacosamide is proposed to have a dual mode of action. First, it enhances the slow inactivation of voltage gated sodium channels during an action potential and thereby reduces the firing in active cells. Second, experiments demonstrated that lacosamide interferes with collapsin-response mediator protein 2 (CRMP-2), a protein which is implicated in the pathophysiology of epilepsy and pain, through its induction of erroneous neuronal outgrowth (26; 141).

The treatment of neuropathic pain remains a big medical challenge. Although there are several drugs available, they often exert an unsatisfying analgesic efficacy (see NNT in Table 1). In clinical trials efficacy on psychological as well as sensory components of pain is considered. Since we investigated reflexive pain behaviour as well as psychological symptoms in chronic constricted rats, this model provides the benefit to investigate the efficacy of novel analgesic drugs not only in neuropathic pain but also in their related symptoms such as anxiety. This may help to characterize the profile of drugs more precisely in the pre-clinical phase of development and differentiate the analgesic and anxiolytic effects of the compounds.

Drug effects of varied compounds on locomotion, pain and anxiety

	Way of action	Vehicle	Motility	MH	Anxiety: time spent in open arms
8-DPAT	5-HT_{1A} agonist	Saline	No effect on locomotion, p = ns.	50 % pain relief, p < 0.01	anxiogenic effect in sham, no effect in CCI
R-715	B1-antagonist	Saline	No effect on locomotion, p = ns	57 % pain relief, p < 0.01	no effect in sham, anxiolytic tendency in CCI
SSR240612	B1-antagonist	90% Natrosol 0.5%, 10% Tween 80 0,1%	No effect on locomotion, p = ns.	42 % pain relief, p = 0.10	anxiogenic effect in sham, no effect in CCI
Tramadol	μ- agonist	Saline	No effect on locomotion, p = ns	64 % pain relief, p < 0.01	anxiolytic tendency in sham, anxiolytic effect in CCI
Mexiletin	Na^+-channel inhibitor	Saline	Sedative effect p < 0.05	No data	No data
Lacosamide	CRMP-2 and Na^+-channel modulator;	90% Natrosol 0.5%, 10% Tween 80 0,1%	No effect on locomotion, p = ns	70 % pain relief, p < 0.01	no effect in sham and in CCI

Table 4. Tested drugs and their effect on locomotion, mechanical hypersensitivity and anxiety-like behaviour;

MH: mechanical hypersensitivity was determined using “electro von Frey algometer”. Significant differences in locomotion between treatment and vehicle group were analysed by unpaired *t-test*. Significant differences in paw withdrawal thresholds between the injured paw of the same animal before and after treatment were analysed using a paired Wilcoxon test.

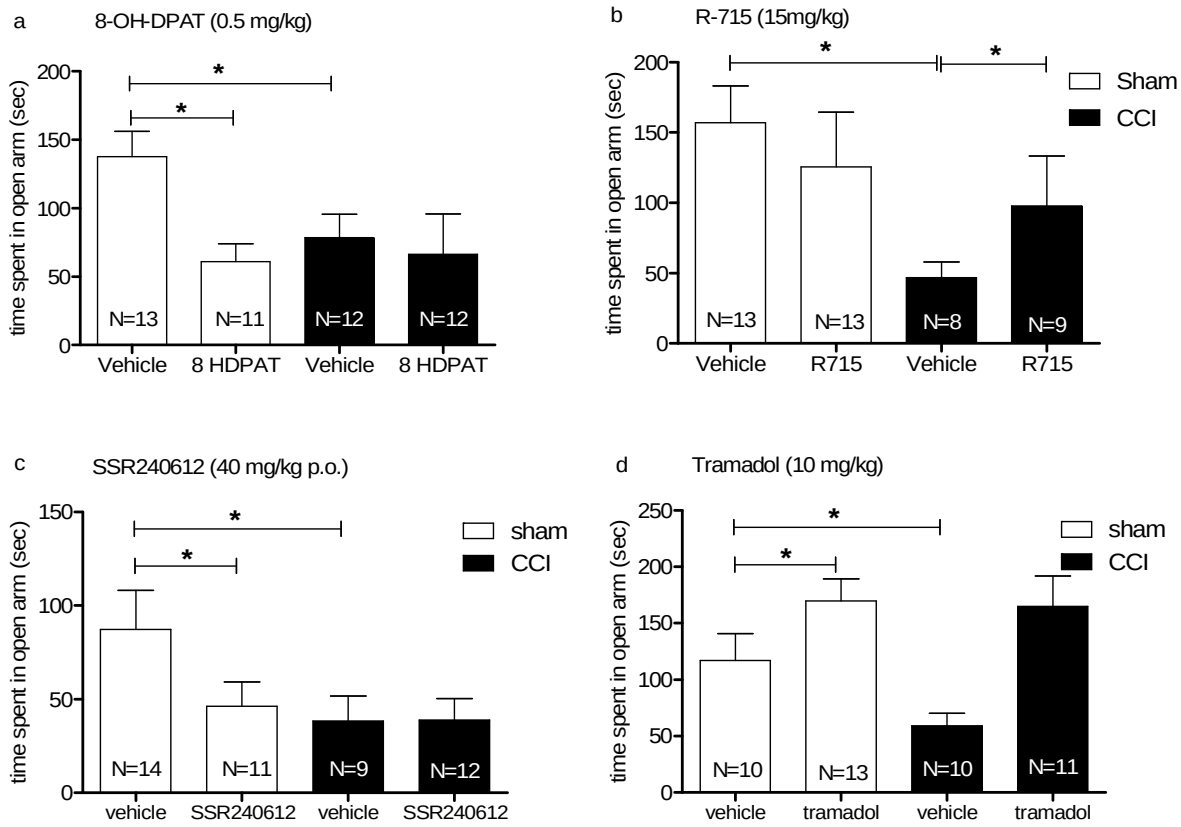


Figure 7. Effect of several drugs on pain-induced anxiety-like behaviour in the elevated plus maze task

8-OH-DPAT (0.5 mg/kg, a), R-715 (15 mg/kg, b), SSR240612 (40 mg/kg, d), tramadol (10 mg/k, c) and lacosamide (10 mg/kg, e) were tested in the EPM. Time spent in open arms after drug treatment is displayed. Data are expressed as means ± SEM. *t-test* adjusted to Bonferroni–Holm indicates statistically significant differences: *P < 0.05; N = 8-13

8-OH-DPAT

The treatment with 0.5 mg/kg 8-OH-DPAT had no effect on locomotion in naive rats. An unpaired *t-test* revealed no significant difference between treatment and control group (P > 0.05). Distance travelled during the observation time of 30 minutes was 33 ± 2 m in the vehicle group (N = 6) and 29 ± 7 m in treated rats (N = 8). The 5-HT agonist had an analgesic effect in CCI animals and reversed the paw withdrawal threshold from 12 ± 0.4 g pre-treatment to 24 ± 2 g (P < 0.001), which corresponds to

a pain relief of 50 %. 8-DPAT did not exhibit an anxiolytic effect in chronic constricted rats but was anxiogenic in sham operated animals (N = 11-13). *Post-hoc* tests yield a significant decrease in time spent in open arms from 137 ± 19 sec in non treated sham animals to 61 ± 13 sec in treated sham animals ($P < 0.05$).

R-715

The peptide R-715, a selective B1-antagonist, (15 mg/kg) displayed no signs of impaired motor function when compared to vehicle treated naive rats (N = 6). The mean distance travelled was 33 ± 4 m in vehicle and 28 ± 3 m in treated animals ($P > 0.05$). A dosage of 15 mg/kg R-715 displayed 57% pain relief in chronic constricted animals and elevated the paw withdrawal threshold from 15 ± 2 g to 23 ± 2 g after 1 h administration ($P < 0.05$). In the elevated plus maze the drug exhibited an anxiolytic tendency in sciatic nerve lesioned rats (N = 8-13). Time spent in open arms was elevated from 47 ± 21 sec to 98 ± 16 sec ($P < 0.05$) in CCI injured rats after treatment.

SSR240612

The synthetic B1-antagonist SSR240612 (40 mg/kg) also exhibited no influence on locomotion and no significant difference in distance travelled between the control group (33 ± 3 m, N = 7) and the treatment group (36 ± 3 m, N = 5) was detected ($P > 0.05$). We observed a 42 % reduction of mechanical hypersensitivity; however, the results revealed only a trend of significance. Paw withdrawal threshold increased from 10 ± 1 g to 18 ± 2 g 1 h after injection ($P = 0.10$). The drug did not reduce anxiety-like behaviour in chronic constricted animals ($P > 0.05$; N = 9-12). In sham operated animals time spent in open arms was significantly decreased from 87 ± 21 sec to 46 ± 13 sec ($P < 0.05$), indicating an innate anxiogenic property of the drug.

Tramadol

No significant changes in distance travelled were observed after treatment with tramadol ($P > 0.05$). Distance travelled amounted to 41 ± 3 m in vehicle treated animals (N = 8) and 32 ± 5 m in tramadol treated naïve animals (N = 7). Mechanical hypersensitivity was reduced about 64 % reflected in an elevated paw withdrawal threshold after treatment. Paired *t-test* revealed a statistically significant increase from 12 ± 1 g up to 24 ± 2 g after treatment ($P < 0.001$). Tramadol displayed an anxiolytic tendency in sham operated rats, and an anxiolytic effect in CCI operated rats (N = 10 – 13). Time spent in open arms increased in sham operated animals

from 116 ± 24 sec to 169 ± 20 sec ($P < 0.1$) and in sciatic nerve lesioned rats from 59 ± 11 sec to 165 ± 27 sec ($P < 0.01$).

Lacosamide

The anticonvulsant drug lacosamide did not show a sign of motor impairment at the dosage of 10 mg/kg ($P > 0.05$). Distance travelled was 45 ± 3 m in the control rats ($N = 9$) and 44 ± 3 m ($N = 7$) in treated animals. Lacosamide lead to 70 % of pain relief and paw withdrawal thresholds were significantly increased from 9 ± 1 g pre-treatment to 19 ± 2 g post-treatment ($P < 0.01$). The compound did not affect anxiety-like behaviour in CCI injured rats. Time spent in open arms was also not influenced in the sham group ($N = 9\text{-}11$; $P > 0.05$).

Mexiletin

The dose of 20 mg/kg mexiletin significantly reduced the distance travelled from 38 ± 2 m in control animals ($N = 7$) to 29 ± 2 m in the treated animals ($N = 7$; $P < 0.05$). Therefore, the effect on mechanical hypersensitivity and the effect on anxiety-like behaviour were not assessed.

Discussion

In Chapter I anxiety-like behaviour due to chronic pain was confirmed in rats with chronic constriction injury. In the present study we characterised several analgesic drugs in this model. We used the elevated plus maze test and the electro von Frey algometer, to dissociate the anti-nociceptive action and the effect of pain-related anxiety of several compounds involved in nociception. The elevated plus maze is commonly used to determine anxiolytic or anxiogenic properties of drugs. When combined with the measurement of mechanical hypersensitivity, sensory abnormalities as well as altered mood status can be examined in an animal with neuropathic pain. The combination of behavioural measures and efficacy measures on sensitivity gives a greater insight in the affective aspects of pain as well as the anxiolytic and analgesic profile of the treated compound. The series of tests provides an advantage for preclinical drug development by predicting not only the efficacy on sensory components but also on pain-related symptoms.

The first compound tested in the model was the 5-HT_{1A} receptor agonist 8-OH-DPAT. We observed an anti-hyperalgesic effect and 50 % pain relief in CCI animals in comparison to the uninjured paw (maximal possible effect). Similar analgesic effects of this 5-HT_{1A} receptor antagonist were already observed in a model for spinal cord injury (81) as well as in the chronic constriction injury model (162). The presence of 5-HT_{1A} receptors in the spinal cord and in the brain stem strengthens a possible involvement of these receptors in pain modulation. Overall, our observations suggest that 8-OH-DPAT attenuates mechanical hypersensitivity in CCI injured rats.

When assessing the effect of 8-OH-DPAT on pain-induced anxiety-like behaviour in CCI rats, we found no change in the time spent in open arms, but an anxiogenic effect in the sham-operated animals, reflected in a reduced time spent in open arms on the EPM. Our data are not in agreement with several pre-clinical studies proposing both anxiolytic as well as anti-depressant-like properties of 8-OH-DPAT (50; 85). Additionally, Blackbourn-Munro and colleagues reported an attenuation of escape/avoidance behaviour after treatment, thereby suggesting that the drug also influences the affective component of pain and reverses pain-related symptoms, which could not be confirmed in our study. However, an anxiogenic effect on the EPM in non-stressed rats after treatment of 8-OH-DPAT was previously observed,

which could be abolished by restrained stress (139) before the test. Accordingly, we assume that the anxiogenic effect in the sham-operated group in this model is coming from the lower stress level in sham animals in comparison to CCI injured animals. This data suggest that 8-OH-DPAT can exhibit a biphasic - anxiolytic as well as anxiogenic – effect depending on the basal stress level of the animal.

The anxiogenic property of 8-OH-DPAT in the elevated plus maze is proposed to be mediated by presynaptically located $5HT_{1A}$ autoreceptors in the dorsal raphe nucleus (44), while an anxiolytic effect seems to be mediated via circuits in the hippocampus (113). Since the compound has its own anxiogenic property, we are not able to detect any anxiolytic efficacy after pain relief. Apparently, the innate anxiogenic property is counteracting the anxiolytic effect. With the $5\text{-}HT_{1A}$ agonist, we were not able to confirm the hypothesis that pain-induced anxiety is susceptible to analgesic drugs, which was due to the biphasic nature of the drug. Therefore, it would be interesting to investigate an antidepressant drug such as duloxetine, a serotonin and noradrenalin reuptake inhibitor. This compound is accumulating serotonin in the synaptic gap, which as well leads to an increased 5-HT receptor activation.

We assessed the effect of the selective B_1-antagonists R-715 and the non-selective B_1-antagonist SSR240612 on the elevated plus maze and determined their analgesic properties. R-715 exhibited a significant pain relief, while SSR240612 only exhibited a tendency of significant pain relief by 42 %. Selective $Bradykinin_1$- antagonists are of potential therapeutic interest for the treatment of chronic inflammation as well as neuropathic pain. Since B_1-receptors are highly inducible by tissue injury and during chronic inflammation, bradykinin represents an important player in the chronic phase of pain. Our results are in agreement with previous studies where an analgesic effect of both B_1-antagonists was shown. R-715 for instance exhibited a significant attenuation of diabetic hyperalgesia while SSR240612 prevented neuropathic thermal pain-induced by sciatic nerve constriction (66; 73). Despite the low level of pain relief of SSR240612 in our study we assume that B_1 receptors represent a valuable target for the management of neuropathic pain.

When tested in the elevated plus maze, R-715 exhibited an anxiolytic tendency in CCI-injured rats while the compound had no effect in the sham group. The low anxiolytic effect of the drug might be due to the partial pain relief of 57 %. R-715 is a

selective peptide which antagonises the B1-receptor. Since it is speculated that the drug is not penetrating the blood brain barrier (unpublished data), the analgesic effect is mainly mediated by peripheral way of action. This is of particular interest, since these data show for the first time, that the anxiolytic tendency is a consequence of peripheral pain reduction. We assume that a higher dosage would have shown a stronger pain relief but sedative effects prevented the use of higher concentrations in the EPM. However, the data already provide the first evidence for an analgesic and anxiolytic effect of R-715 on mechanical hypersensitivity and pain associated anxiety. Taken together, this example strengthens our hypothesis that pain relief also results in a reduction of the secondary symptom anxiety in rats.

In contrast to the selective peptide R-715, the synthetic B_1-antagonist SRR240612 displayed no effect in the EPM in the CCI animals and an anxiogenic effect in the sham group, reflected in a reduced time spent in open arms. These contrary results can not only be explained by the reduced analgesic property but also by the different binding profile of the drug. While R-715 is a selective B_1-antagonist, exerting his analgesic property by inhibiting the pain B_1 receptor, SRR240612 has been reported to bind not selectively to B_1-receptors. Unpublished data suggest also an antagonistic property for instance at the muscarinic receptor M_1 and M_2, bradykinin receptor 2, neuropeptide Y receptor 1 and 2 and others. Since an injection of neuropeptide Y produces anxiolytic effects (253; 254) it is likely that the mediated action of SRR240612 via neuropeptide Y receptors may contribute to the anxiogenic effect seen in the EPM test.

This is to our knowledge the first study which examined the effect of B_1-antagonists on the affective component of pain. The data demonstrated different effects of B_1-antagonists on pain-related behaviour potentially depending on the binding profile of the drug. However, we could successfully show that the analgesic property of R-715 leads to a reduction of pain-related anxiety, thereby supporting our hypothesis that pain relief also causes reduction of pain-induced anxiety.

We found an anti-hyperalgesic effect after treatment of CCI rats with 10 mg Tramadol, reflected in a 64 % pain relief. Without showing an impairment of locomotion, the compound also displayed an anxiolytic effect on the elevated plus maze in CCI operated rats and an anxiolytic tendency in sham-operated rat. Intriguingly, this weak

opioid compound exerts a slightly different profile than the strong opioid morphine, which was investigated in chapter I. The different results of both compounds might be mechanism dependent. While morphine exerts its effects via different opioid receptors, tramadol is believed to mediate an analgesic effect only via the μ-opioid receptor. Additional targets of tramadol, such as 5-HT transporter inhibition and G-coupled protein receptors modulation, are supposed to contribute to the analgesic effect of the compound. Furthermore, tramadol blocks serotonin and norepinephrine reuptake, also observed in tricyclic antidepressants. Oliva and colleagues (158) for instance showed an antinociceptive effect of tramadol in the mouse formalin test which was probably mediated by 5-HT_{2C} receptors. This is of particular interest, since 5-HT_{2C} receptor antagonism has been postulated to play a role in the generation of obsessive-compulsive disorder (OCD), a severe and disabling anxiety disorder (184). Therefore, it is not surprising that tramadol does not only act as an antidepressant drug, but also reduces symptoms in OCD (185). We speculate that the observed reduction of time spent in open arms in the EPM after treatment of tramadol is mediated via the 5-HT system and the opioid system, thereby suggesting that tramadol has an own anxiolytic component. Although we cannot completely support our hypothesis, that pain relief also attenuates pain-induced anxiety with the data from tramadol, our model successfully demonstrated the anxiolytic and analgesic properties of the drug. Yet the study provided evidence that this compound is a good therapeutic option to treat neuropathic pain as well secondary symptoms.

When we examined the profile of lacosamide, a newly developed anti-epileptic drug, an analgesic effect with 70 % pain relief was observed. Previous literature reported the use of anticonvulsant drugs for the management of neuropathic pain (198). Lacosamide exhibited not only antiepileptic effects in different rodent seizure models but also antinociceptive properties in animal models for chronic inflammatory and neuropathic pain. It is suggested that lacosamide has a dual mode of action underlying its anticonvulsant and analgesic effects. The drug enhances the slow inactivation of voltage-gated sodium channels, thereby controlling neuronal hyperexcitability without affecting physiological activity. Furthermore, lacosamide was identified as a binding partner of the CRMP protein, which is involved in axonal outgrowth induced by neurotrophic factors (NT) such as brain-derived neurotrophic factor (BDNF) (247). BDNF is directly implicated in the development of pain processes by inducing rearrangement of neuronal outgrowth (26). Both specific

actions are proposed to be responsible for the analgesic effect of the drug. However, the drug, which did not cause any motor impairments or marked sedative effects at the dose of 10 mg/kg, could not reverse pain-induced anxiety-like behaviour in CCI rats.

We already investigated the effect of another antiepileptic and analgesic drug on pain-induced anxiety-like behaviour. In comparison to lacosamide, gabapentin reversed anxiety behaviour in the EPM and displayed a complete pain relief. Interestingly, gabapentin is not only effective in reducing evoked pain but also in attenuation of the so called spontaneous pain or ongoing pain, which is a common symptom of neuropathic pain patients. Joshi *et al.* for instance showed that gabapentin fully reversed evoked pain as well as spontaneous flinching behaviour in rats (102). The efficacy of gabapentin on spontaneous pain behaviour has also been found in models using patients with neuropathic pain (79). We assume that in our model particularly persistent spontaneous pain which occurs without painful stimuli is responsible for the development of pain-induced anxiety. We did not observe a reduction of anxiety behaviour after the treatment with lacosamide. This observation could support the fact that the drug indeed has analgesic properties to reverse evoked pain but it has no efficacy in reducing spontaneous pain. This is in agreement with the literature were to our knowledge no results concerning the efficacy on spontaneous pain are published. Different effects on ongoing pain and evoked pain can be explained by unequal sites of actions of both compounds. While gabapentin mediates its effect via the $\alpha_2\delta$-subunit of voltage-gated Ca^{2+}-channels, lacosamide acts via voltage-gated Na^{+}-channels and the modulation of NT-mediated processes. Taken together we speculate that lacosamide is an analgesic compound which shows no relief of pain-induced anxiety due to the lack of efficacy on ongoing pain in the rats. This might be a disadvantage in comparison to the antiepileptic drug gabapentin which reversed also the secondary symptom anxiety in CCI-injured rats following pain relief.

The sodium blocker mexiletin was not assessed in the whole number of tests. This example highlights the limitations of the present model. The compound mexiletin displayed a reduced ambulation at the range where it also was described to have analgesic effects and therefore was not evaluated in the EPM paradigm. Since the elevated plus maze is a locomotor-based paradigm, an anxiolytic effect can only be

observed with non sedative dosages of the selective drug. Therefore, it is likely that an anxiolytic effect of drugs can not be identified.

In humans, neuropathic pain is associated with significant co-morbidities, including anxiety and depression, which strikingly reduce quality of life and daily activities. In clinical studies all these domains are considered and therefore provide a large evaluation of neuropathic pain symptoms in patients including subjective, qualitative and psychological components. However, to date the investigation of such co-morbid behaviour in rodent models of neuropathic pain is limited and mainly disregarded. We used the well characterized anxiety indicating parameter, elevated plus maze, to determine whether peripheral neuropathy in nerve injured rats triggers anxiety-like behaviour. The anxiety paradigm was applied to successfully resemble the secondary symptoms often observed in neuropathic pain patients. Several subclasses of neuropathic pain compounds were tested in this model. Tramadol, which has already been assessed in clinical trials and is used in pain therapy, provides a potential candidate to compare the preclinical and clinical efficacy on anxiety and to draw parallels with the clinical scenario. Tramadol was effective in both paradigms of our model. The drug exhibited a strong pain relief and improved pain associated symptoms in rats with sciatic nerve injury. Similar effects were observed in humans: In two double-blind, placebo-controlled studies for either diabetic neuropathy or cancer-associated neuropathic pain, tramadol exerted not only analgesic properties but also an improvement of daily life activities. Furthermore, efficacy in the anxiety disorder OCD could be shown (7; 84; 184). These data suggest that the validated model is a potential possibility to predict the efficacy of compounds more precisely and to compare the results with clinical data by considering sensory aspects as well as associated symptoms of pain as endpoint measures.

The current study clearly demonstrated that the used series of tests is a predictive tool to assess not only the efficacy of drugs on sensory components of pain but also on pain-associated anxiety. Through the integrated behavioural paradigm elevated plus maze, we could additionally appreciate the diverse symptomatology of persistent pain. With the B_1-antagonist R-715, we could confirm our hypothesis, that pain-induced anxiety is susceptible to peripheral analgesic compounds. Since information regarding emotional or subjective aspects of pain is commonly measured in clinical

trials, it is advantageous to also include such endpoint measures in pre-clinical analyses to draw closer parallels to the clinical scenario. We succeeded to show similar efficacy of tramadol on anxiety and pain in comparison to clinical studies, thereby improving the predictability of the model.

Taken together, this experiment provided further evidence that pain relief also results in reduced anxiety in neuropathic rats. The sensory test in combination with the behavioural paradigm gave a better insight into the analgesic and anxiolytic profile of the utilized drugs and could predict the efficacy of new analgesic compounds on pain-related anxiety.

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CHAPTER III

Role of oxytocin and vasopressin in pain-induced-anxiety in rats with sciatic nerve lesion

In Chapter I we reported that rats subjected to neuropathic pain exhibit pain-induced anxiety-like behaviour three weeks after surgical trauma. With these results we demonstrated the association between pain and anxiety-like behaviour in animals. Despite a number of studies indicate a link between pain and anxiety, the pathophysiology, the implicated neuropeptides and involved brain areas are still not completely identified. The current study was performed to assess, whether pain-induced anxiety-like behaviour causes a change of mRNA levels of oxytocin and vasopressin in the amygdala of rats with neuropathic pain.

Amongst others, the amygdala appears to be one of the most important forebrain structures which is receiving, processing and integrating sensory as well as affective information such as pain and anxiety (155). The limbic structure is not only involved in the transmission of antinociception in the rat formalin or tail-flick test (133; 134), but is also implicated in emotional responses in humans and animals (92; 231). These data suggest that pain and anxiety are at least partially processed in the amygdala. In this brain area, which is connected to other pain and anxiety mediating structures such as periaquaeductal grey (PAG) and stria terminalis, the neuropeptides vasopressin and oxytocin act as important neuromodulators. Both neuropeptides are frequently supposed to play a key role in anxiety states as well as in modulation of nociceptive information (Chapter: “Link between pain and anxiety”). Gu and colleagues demonstrated for instance an analgesic effect after oxytocin injection in the nucleus accumbens (78), while Yang observed that vasopressin exerts an anti-nociceptive property in the nucleus raphe magnus (243). Moreover, a repeated stressor triggered the vasopressin release within the hypopthalamus (142), and it has been shown that the oxytocin receptor is very important for reduced anxiety behaviour in postpartum rats (63).

We investigated in the current study whether there are changes in the level of mRNA of vasopressin and oxytocin in the amygdala caused by pain-induced anxiety-like

behaviour in chronic constricted rats (CCI) in comparison to sham operated rats. We quantified the mRNA levels of oxytocin and vasopressin in the amygdala three weeks after sciatic nerve lesion. Furthermore, we assessed whether vasopressin and oxytocin are involved in mechanical hypersensitivity and pain-induced anxiety-like behaviour by administering the corresponding antagonists. Here we provide the first evidence that increased oxytocin levels in the amygdala contribute to pain-induced anxiety, while the inhibition of vasopressin did not exhibit any effect.

Oxytocin is upregulated in the amygdala of rats with neuropathic pain

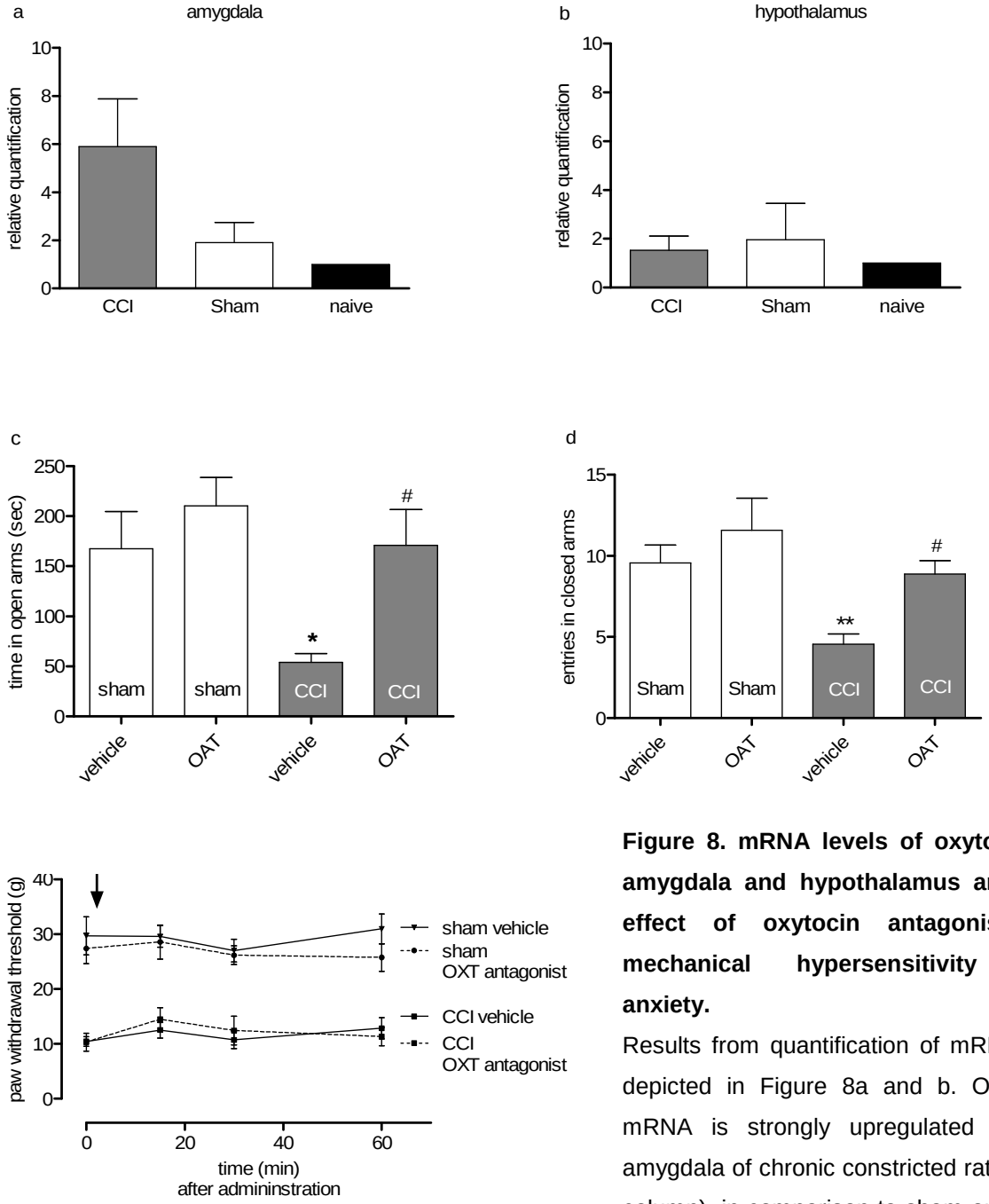


Figure 8. mRNA levels of oxytocin in amygdala and hypothalamus and the effect of oxytocin antagonist on mechanical hypersensitivity and anxiety.

Results from quantification of mRNA are depicted in Figure 8a and b. Oxytocin mRNA is strongly upregulated in the amygdala of chronic constricted rats (grey column), in comparison to sham-operated and naive rats. No upregulation in the hypothalamus was observed. After the treatment of oxytocin antagonist no effect on mechanical hypersensitivity (Figure 8e) was observed but the antagonist could significantly decrease the anxiety-like behaviour in the lesioned rats (Figure 8c-d). Taq-man analysis was performed twice and results from both experiments were pooled. Behavioural studies were performed with 7-9 animals per group. Data are expressed as means ± SEM. *t-test* adjusted to Bonferroni–Holm indicates statistically significant differences: # $P = 0.05$ CCI vehicle group vs. CCI treatment group, *$P < 0.05$ CCI vehicle group vs. sham vehicle group.

The amygdala of CCI rats with significant hyperalgesia and anxiety-like behaviour was isolated and mRNA analysis was performed. CCI rats exhibit a pronounced upregulation of the neuropeptide OXT (6-fold) in the amygdala in comparison to the sham and naive animal group (Figure 8a). In the hypothalamus no increase of mRNA levels could be detected. The data demonstrate that neuropathic pain and the associated anxiety-like behaviour apparently leads to an increase of mRNA of OXT in the amygdala.

Effect of oxytocin antagonist on mechanical hypersensitivity

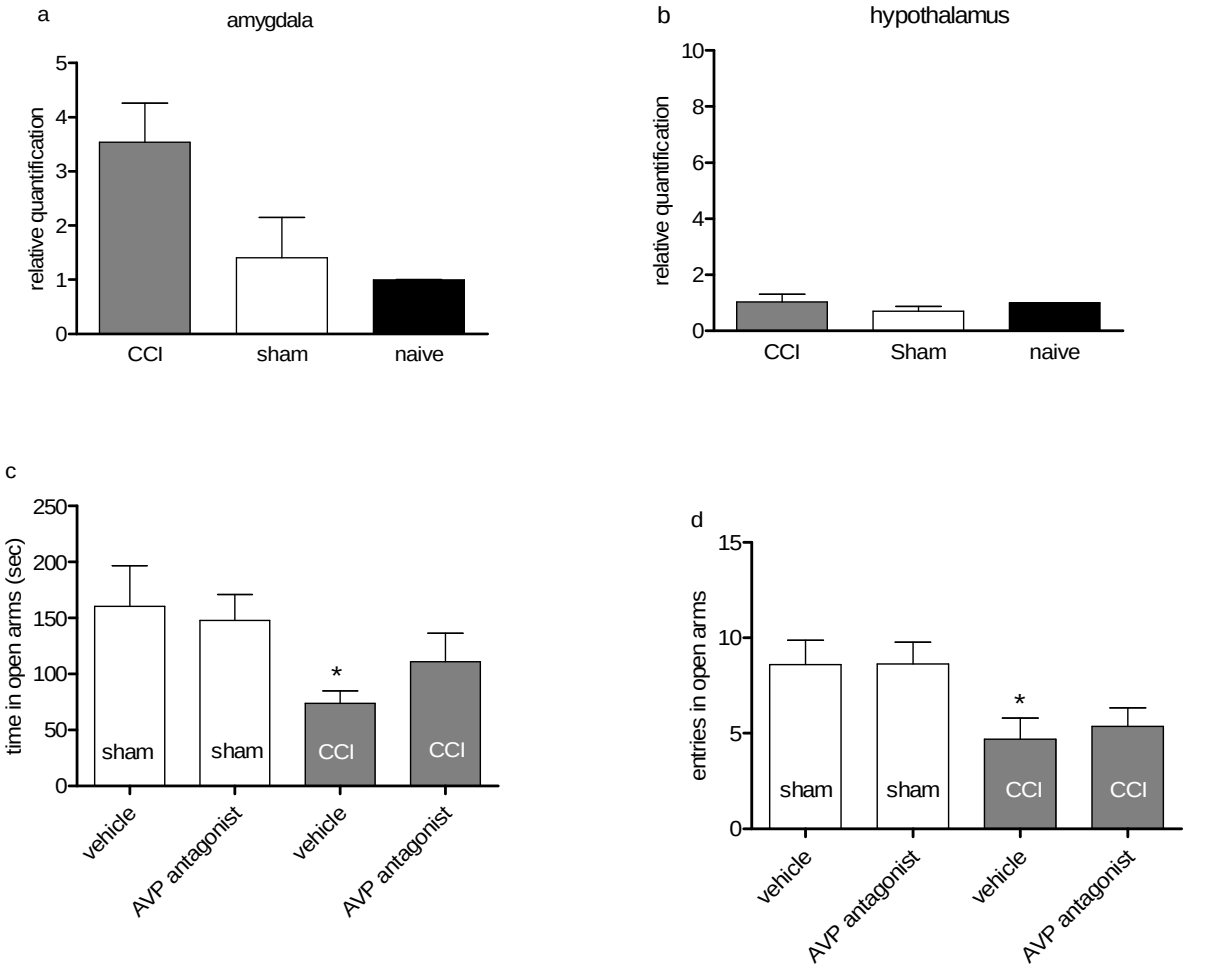
Having demonstrated, that OXT mRNA is upregulated in the amygdala, we assessed whether the high level of oxytocin affects mechanical hypersensitivity or anxiety-like behaviour (Figure 8b). Therefore, the effect of OXT antagonist after applying mechanical stimuli was measured over a period of 1 hour post application. A multivariate ANOVA comparison revealed statistically significant main effects for surgery ($P < 0.001$). *Post-hoc test* revealed a significant decrease of paw withdrawal threshold from 29.7 ± 3.5 g in sham operated rats to 10.4 ± 1 g in CCI operated animals ($P < 0.001$). The OXT antagonist could not reverse the strong mechanical hypersensitivity in the CCI injured rats, indicating that OXT is not responsible for the strong pain responses in these animals.

Effect of oxytocin antagonist on pain-induced anxiety-like behaviour

After assessing the effect of oxytocin antagonist on mechanical hypersensitivity we additionally wanted to know whether the drug is able to affect the pain-induced anxiety behaviour of CCI rats on the EPM. Two-way ANOVA comparison revealed statistically significant main effects in entries in the open arms for treatment ($P < 0.05$) and surgery ($P < 0.01$), and in time spent in open arms for treatment ($P < 0.05$) and surgery ($P < 0.05$). We observed significant anxiogenic behaviour in CCI rats in comparison to sham-operated animals. *Pos- hoc* test yield a significant decrease of time spent in open arms form 167 ± 37 sec to 54 ± 9 sec ($P < 0.05$) and entries in open arms reduced from 9.6 ± 1 in sham to 4.6 ± 0.6 ($P < 0.01$) in CCI lesioned rats, respectively (Figure 8c-d). After blocking the effect of oxytocin by injection the corresponding antagonist into the amygdala, time spent and entries in open arms are significantly increased in the CCI lesioned animals to a value of 171 ± 36 sec ($P < 0.05$) and 9 ± 1 ($P < 0.05$), respectively. Notably, this anxiolytic effect

was only observed in animals with neuropathic pain, while the values in the sham-operated rats remained unchanged after treatment. These results demonstrate clearly that the increased oxytocin level in the amygdala is relevant for pain-induced anxiety-like behaviour.

Vasopressin is upregulated in the amygdala of rats with neuropathic pain



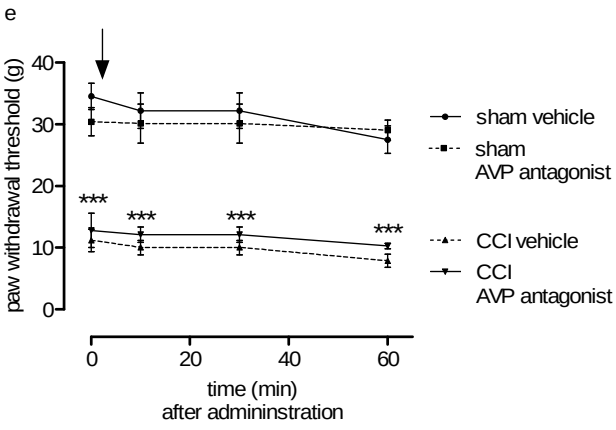


Figure 9. mRNA levels of vasopressin in amygdala and hypothalamus and the effect of vasopressin antagonist on mechanical hypersensitivity and anxiety. Results from quantification of mRNA are depicted in Figure 9a and b. Vasopressin mRNA is strongly upregulated in the amygdala of chronic constricted rats (grey column), in comparison to sham-operated and naive rats. No change of mRNA was observed in the hypothalamus. After the treatment of AVP antagonist neither an effect on mechanical hypersensitivity (Figure 9d) nor anxiety-like behaviour was observed in the lesioned rats (Figure 9c-d). Taq-man analysis was performed twice and results from both experiments were pooled. Behavioural studies were conducted with 10-11 animals per group. Data are expressed

as means ± SEM. *t-test* adjusted to Bonferroni–Holm indicates statistically significant differences: *P < 0.05 CCI vehicle group vs. sham vehicle group.

Within the investigation of molecular biological changes due to suffering from persistent pain we additionally investigated the mRNA levels of the neuropeptide vasopressin. CCI rats exhibit a pronounced upregulation of the neuropeptide vasopressin (4-fold) in the amygdala in comparison to the sham and naive animal group while in the hypothalamus no increase of mRNA levels could be detected. The data demonstrate that neuropathic pain and the associated anxiety-like behaviour apparently leads to an increase of mRNA of AVP in the amygdala.

Effect of vasopressin on mechanical hypersensitivity

Similar to the experiment with an oxytocin antagonist, we determined the effect of a vasopressin antagonist on pain behaviour and pain-induced anxiety in the rats with sciatic nerve lesion. A multivariate ANOVA comparison revealed statistically significant main effects only for surgery ($P < 0.001$). We confirmed a mechanical hypersensitivity in the chronic constricted animals 3 weeks post surgery. A post-hoc test yield a statistically significant reduction of the paw withdrawal threshold in sham rats from 30.4 ± 4 g to 11.2 ± 2 g in the CCI injured rats ($P < 0.001$). However, we did not observe a significant reduction of pain response after treatment with vasopressin antagonist during a period of 1 hour post-treatment.

Effect of vasopressin on pain-induced anxiety-like behaviour

Thereafter the effect of vasopressin antagonist on anxiety-like behaviour was determined. As a prerequisite for the experiment anxiety-like behaviour had to be verified in the rats with neuropathic pain. A two-way ANOVA comparison revealed statistically significant main effects in entries in the open arms for surgery ($P < 0.01$) as well as in time spent in open arms for surgery ($P = 0.05$) but neither for interaction nor for the treatment. A pairwise *t-test* revealed a significant reduction in time in open arms form 160 ± 36 to 74 ± 10 sec ($P < 0.05$) and entries in open arms reduced from 8.6 ± 1 in sham to 4.7 ± 1 ($P < 0.05$) in CCI lesioned rats, respectively. The anxiogenic behaviour in the rats with neuropathic pain could not be reversed by the AVP antagonist. These results indicate no contribution of AVP to pain behaviour or pain-induced anxiety.

Discussion

In the present study we observed an upregulation of oxytocin and vasopressin mRNA in the amygdala of rats with neuropathic pain and associated anxiety-like behaviour. Furthermore, we assessed the influence of oxytocin and vasopressin antagonist on pain-induced mechanical hypersensitivity and anxiety-like behaviour in rats with sciatic nerve lesion. Our findings indicate that oxytocin but not vasopressin is implicated in the occurrence of anxiety-like behaviour in rats with neuropathic pain, while both neuropeptide systems do not affect mechanical hypersensitivity in CCI injured animals. These results provided new insights in the pathophysiology of pain-induced anxiety like behaviour.

The upregulation of vasopressin and oxytocin mRNA in the amygdala of chronic constriction injured animals was observed three weeks post-injury. We hypothesise that the increased levels of mRNA might be a result of chronic pain or its associated symptoms. This is based on the fact that persistent noxious stimulation leads to several biochemical, physiological and morphological changes in the peripheral as well as in the central nervous system. Synaptic plasticity in the amygdala and pain associated sensitization has for example been demonstrated by Neugebauer, in an model for prolonged pain (154). Previous reports provided evidence for an activation of neuroendocrine responses after formalin injection reflected in a stronger release of corticotrophin-releasing hormone (CRH) and arginine vasopressin levels (117). Anxiety is also associated with several alterations in the central nervous system. After exposure to stress, $GABA_A$ receptors in the cerebral cortical membranes and their subunits are for instance modified in their efficacy and binding potency (22; 88). Other studies found that acute stress also produces an increase in CRF mRNA levels within the amygdala, whereas chronic foot shock significantly increases CRF mRNA in the hypothalamic paraventricular nucleus (97). In Chapter I it was demonstrated that rats with neuropathic pain also develop anxiety-like behaviour. Therefore, the observed upregulation of vasopressin and oxytocin could not only be a consequence of chronic pain but may also be due to pain-related anxiety.

The amygdala is involved in integrating, encoding and receiving pain information (155). The nociceptive-specific information is mediated via the spino-parabrachio-amygdaloid pathway to the latero-capsular central amygdala coming from the spinal cord and brainstem. Further evidence from imaging studies or behavioural

experiments suggest that the amygdala is part of the descending pain pathway (68; 147; 168; 180). The forebrain structure also has a well-documented role in affective states and related disorders. As an output nucleus the central amygdala possesses connections back to forebrain areas such as hypothalamus and brainstem, to process emotional behaviour (27; 48; 126). Through its well located position and its distinct connection to different nuclei, which are involved in emotional processes, cognition, autonomic function, nociception and stress the amygdala is thought to play an important role in processing pain and emotional-affective information (126; 133; 134; 155). Our results concerning pain-related upregulation of oxytocin and vasopressin mRNA support previous findings that the amygdala is critically involved in the affective component of pain and emotional pathways.

Since it was not clear whether the high mRNA levels of oxytocin affect pain and anxiety, we determined anxiety-like behaviour and mechanical hypersensitivity after treatment with the corresponding antagonist. The oxytocin antagonist exhibited no effect on pain responses, thereby suggesting that oxytocin in the amygdala is not involved in the development of mechanical hypersensitivity. In the literature an anti-nociceptive effect of oxytocin is proposed from in-vivo experiments (131; 149). Exogenous oxytocin administered via i.c.v. application or intrathecal injection reduced pain responses in the tail flick test (244) and microinjection into the paraventricular nucleus of the hypothalamus showed a diminished pain behaviour after mechanical and thermal stimuli (149). Although there is no direct evidence for an analgesic effect of oxytocin mediated by the amygdala, it is shown by autoradiography and histochemical studies that oxytocin receptors are expressed in this area (14; 246). Nevertheless, oxytocin might possibly be part of an endogenous analgesic system which counteracts the hypersensitivity in rats with neuropathic pain. In such a case one would expect that an antagonist even decreases the pain threshold after mechanical stimulation. However, under our conditions we did not see an increase of pain response neither in sham nor in CCI injured animals. The antagonist did not display an effect in sham animals, since maybe the basal level of oxytocin was to low to cause an attenuation of hypersensitivity. In the very severe chronic constriction model a ceiling effect of detectable hypersensitivity might have been the limited factor. This aspect has to be investigated in further studies, since it was not the main goal in the present experiment.

In the current experiment we investigated the effect of oxytocin antagonist on anxiety-like behaviour in CCI injured animals. We determined an anxiolytic effect after treatment of an oxytocin antagonist, thereby suggesting that oxytocin upregulation and its possible increased release might be responsible for pain-induced anxiety-like behaviour. This finding, that oxytocin is involved in anxiety behaviour is in agreement with several other animal studies. Mantella for instance observed a decreased anxiety-like behaviour in oxytocin deficient mice (135). Furthermore, an increased release after stress-inducing forced swim test could be shown, thereby suggesting that the neuropeptide is critically involved in symptoms of psychiatric disorders (236). There are some contradictory studies, showing an anxiolytic effect after oxytocin release or oxytocin receptor activation (24). In particular, Figueira observed a reduction of anxiety at high levels of oxytocin, while a low oxytocin level increases anxiety (63). Thus, it should be considered that the effect of neuropeptides in the CNS is dependent on the located area and the concentration of the neuropeptide.

In the present study we also focused on the expression of vasopressin and its effect on pain and anxiety-like behaviour in lesioned rats. Vasopressin was upregulated less pronounced than oxytocin in the amygdala of CCI-injured rats. After treatment of vasopressin antagonist, no change of pain thresholds was observed in rats with neuropathic pain. This leads to the conclusion, that vasopressin in the amygdala is not involved in pain transmission after sciatic nerve lesion.

Several studies described a strong implication of vasopressin in chronic pain. For instance it is shown that intra-ventricular injection (252) or microinjection of vasopressin in different brain areas such as nucleus raphe magnus (243), central amygdala (3) or caudate nucleus (242), exhibit anti-nociceptive effects in the rat. Additionally, histological studies demonstrated a dense expression of V1 receptors in brain areas strongly associated with pain processes such as raphe magnus, PAG, locus coeruleus and amygdala (55; 165; 166). Since vasopressin is reported to have analgesic properties, it might also be a part of the endogenous pain-modulating system. A decrease of mechanical hypersensitivity could not be measured in CCI-injured or sham-operated animals after the treatment of the vasopressin antagonist. The reasons are similar to those from the oxytocin study. The CCI model is a very severe neuropathic pain model and it might be not possible to detect an even higher

mechanical hypersensitivity. To address this question a pain test with higher sensitivity needs to be used.

Finally, we investigated the effect of the vasopressin antagonist on anxiety-like behaviour. An implication of vasopressin in anxiety was shown by Ebner, who found an increased vasopressin synthesis and release in the amygdala upon exposure to stress (61). Furthermore, V_{1a} receptors are enriched in the amygdala (82). Despite some studies show an implication of vasopressin in anxiety, we did not measure an increase in time spent or entries in open arms after the inhibition of the vasopressin receptors, as it was the case with the oxytocin antagonist. The data suggest that in contrast to oxytocin, vasopressin in the amygdala is not implicated in modulating pain–induced anxiety in neuropathic animals. Furthermore, it indicates, when compared to the results from Ebner that the location of vasopressin release might be different from the area where the neuropeptide exerts his effect.

Our present findings show that neuropathic pain triggers an upregulation of oxytocin and vasopressin in the amygdala of rats with sciatic nerve lesion. We provided evidence that the oxytocin contributes to pain-induced anxiety-like behaviour, since the treatment of the oxytocin antagonist but not vasopressin antagonist reduces pain-induced anxiety-like behaviour in chronic constricted rats. After injection of the corresponding antagonists, changes in pain threshold are not detectable, thereby suggesting, that both neuropeptides are not involved in mechanical hypersensitivity of sciatic nerve lesioned rats.

CHAPTER IV

Effect of pre-existing anxiety on the development of neuropathic pain in rats

In the first chapter we investigated, whether neuropathic pain causes anxiety-like behaviour as a consequence of suffering from chronic pain. In fact we experimentally proved that rats with neuropathic pain display pain-induced anxiety-like behaviour. It is also suggested that anxiety itself can influence pain responses or pain sensitivity in humans (43). Post-traumatic stress disorder patients exhibited a more intense pain response, which is correlated with the severity of the disorder (52). Furthermore, it is known that patients with persistent pain have a high incidence for the comorbid anxiety (207). Despite the numerous evidences for a close relationship between anxiety and pain in humans, the situation in animals has hardly been assessed.

Therefore, in the following studies we asked, whether pain response can be affected by anxiety levels and investigated, whether rats with a high level of anxiety are more sensitive to mechanical stimuli than rats with low level of anxiety following nerve injury. Certain authors distinguish personality-based (permanent) “trait” anxiety to fear-induced “state” anxiety. Since we were interested in both kinds of anxiety, we selected a genetically based model for mimicking “trait” anxiety (Chapter IV) and a pharmacologically induced model for imitating the “state” anxiety (Chapter V).

In the first part trait anxiety was evaluated by using rats selectively bred for extreme high and low anxiety (120). High anxiety behaviour rats (HAB) display a robust and consistent trait anxiety, are more susceptible and vulnerable to stressor exposure and show signs of hyper-active hypothalamic-pituitary-adrenocortical (HPA) axis, thus resembling anxiety disorder patients. Intriguingly, microdialyse experiments revealed a significantly higher release of vasopressin in the HAB rats compared to LAB rats (low anxiety behaviour), thereby suggesting that vasopressin is critically involved in the excessive anxiogenic phenotype (120).

In the current study the behaviour on the elevated plus maze was taken to characterise anxiety-like behaviour in both strains. HAB and LAB rats were ligated at the sciatic nerve and mechanical hypersensitivity was measured over a period of 57

days post-operatively. Additionally, the efficacy of gabapentin on PWT following mechanical stimulation in both strains was assessed. To our knowledge, this is the first study showing an influence of anxiety-like behaviour on pain responses in HAB rats with sciatic nerve lesion.

Characterisation of high and low anxiety-like behaviour in the two strains

To characterise the different levels of trait anxiety in HAB and LAB rats, the animals were tested in the elevated plus maze before inducing mechanical hypersensitivity. LAB rats spent significantly more time in the open arms (172 ± 8 sec; $p < 0{,}0001$) in comparison to HAB animals (4 ± 2.5 sec). Also the number of entries in open arms was significantly higher in LAB rats (8.6 ± 0.5; $p < 0.0001$) than in their high anxious counterpart (0.9 ± 0.4; data not shown). After separating the animals due to their anxiety behaviour, sciatic nerve lesion was performed.

Different levels of mechanical hypersensitivity in HABs and LABs with sciatic nerve lesion

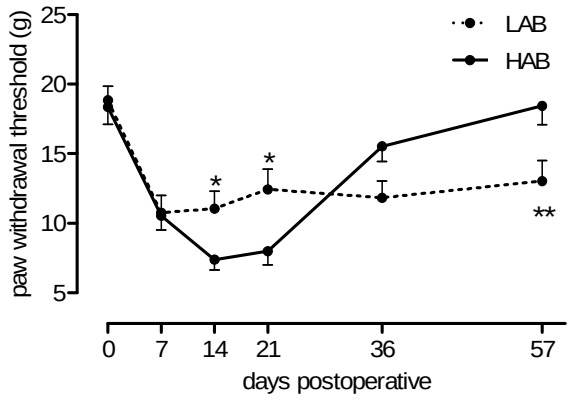


Figure 10. Time course of mechanical hypersensitivity in HAB and LAB rats after sciatic nerve injury.
A noxious mechanical stimulus was applied to the hind paw and the paw withdrawal thresholds were determined. Pain responses were assessed one day before surgery and at day 7, 14, 21, 36 and 57 post-injury in both strains. Data are expressed as mean ± SEM. N = 10 – 15; * $p < 0.05$, ** $P < 0.01$ injured HABs vs. injured LABs.

Mechanical hypersensitivity in rats with low (LAB) and high anxiety (HAB) behaviour was observed for a time period of 57 days after surgery. A two way ANOVA analysis revealed a significant main effect for time ($p < 0.0001$), group ($p < 0.0001$) and group x time interaction ($p < 0.0001$), indicating that mechanical hypersensitivity differed between the two rat strains at some but not all time points (Figure 10).

One day before surgery no baseline difference of the thresholds in both strains was observed. PWT of the non-lesioned paw remained constant throughout the

experiment in both strains and was not significantly different. *Post hoc* tests revealed highly significant decrease of PWT in the lesioned paw of LAB and HAB rats in comparison to the contralateral side ($P < 0.001$ for all days in both strains except day 36 and 57 for HAB rats: p not significant), indicating the development of mechanical hypersensitivity in the injured paw of LAB and HAB rats and an accelerated recovery in HAB rats. However, the two strains developed a different temporal pattern of pain responses to mechanical stimuli. As depicted in Figure 10, HAB animals displayed an even lower PWT than LAB animals at day 14 ($P < 0.05$) and 21 ($P < 0.05$). PWT of LAB rats amounts to 11 ± 1 g at day 14 and 12 ± 1 g at day 21, while the values of HAB rats were 7 ± 1 g at day 14 and 8 ± 1 g at day 21. From day 21 onward the HAB rats show a stronger recovery process. The PWT was 18 ± 1 g in the high anxiety animals at day 57 and did not reveal a significant difference compared to baseline level. The LAB value remained at a significant lower value of 13 ± 1 g. These findings provide evidence for the influence of trait anxiety on a simple pain behaviour (paw withdrawal).

Anti-nociceptive effect of gabapentin in HAB and LAB rats with sciatic nerve injury

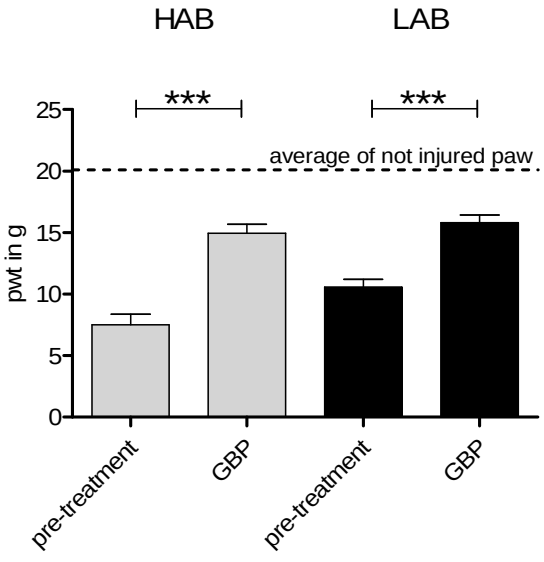


Figure 11. Analgesic efficacy of gabapentin in injured HAB and LAB rats.
Mechanical hypersensitivity was determined 1 h after treatment of 30 mg/kg (i.p.) gabapentin in injured HAB (grey) and LAB (black) rats 23 days after nerve injury. Pain sensitivity was significantly reduced with the similar efficacy in the LAB and HAB rats. Mean of PWT of the not injured paws are indicated by the dotted line. Data are expressed in mean ± SEM. N = 14 – 15. *** $P < 0.001$.

The effect of gabapentin on mechanical hypersensitivity was measured at day 23 post-injury, two days after the last determination of mechanical hypersensitivity, in order to minimise habituation to the test procedure. In Chapter I we selected the dose of 30 mg/kg gabapentin due to its analgesic effect and its lack of motor impairment. Paw withdrawal threshold to mechanical stimuli in the injured hind paw were

significantly decreased in both rat strains, when compared to the non-injured contralateral paw. One hour after administration of gabapentin (30 mg/kg; i.p.), the compound significantly reduced the pain sensitivity in the LAB and HAB rats with the similar efficacy. As illustrated in Figure 11 PWT increased from 7.5 ± 1 g to 15 ± 1 g (P < 0,001) in the HAB animals, while in LAB animals the PWT was elevated from 10.6 ± 1 g to 15.8 ± 1 g (P < 0,001). When expressed as percentage of maximum possible effect change in PWT was 55 % in LABs and 59 % in HABs, respectively. Our data indicate that the efficacy of gabapentin is independent of the level of anxiety in the rats.

Neuropathic pain-induced anxiety-like behaviour in LAB rats after sciatic nerve injury

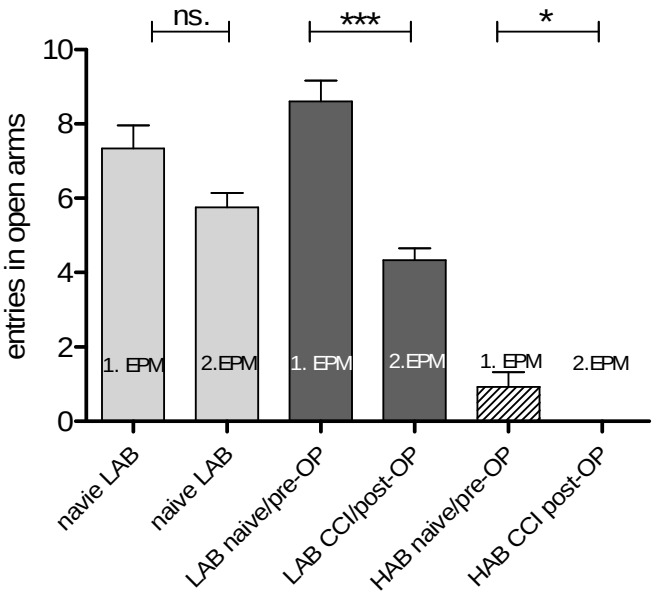


Figure 12. Anxiety behaviour from naive LAB and sciatic nerve injured HAB and LAB before and 4 weeks after surgery, respectively.

Entries in open arms as an indication for non-anxious behaviour was determined in naive (bright column) and CCI-operated LAB (dark column) and HAB animals (diagonal line column). All rat groups were measured at the age of 8 weeks (1. EPM) and a second time at day 36 post-injury (2. EPM). Entries in open arms were significantly reduced in lesioned LAB and HAB rats as a consequence of suffering from neuropathic pain. Data are expressed as mean ± SEM. N = 12 – 15 , *** P < 0.001; * P < 0.05.

We were interested, whether LAB and HAB animals exhibit an increased anxiety behaviour after CCI injury, which was already observed in Wistar rats with CCI in Chapter I. At day 36 post-injury, when mechanical hypersensitivity was still pronounced in the LAB and HAB rats (both p < 0.001 vs. non-injured contralateral paw, but not significantly different between strains, Figure 10) we determined anxiety-behaviour a second time (2. EPM). The first EPM (1. EPM) test had been determined before sciatic nerve lesion to verify HAB and LAB phenotypes. As depicted in Figure 12 naive LAB rats did not exhibit an increased anxious behaviour at 2. EPM. The rats

entered the open arms of the EPM 7 ± 0.6 times in the first session and 6 ± 0.4 times in the second session ($P > 0.05$). A paired *t-test* analysis revealed a marked and significant decrease of entries in open arms in lesioned LAB rats from 9 ± 0.6 to 4 ± 0.3 ($P < 0.0001$). HAB rats already exhibited very few entries in open arms in the first session (0.9 ± 0.4). When tested after CCI injury, entries were significantly reduced even further to 0 ± 0 ($P < 0.05$). The results clearly show that persistent pain triggers anxiety-like behaviour as a consequence of suffering from chronic pain.

Discussion

In the current study we investigated whether trait anxiety affects pain responses in rats with sciatic nerve injury. Pain responses after mechanical stimulation in high anxiety behaviour (HAB) and low anxiety behaviour (LAB) rats were determined after sciatic nerve lesion. We observed that HAB rats exhibited a stronger pain response than LAB rats two and three weeks post-injury, while baseline sensitivity in both strains was not different. These data provide the first evidence that trait anxiety can modify pain sensation in rats with neuropathic pain.

Baseline sensitivity of HAB and LAB rats

In order to investigate the development of mechanical hypersensitivity after sciatic nerve lesion we determined the PWT in injured LAB and HAB rats over 8 weeks. Before CCI surgery there was no significant difference in mechanical sensitivity in HAB and LAB rats on the left and right hind paw, although the two rat strains have a predisposition for high and low anxiety-related behaviour, respectively (98; 127; 233; 234; 245). In a similar experiment, Wilson and colleagues (235) characterised naive Wistar rats with the EPM task and separated them into high and low anxious animals. Despite their different basal levels of trait anxiety, rats did not exhibit different levels of mechanical hypersensitivity. These studies indicate that sensitivity to phasic painful stimuli is not influenced by high or low trait anxiety levels.

Contrary to our results, Jochum and colleagues observed an increase of heat pain threshold in HAB rats compared to normal Wistar animals (101). This discrepancy might be due to the use of different pain modalities and pain tests: while in the current study mechanical stimuli were applied, Jochum and colleagues measured responses to heat stimuli, which usually are of longer duration than mechanical stimuli and induce both peripheral and central sensitization and habituation processes (76; 163). Moreover, Ramos and colleagues found that the anxious inbred rat strain LEW, another useful genetic model for the assessment of anxiety, displayed more nociceptive responses after formalin injection than the less anxious SHR strain (177). Although the formalin test is often considered a model for acute pain, it causes pronounced changes in the responsiveness of the nociceptive system, and in particular its second phase is a model of rapid pain plasticity rather than acute pain. These heterogeneous data demonstrate that the pain response in naive rats may be

dependent on the pain modality used and the extent to which pain plasticity is invoked.

Mechanical sensitivity in HAB and LAB rats with sciatic nerve injury

Rats subjected to CCI lesion exhibit pronounced pain responses after applying evoked mechanical stimuli (20; 58). We demonstrated that CCI injury provokes a significant mechanical hypersensitivity of the same magnitude at day 7 post-injury in HAB and LAB rats. Thus, the differences in trait anxiety between HAB and LAB rats and their underlying mechanisms came only into effect at a later stage of this neuropathic pain model.

We observed that high anxious rats exhibit a stronger pain response than low anxiety animals at day 14 and 21 postoperatively, reflected in a decreased PWT to mechanical stimuli, whereas beyond day 36, HAB rats exhibited a weaker pain response that LAB rats. This study is the first showing a change in pain thresholds after sciatic nerve lesion in constitutively anxious animals. We therefore conclude that pre-existing anxiety affects the development of chronic neuropathic pain. This pro-nociceptive effect of anxiety differs from the anti-nociceptive effects of fear (180), which occur in the context of stress-induced analgesia (5; 35; 123).

The concept that anxiety causes stronger nociceptive responses is not only detected in animals but is also observed in humans. For instance a decrease of pain thresholds and stronger pain responses have been reported in patients suffering from anxiety disorders (98). Moreover, anxiety has been closely associated with negative pain experiences in chronic settings (11) and pain sensitivity increases with higher level of anxiety (43; 56; 180). Taken together, these data support our finding, that trait anxiety of the HAB rats notably influences nociception.

Potential mechanisms of differences between HAB and LAB rats in the CCI model of neuropathic pain

HAB and LAB rats were selectively bred for differences in anxiety- and depression-related behaviour. Candidate genes for this behavioural difference include vasopressin (120; 234). It was for instance found that HAB rats exhibit a stronger synthesis of the neuropeptide vasopressin in parvo- and magnocellular neurons of

the paraventricular nucleus. Treating HAB rats with a V_1-receptor antagonist reduced anxiety behaviour in these rats. These data highlighted that vasopressin is a candidate gene for anxiety and contributes to the anxious phenotype of the HAB rats. On the other hand several lines of evidence suggest a role of vasopressin in pain modulation. In an animal model of complex regional pain syndrome (CRPS), nociceptive behaviour was enhanced by vasopressin (238). Likewise, in an experimental human model of CRPS, vasopressin produced thermal hyperalgesia (59). Thus, enhanced vasopressin release may be responsible for both increased anxiety and increased mechanical hypersensitivity following CCI injury in HAB rats. On the other hand, microinjection studies provide evidence for an analgesic effect of vasopressin in the caudate nucleus and the raphe magnus (242; 243). These data indicate that this neuropeptide may have both pro-algesic and analgesic effects via different circuits in multiple central brain areas. We therefore assume that vasopressin may contribute to the biphasic effect observed in HAB in comparison to LAB rats during 8 weeks after CCI lesion.

HAB rats are also more susceptible to emotional stressors than LAB rats (119). Furthermore, HAB rats have a hyperactive hypothalamo-pituitary-adrenal axis with the consequence of a higher plasma concentration of prolactin and ACTH upon stressors (120). If the hyperalgesic state in the chronic constriction injury model acts as a stressor, HAB rats are likely to develop an elevated plasma level of glucocorticoids, which may explain the accelerated recovery. Systemic or intrathecal glucocorticoid application is known to inhibit the development and maintenance of mechanical hypersensitivity in animal neuropathic pain models (210; 240). There is even a small clinical trial (114) showing an effect of intrathecal glucocorticoid injections on intensity and area of dynamic mechanical allodynia in post-herpetic neuralgia. Interestingly, the anti-allodynia was associated with reduction in ongoing pain of nearly the same magnitude.

Efficacy of gabapentin on mechanical and thermal hypersensitivity

After showing that HAB and LAB rats vary in their pain sensation due to the different levels of anxiety, our second aim was to assess the anti-nociceptive efficacy of gabapentin on mechanical hypersensitivity in both strains at day 23 post-injury. The anticonvulsant drug gabapentin displayed efficacy in rat neuropathic pain models, the CCI model, and is currently utilized for treating neuropathic pain patients (152). In our

study we demonstrated that gabapentin attenuated the pain sensitivity in HABs and LABs with similar efficacy compared to the mean of maximal possible effect. These data suggest that despite their different emotional properties and the genetic predisposition for anxiety the efficacy of gabapentin is not influenced.

Pain-induced anxiety in LAB and HAB rats with neuropathic pain

An association between anxiety and pain sensation has widely been accepted. In Chapter I we already showed that neuropathic pain induces anxiety-like behaviour in Wistar rats three weeks after being subjected to sciatic nerve injury. Our data supported work from other authors who also assessed pain-induced symptoms mainly including the affective-motivational component of pain (94; 177; 226). For instance Wallace and colleagues showed that in a model for HIV-associated neuropathic pain anxiety-like behaviour can be observed (227). We now demonstrated that the low trait anxiety level in LAB rats did not prevent them from developing pain-related anxiety-like behaviour in the elevated plus maze after chronic constriction injury. In HAB rats, pain-related anxiety occurred on top of the already high level of trait anxiety. These findings suggest that pain-related modulation of state anxiety was preserved in both rat strains.

Several publications pointed out that anxiety strongly influences pain sensation. In the present experiment we could confirm this hypothesis, since we found that a genetic model for trait anxiety exhibit a more pronounced sensitivity after sciatic nerve lesion, when compared to a low anxiety model. Since anxiety is thought to amplify the intensity of emotional reactions and the sensitivity to such sensations (45), it is possible, that anxiety even exacerbates components of chronic pain and vice versa. In particular, the data support the hypothesis that anxiety and pain may end up in a vicious cycle where strong pain increase anxiety behaviour and an increase of anxiety leads to stronger pain sensation. Similar results were observed by Rhudy and colleagues in a recent study where people reported about more pain after their anxiety had been raised (180). This correlation provides evidence that our hypothesis is also transferable to the human situation and represents therefore a suitable model to further investigate the link and the underlying circuits of pain modulation and anxiety states.

Conclusion

Taken together these results provided a more detailed understanding of the relationship between trait anxiety and pain sensitivity in rats. The level of pre-existing anxiety influenced the development of mechanical hypersensitivity in rats subjected to a standard neuropathic pain model (sciatic nerve lesion). Rats with high anxiety behaviour exhibited a stronger pain response to mechanical stimuli at day 14 to 21 post-injury. In turn, rats with a sciatic nerve lesion displayed more anxiety-like behaviour than rats without such a lesion. The strong effect of trait-anxiety even on a simple nociceptive behaviour and the mutual enhancement of anxiety by pain and vice versa suggested that a vicious cycle of pain and anxiety may contribute to chronic pain states. Baseline mechanical pain sensitivity and analgesic efficacy of gabapentin were not influenced by the emotional state of the animals.

CHAPTER V

Effect of pharmacologically induced state anxiety on mechanical hypersensitivity

Several clinical studies demonstrate that pain responses are increased by elevated anxiety e.g. in anxiety disorder patients (52). However, the preclinical situation has hardly been investigated. Only limited experiments are published which examined changes in pain responses in an animal model for anxiety. In chapter IV***CHAPTER IV*** we successfully demonstrated, using a genetical model for pre-existing anxiety that pain responses in rats with sciatic nerve lesion are significantly reduced in comparison to low anxiety behaviour rats. In order to further examine the effect of an acute and more temporary anxiety state on pain sensation in animals, mechanical hypersensitivity was investigated in rats with pharmacologically induced anxiety.

A link between anxiety and pain in humans is mainly simulated and assessed experimentally in healthy subjects, by applying a single method of noxious stimulation (108). To also resemble uninjured volunteers and to compare the observation in humans with findings in animals, we investigated pain responses in naive rats, after inducing anxiety with a pharmacological agent. In the following study state anxiety was induced by oral injection of the compound pentylentetrazol (PTZ). This $GABA_A$ antagonist was described as an anxiogenic compound which can induce acute or sub-chronic states of anxiety. Animals were treated once (acute) or three times a day over a period of 9 days (sub-chronic) and mechanical hypersensitivity was measured at several time points. Furthermore, we determined the effect of the analgesic drug gabapentin in this model. To find out whether an anxiolytic drug reverses PTZ-induced mechanical hypersensitivity we treated the animals with the $GABA_A$ agonist midazolam. An analgesic effect after midazolam treatment would therefore support our hypothesis that anxiety triggers the elevated pain response. These studies will give further insights in the impact of state anxiety on pain sensitivity in rats.

Effect of acute PTZ treatment on mechanical hypersensitivity and anxiety-like behaviour

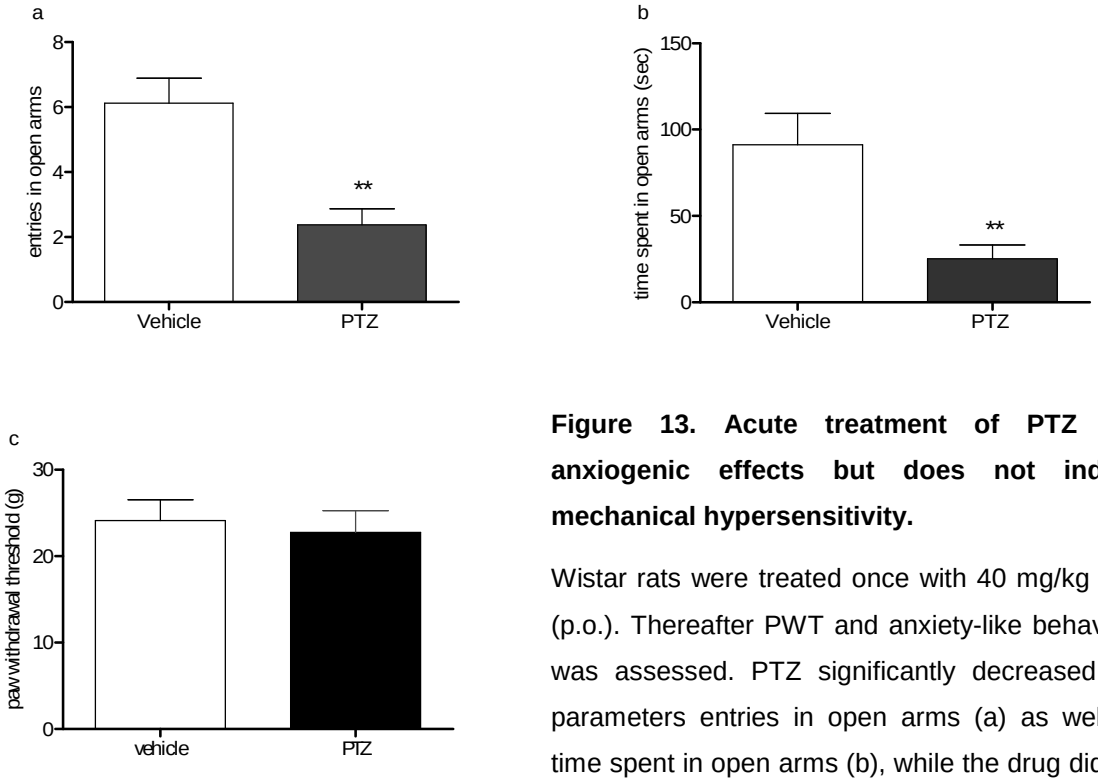


Figure 13. Acute treatment of PTZ has anxiogenic effects but does not induce mechanical hypersensitivity.

Wistar rats were treated once with 40 mg/kg PTZ (p.o.). Thereafter PWT and anxiety-like behaviour was assessed. PTZ significantly decreased the parameters entries in open arms (a) as well as time spent in open arms (b), while the drug did not affect pain response (c). Data are expressed in mean ± SEM. N = 8. ** $p < 0.01$.

After the acute treatment of (40 mg/kg, per os) PTZ, anxiety and PTZ-induced mechanical hypersensitivity were measured using EPM task and electronic von Frey, respectively (Figure 13). An unpaired *t-test* revealed a statistically significant anxiogenic effect 15 minutes after single PTZ treatment. Entries in open arms reduced from 6 ± 1 in the vehicle treated rats to 2 ± 1 in the PTZ treated group ($p < 0.01$). The second parameter time spent in open arms diminished from 91 ± 18 sec in control animals to 25 ± 8 sec in the PTZ group ($p < 0.01$). Mechanical sensitivity was not changed in the PTZ-treated rat group in comparison to control animals, thereby suggesting that the acute anxiogenic effect of the $GABA_A$ antagonist does not affect pain responses.

Effect of sub-chronic PTZ treatment on hypersensitivity and anxiety-like behaviour

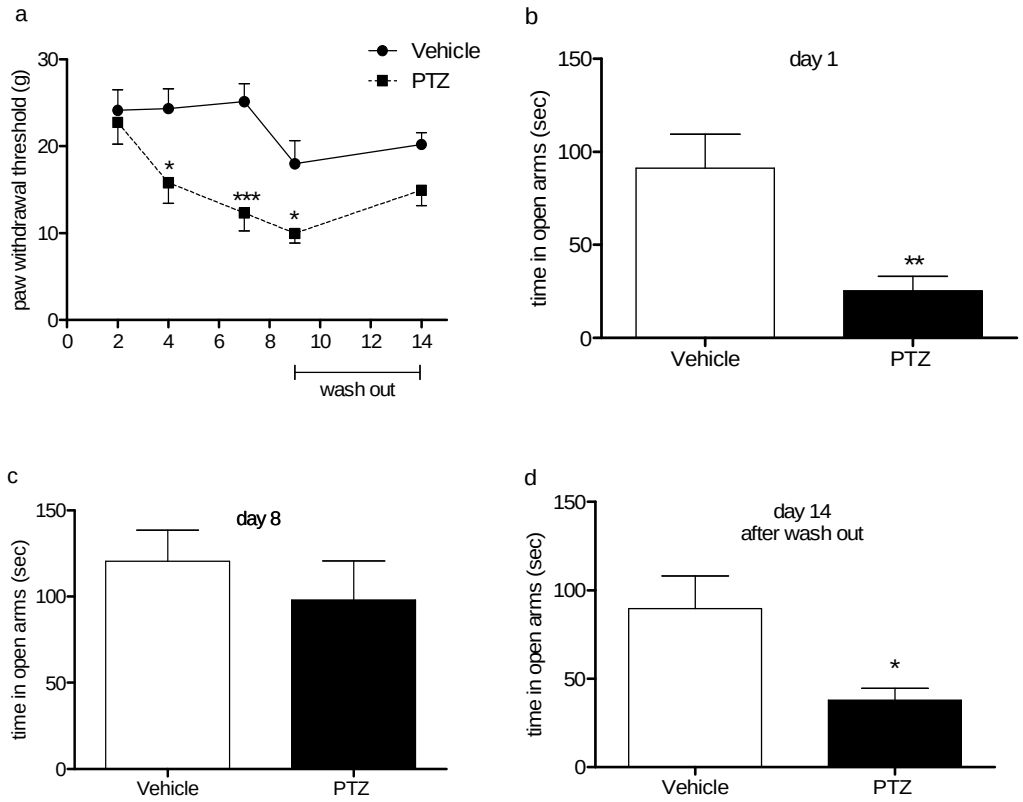


Figure 14. Treatment of pentylentetrazol over a period of one week has anxiogenic effects and induces mechanical hypersensitivity.

Wistar rats were treated with 40 mg/kg PTZ (p.o.) three times daily and PWT was assessed at day 1, 4, 7 and 9 and 14. A 6 day washout phase was kept from day 9 to day 14. PWTs significantly decreased after mechanical stimuli at day 4, 7 and day 9 (a). The withdrawal of the anxiogenic compound returned mechanical sensitivity. Anxiety-like behaviour was confirmed at day 1, 8 and 14. The anxiogenic compound significantly decreased the entries in open arms at day 1 and day 14, but at day 8 a tolerance effect occurred (b-d). Data are expressed in mean ± SEM. N = 8. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In the following experiment we investigated, whether a sub-chronic treatment of the anxiogenic compound PTZ influences the PWT of naive rats. PTZ was given orally 3 times daily for a period of 9 days. A two-way ANOVA revealed a statistically significant main effect in mechanical hypersensitivity for treatment ($p < 0.001$) and for time ($p < 0.001$), but not for interaction. The development of mechanical hypersensitivity has been observed at day 4 which was even more pronounced on the following days. After PTZ treatment we determined a significant decrease of PWT to 17 ± 2 g at day 4 ($p < 0.05$), 12 ± 7 g at day 7 ($p < 0.01$) and 10 ± 7 g at day 9

($p < 0.05$). PWT of the PTZ group slightly increased to 15 ± 2 g after washout phase. This value was still significantly different from baseline value at day 1 ($p < 0.001$). PWT of the control group was stable in the first week and values amounted to 24 ± 19 g at day 1 and 25 ± 20 g at day 4 and 7, respectively. Interestingly, the control group showed a decrease of PWT to 18 ± 3 g at day 9, which was not significant in comparison to day 1 or day 7 ($p = 0.07$ vs. day 7, $p = 0.10$ vs. day 1). After the 6-days washout phase the PWT amounted to 20 ± 1 g ($p = 0.31$ vs. day 1). Results depicted in Figure 14a clearly demonstrate, that pharmacologically induced anxiety affects the mechanical sensitivity in naive rats, which is reflected in a decrease of PWT in comparison to vehicle treated rats.

It is necessary to experimentally prove whether PTZ in fact induces anxiety behaviour after the sub-chronic treatment. Figure 14 b-d shows the results of the EPM at different days after PTZ treatment. The acute treatment (day 1) of PTZ exhibited an anxiogenic effect which is reflected in a reduced time spent in the open arms ($p < 0.01$). At day 8 the animals did not exhibit anxiety-like behaviour since the compound showed a tolerance effect ($p < 0.44$). After the washout phase, PTZ is able to trigger anxiety behaviour. Time spent in open arms was reduced from 89 ± 18 sec in the control group to 38 ± 7 sec in the treatment group ($P < 0.05$).

Effect of gabapentin on PTZ-induced mechanical hypersensitivity and anxiety-like behaviour

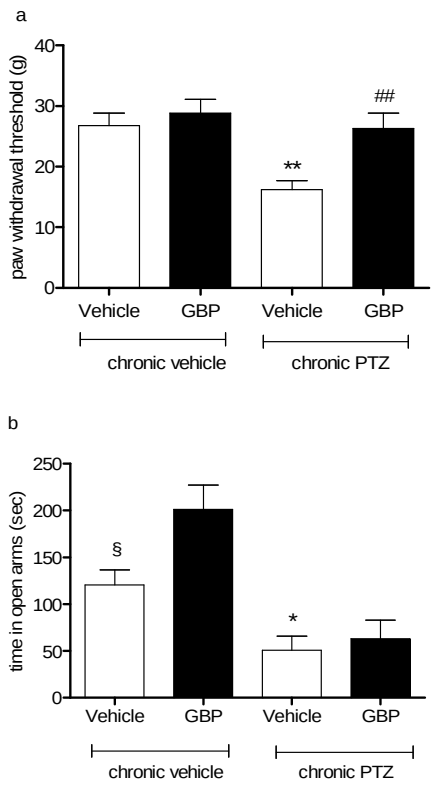


Figure 15. Effect of gabapentin on PTZ-induced sensitivity and anxiety-like behaviour.
Sub-chronic treatment of pentylentetrazol (40 mg/kg; three days; 3 x daily, p.o.) induced a significant mechanical hypersensitivity, which could be reversed by the analgesic drug gabapentin (GBP; a). PTZ also had an anxiogenic effect on the time spent in open arms (b) which was not affected after gabapentin treatment. Data are expressed in mean ± SEM. N = 7-8. Post-hoc test adjusted to Bonferroni-Holm: * $P < 0.05$; ** $P < 0.01$ chronic PTZ/acute vehicle vs. chronic vehicle/acute vehicle; ## $P < 0.01$ chronic PTZ/acute GBP vs. chronic PTZ/acute vehicle; § $P < 0.05$ chronic vehicle/acute vehicle vs. chronic vehicle/acute GBP.

In the next study we addressed the question, whether the analgesic drug gabapentin (30 mg/kg, i.p.) has any effect on PTZ-induced mechanical hypersensitivity or anxiety-like behaviour. Due to the tolerance effect and the change of PTW in the vehicle group in the previous study, we treated animals on 3 following days (3 x daily, p.o.) with PTZ. At day 4 anxiety behaviour and mechanical sensitivity was determined by using the EPM task and “electronic von Frey test”, respectively. In Figure 15a the analgesic effect of gabapentin on PTZ-induced mechanical hypersensitivity is depicted. The reduced PWT was significantly elevated from 16 ± 1.5 g to 26 ± 2.5 g ($p < 0.01$), suggesting that anxiety-induced pain can be reversed by the analgesic drug gabapentin. Anxiety-like behaviour could be significantly induced by sub-chronic treatment of PTZ ($p < 0.05$). Time spent in open arms decreased from 120 ± 16 sec in the vehicle treated control group to 51 ± 15 sec in the chronically treated PTZ group. Gabapentin exhibited an anxiolytic effect in the vehicle group ($p < 0.05$), while it could not attenuate the anxiogenic behaviour in the PTZ group (Figure 15b).

Effect of midazolam on PTZ-induced mechanical hypersensitivity and anxiety-like behaviour

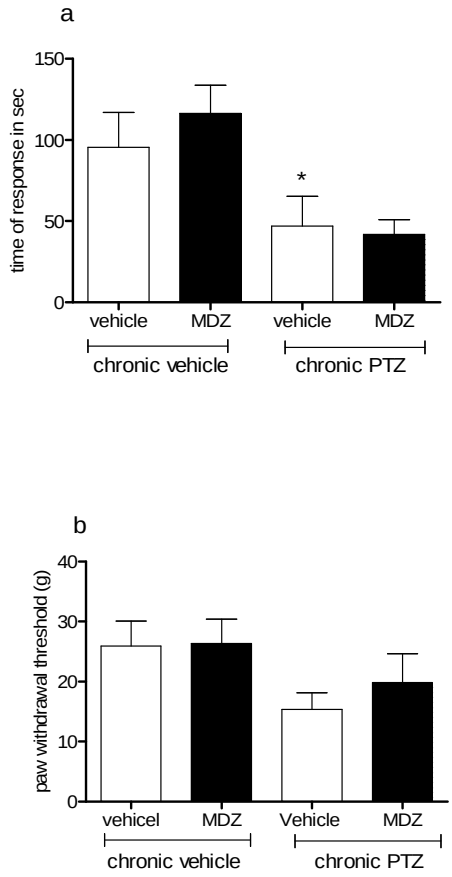


Figure 16. Effect of midazolam on PTZ-induced mechanical hypersensitivity or anxiety-like behaviour.

Sub-chronic treatment of PTZ (40 mg/kg; three days; 3 x daily, p.o.) had an anxiogenic effect on the EPM which was not affected after midazolam treatment (a). Pentylentetrazol induced a significant mechanical hypersensitivity, which could not be reversed by the anxiolytic drug midazolam (MDZ; b). Data are expressed in mean ± SEM. N = 7-9. *Post-hoc* test adjusted to Bonferroni-Holm: * $p < 0.05$ chronic PTZ/acute vehicle vs. chronic vehicle/acute vehicle;

Having demonstrated that the analgesic drug gabapentin was not able to reverse PTZ-induced anxiety, we assessed the effect of an acute anxiolytic treatment in order to reverse mechanical hypersensitivity by blocking anxiogenic PTZ effect. The anxiolytic drug midazolam was administered after an induction of anxiety by pentylentetrazol (3 times daily for 3 days). A two-way ANOVA comparison revealed a statistically significant main effect in time spent in open arms for chronic treatment ($p < 0.001$) but not for acute drug treatment or interaction. A pairwise t-test revealed a statistically significant decrease in number of entries into the open arms after three days of PTZ treatment from 9 ± 1 to 4 ± 1 ($p < 0.05$, data not shown) as well as in time spent in open arms from 99 ± 20 sec to 47 ± 19 sec ($p < 0.0$; a), indicating that PTZ induces anxiety-like behaviour. However, midazolam could not display a significant anxiolytic effect in any of the groups.

As a next step the effect of the anxiolytic drug midazolam on PTZ-induced mechanical hypersensitivity was assessed (b). A two-way ANOVA comparison revealed a statistically significant main effect for the chronic treatment ($p < 0.01$). Post-hoc analysis yield a significantly lower paw withdrawal threshold in sub-chronic PTZ treated animals (14 ± 2 g) in comparison to vehicle treated rats (28 ± 3 g;

$p < 0.05$). Midazolam could neither antagonize the PTZ-induced hypersensitivity nor blocking the PTZ –induced anxiety.

Discussion

The purpose of the current study was to examine the effect of state anxiety on pain responses in non-lesioned rats. Anxiety was induced acute or sub-chronical via injection of the anxiogenic compound pentylentetrazol and hypersensitivity was determined by applying mechanical stimuli. We observed an increase of mechanical hypersensitivity after the sub-chronic treatment of the $GABA_A$-antagonist while no change after acute treatment was detected. Furthermore, we found that gabapentin reversed pharmacologically induced hypersensitivity, but did not exhibit any effect on anxiety-like behaviour. The anxiolytic drug midazolam could neither significantly reverse PTZ-triggered anxiety nor affect PTZ-induced mechanical hypersensitivity. These data suggest an influence of state anxiety on pain sensation in healthy rats, and contributes to the elucidation of the relationship between anxiety and pain.

Model of state anxiety

The first challenge in this study was to find a proper model, resembling state anxiety behaviour. In chapter IV we used the genetically established HAB rat strain, which reflects the existence of stable trait anxiety. State anxiety is defined as a temporary emotional condition characterized by tension and fear about a particular situation and accompanied by behavioural indications such as freezing. Here we resembled state anxiety via per os injection of the compound pentylentetrazol. Pentylentetrazol is known as a potent anxiogenic drug, mediating its effects via specific interaction with $GABA_A$ receptors. Initially it was described as a convulsant drug which induces seizures. Later animal studies confirmed anxiety-like properties in several behavioural tasks such as elevated plus maze and Vogel conflict test (53; 70). The compound not only reduced the time spent in open arm as a classical indication for anxiety in the EPM, but we also observed some emotive components such as freezing, startle response and retirement in their home cages, before treatment. Therefore, we selected this drug to induced temporal limited and reversible anxiety-like behaviour. The selected dosage of 40 mg/kg was evaluated in pilot studies and adapted with the published literature (19).

Sensitivity in rats after acute and sub-chronic PTZ treatment

We investigated the effect of an acute treatment of PTZ on anxiety-like behaviour and mechanical hypersensitivity. Pentylentetrazol induced a strong anxiogenic effect after

one-time application, but there was no change of PWT after mechanical stimulation. These data are in agreement with our previous experiment conducted with the LAB and HAB rat strain, where also no effect on mechanical hypersensitivity was observed in healthy animals before they were subjected to sciatic nerve injury. The findings suggest that a single dose of PTZ, which indeed has anxiogenic effect, is not sufficient to change the pain response in naive animals.

The effect of a sub-chronic treatment of PTZ (three times daily) resulted in a strong induction of mechanical hypersensitivity, reflected in a decrease of paw withdrawal threshold at day 4, 7 and 9 post-treatment. These data support the theory that anxiety is an important factor for pain modulation and that pharmacologically induced anxiety is able to trigger mechanical hypersensitivity. Several clinical experiments already provided evidence that anxiety is associated with elevated pain. Data derived not only from experiments with healthy subjects, which are confronted with anxious situation and painful stimuli (107; 108), but also from patients with anxiety disorders, reporting about more severe pain (10; 116). Koegh and colleagues found that anxiety was positively correlated with sensory and affective pain ratings in response to cold pressure task (108). We conclude that the PTZ-induced anxiety is a proper model to mimic mechanical hypersensitivity triggered by an anxious behaviour and can be used to further investigate the link between pain and anxiety.

There are a few studies, which examined the interaction between anxiety and pain in animals. In contrast to our data, Jimenez-Velazquez and colleagues could not observe a change of pain response after the induction of anxiety-like behaviour by the anxiogenic drug yohimbine (100). The α_2- adrenoceptor antagonist even reversed nociception in a model for arthritis resembling stress-induced analgesia. Apparently there are divergent mechanisms to induce anxiety-like behaviour and furthermore, they also lead to different impacts on pain responses. While the $GABA_A$ receptor system seems to be more involved in pain-induced hyperalgesia, α_2 receptors mediate stress-induced analgesia. To confirm this hypothesis further studies needed to be conducted, i.e. blocking the effect of PTZ with a corresponding agonist.

To assess the effect of anxiety-like behaviour on pain thresholds, rats were tested in the EPM at day 1, 8 and 14. (at time points, which were close to the measurements of pain thresholds). An anxiogenic effect of PTZ could be determined at day one and at day 14. A tolerance effect due to the frequent treatment occurred at day 8, which

was already demonstrated by Buczek and colleagues (32). Therefore, in further studies acute treatment of gabapentin and midazolam was given after a sub-chronic treatment of 3 days.

Efficacy of gabapentin on PTZ-induced mechanical hypersensitivity

We assessed the effect of gabapentin on PTZ-induced anxiety and pain sensitivity at a dose which already showed analgesic effects in our previous studies (30 mg/kg). PTZ-induced mechanical hypersensitivity could be reversed after treatment of gabapentin, which was reflected in a significant increase in PWT, while it was not able to exert anxiolytic effects in the PTZ group. These data suggest that hypersensitivity can be reversed after an acute treatment of an analgesic drug. Intriguingly, we observed an anxiolytic effect of gabapentin in the vehicle treated group. This is of particular interest, since in a previous study and in former experimental conditions (CCI rats and sham operated rats in chapter I) we never found anxiolytic properties of gabapentin. In a pilot study, two animal groups were compared, resembling the CCI study (Chapter I) and the current PTZ study. One group has been treated once and the second group over a period of 4 days with vehicle or gabapentin (data not shown). The results showed that the sub-chronic treated rats, which mimic the PTZ study, exhibit an anxiolytic effect of gabapentin, while after one-time application gabapentin did not display an anxiolytic effect. Apparently, rats get used to the treatment procedure and display a diminished stress level. In these conditions gabapentin is able to exert anxiolytic properties, while the one-time application exerts a more stressful situation. Therefore the anxiolytic effect of the compound is not detectable. In the PTZ treated rats, gabapentin can not overcome or defeat the strong anxiogenic effect following PTZ injection. Similar results were observed from de-Paris and colleagues, who found an anxiolytic effect of gabapentin under less stressful test conditions (51). One might suggest that the effect of gabapentin is dependent on the initial anxiety or stress state of the animals.

Efficacy of midazolam on PTZ-induced mechanical hypersensitivity

Finally the effect of midazolam on anxiety-induced hypersensitivity was examined. The anxiolytic drug did not modify nociceptive thresholds. Our data are in agreement with the behavioural study performed by Rivat and colleagues. They found that hypersensitivity, caused by a stressful social defeat procedure, was not blocked by

the anxiolytic drug chlordiazepoxide (183). Interestingly, midazolam was also not able to reverse PTZ-induced anxiety-like behaviour, even both compounds mediate their effects via $GABA_A$ receptors. However, antagonistic effects of MDZ have been demonstrated for example by Vivian and colleagues. Anxiety state, which was induced by 20 mg/kg PTZ, could be reversed by a 0.5 mg of MDZ (comparable to our study) (221). The reason for lack of efficacy in the current study after MDZ treatment might be the high dose of PTZ and the sub-chronic treatment, which apparently induces a very strong anxiety-like behaviour in the rats. This presumption is furthermore based on our results from a pilot study in which MDZ could reverse PTZ-induced anxiety (sub-chronic treatment of 20 mg/kg; data not shown). However, the development of mechanical hypersensitivity was very low and unstable at the dose of 20 mg. As we were primarily interested in changes of pain responses, all studies were performed in the higher PTZ dose.

Role of gabaergic system in PTZ-induced anxiety and mechanical hypersensitivity

$GABA_A$ is a very important inhibitory neurotransmitter of the central nervous system, which is modulating also the descending systems in the spinal cord controlling and modulating pain information (218). It is known that activation of spinal ionotropic $GABA_A$ receptors leads to pain relief. Furthermore, gabaergic pathways exert an inhibitory influence upon the release of many neurotransmitters known to mediate anxiogenic actions (136). We investigated the development of anxiety after sub-chronic treatment of the PTZ antagonist and an associated increase of mechanical hypersensitivity. However, t no anxiogenic effect was detected at day 8 of PTZ treatment anymore (Figure 14c). This tolerance effect of the compound in the anxiety paradigm is of particular interest, as no tolerance was observed in the pain test. In contrast mechanical hypersensitivity even increased to a maximum level at day 9. It has proposed that a long-term exposure of $GABA_A$-receptor ligands lead to changes in corticolimbic populations of $GABA_A$ receptors which are related to the progressive development of tolerance and dependence (237; 249). Single and repeated treatment of PTZ (30 mg/kg) results in a decrease in GABA binding and in ion uptake (41; 42). Another study reports a decrease of $GABA_A$ receptor mRNA after PTZ treatment which leads also to a reduction in gabaergic inhibition. These results lead to the assumption that tolerance might induce changes in the gabaergic system which exerts different effects on anxiety and pain behaviour. While the cellular

changes lead to a reversal of anxiety, mechanical hypersensitivity is not influenced. Furthermore, the data suggest that both processes are not directly linked via the gabaergic system.

One might argue that the long term alterations of the $GABA_A$ receptors are responsible for the hypersensitivity since PTZ causes a reduction of descending inhibition in the spinal cord. This implies that mechanical hypersensitivity is not a result of increased anxiety-like behaviour but is due to inactivation of inhibitory neurons in the spinal cord. However, as we did not observe a decrease of paw withdrawal threshold after acute treatment of PTZ, it is likely that the mechanical hypersensitivity is not mediated by the blockage of the gabaergic pathways in the spinal cord. Furthermore, the studies of reduced expression of $GABA_A$ receptors have been seen in the cerebellum, the cortex and hippocampus but not in the spinal cord, which is the main area for descending inhibition. Since the calcium^{2+} channel modulator gabapentin significantly reversed the anxiety-induced hypersensitivity, it is more likely that the anxiety-induced pain process is independent from $GABA_A$ receptors. Furthermore, rats did not exhibit a tolerance effect in mechanical hypersensitivity, thereby suggesting that both processes are not directly linked to each other and different nervous system areas are involved. Although we can not completely exclude that the block of $GABA_A$ receptors leading to the elevated pain response, we mainly propose that the present hypersensitivity is a result of the high anxiety behaviour in the rats. Further studies may provide insights into the mechanisms which are responsible for the PTZ-induced mechanical hypersensitivity.

Taken together these studies suggest that mechanical hypersensitivity can also be triggered by pharmacologically induced anxiety using a sub-chronic treatment of PTZ. Furthermore, the data indicate that the mechanical hypersensitivity can be reversed with an analgesic drug. We clearly showed that also state anxiety can influence pain responses in naive rats. The model not only supports the finding from the former study but also provides a second model to investigate the relation between pain and anxiety.

SUMMARY AND GENERAL DISCUSSION

In the current study the following results were achieved:

- Neuropathic pain induces anxiety-like behaviour in rats with a chronic constriction injury.

- Pain-induced anxiety can be reversed by the pure analgesic drugs morphine and gabapentin and by the anxiolytic drug midazolam in CCI injured rats.

- Several drugs were tested in the CCI model. Different anxiolytic and analgesic effects were determined in the EPM and after applying mechanical stimuli, respectively.

- Oxytocin and vasopressin are upregulated in the amygdala of CCI-injured animals. After the intra-amygdala treatment of oxytocin antagonist but not vasopressin antagonist, pain-induced anxiety-like behaviour was reduced. Both drugs had no effect on mechanical hypersensitivity.

- Trait anxiety in rats increases pain behaviour after applying mechanical stimuli 2 - 3 weeks after sciatic nerve lesion.

- Pharmacologically induced anxiety in rats increases pain responses after applying mechanical stimuli.

Rodent models for preclinical pain research have been studied extensively to characterise the behavioural signs of neuropathic pain. However, the majority of these models only assess reflex responses after evoked painful stimuli but not the associated emotional components and psychological comorbidities and quality of life aspects (e.g. sleep disturbances, anxiety, depression), which are often reported in clinical trials. The classical tests measure evoked nociceptive behaviours, which result from activation and modulation of circuits in the spinal cord that are different from pain transmission pathways and convey little information about other aspects of pain (220). To meet this requirement there is an urgent need for the development of pre-clinical models which also focus on the complex behavioural symptoms of pain.

Therefore, we established an animal model of neuropathic pain in chapter I, which also induces pain-related components such as anxiety. We demonstrated that sciatic nerve injured rats display a reduced time spent in open arms, which is not a consequence of locomotor impairment (Figure 4d). Furthermore, we observed that the analgesic drugs morphine and gabapentin diminished pain-related anxiety-like behaviour in this model. These results are of particular interest, since we determined not only the sensory component of pain but also the more affective and emotional aspects by using the EPM paradigm. We conclude that our model is not only an useful approach for modelling the reflexive and the emotional components of pain, but also a potential tool for a more successful analgesic drug profiling. Recently, a number of groups have started to establish animal models, combining the sensory as well as affective component of pain. Behavioural paradigms such as the open field (86; 226) as well as the place avoidance test (94) were successfully used to show pain-related comorbidities in several neuropathic pain models.

The effects of the analgesic drugs gabapentin and morphine in CCI rats in comparison to the anxiolytic drug midazolam were assessed to differentiate between their anti-nociceptive and anxiolytic effects. Anxiety-like behaviour was normalized by midazolam in sham and CCI-injured rats without affecting nociception. Of interest, a recent study also observed similar results with the anxiolytic drug diazepam in a model for HIV associated neuropathy, thereby confirming that benzodiazepines are rather acting as an anxiolytic drug than as an analgesic drug (227).

We demonstrated that gabapentin and morphine significantly reversed mechanical hypersensitivity in CCI-injured rats. After excluding a sedative effect of the drug by

using a locomotion-based test, the compounds were assessed in the EPM. Gabapentin is reported to be efficacious in reducing not only human neuropathic pain conditions (90) but also pain-like behaviour in rodent models (89). Morphine is also well known to reduce pain behaviour in rats, which could be demonstrated in our experiments. When tested in the EPM, both analgesic drugs exhibited anxiolytic efficacy in sciatic nerve-injured animals without affecting the control group. In line with our results is a study from Wallace at al (227), which also demonstrated anxiolytic effects of gabapentin and morphine in a model for HIV-associated neuropathy without affecting the control group. Our data support the hypothesis that anxiety-like behaviour measured in the present study is a consequence of neuropathic pain and is sensitive to analgesic compounds. It appears that this model provides a potential tool for determining the affective component of pain. Information concerning the emotional symptoms of neuropathic pain, which can be determined by using behavioural measures such as the EPM or the open field, will certainly help to close the gap between preclinical and clinical pain research.

After establishing the CCI model for assessing pain-induced anxiety-like behaviour by using the reference compounds morphine and gabapentin, we characterised several drugs in the CCI rats, which are potentially implicated in the modulation of neuropathic pain. This study was performed to assess, whether the release of pain-related symptoms is restricted to the two reference compounds or if it can also been achieved by other pain modulating drugs. A broad spectrum of different acting drugs was investigated. Table 4 shows an overview of all tested compounds and their efficacy on locomotion, pain relief and reduction of pain-induced anxiety.

As a prerequisite for assessing the effect of the selected drugs in the EPM, they needed to show an analgesic effect on mechanical hypersensitivity and no impairment on locomotion. Sedative effects were observed after treatment of the sodium channel blocker mexiletin, which was therefore not tested in the EPM. The other compounds did not exhibit an impairment of locomotion and an adequate pain relief was observed. Varied effects in sham and CCI-injured rats were examined:

An anxiolytic tendency was measured after treatment of R-715 in CCI rats and after treatment of tramadol in CCI- and sham-operated rats. Intriguingly, R-715 is mainly mediating its effect in the periphery, which strengthens the hypothesise that anxiety is in fact reduced as a consequence of pain relief and not due to central effect of the

compound. Tramadol mainly exert its effects via the 5-HT system and the μ-opioid receptor. We assume that a more anxiolytic effect in the sham group is caused by serotonin component of the drug, which was not observed in the pure opioidergic drug morphine. However, in conclusion the results of both drugs support our hypothesis that pain relief results in a reduction of anxiety-like behaviour following sciatic nerve lesion.

Lacosamide displayed no effect in sham or CCI rats and a reduced time in open arms was observed after the treatment of 5HT-$_{1A}$ agonist 8-OH-DPAT as well as after injection of the B_1-antagonist and SSR240612. We assume that lacosamide is only effective in sensory pain relief but not in the anxiety-inducing ongoing pain. The 8-OH-DPAT and SSR240612 exhibit an innate anxiogenic property due to their mixed binding profile, which prevents the anxiolytic effect of the compound in the EPM. Although, we cannot draw a conclusion concerning the reduction of pain-related behaviour with all investigated drugs, the model provides a useful tool for assessing the analgesic profile of a drug more precisely.

We provided evidence that pain relief also causes reduction of the secondary symptom anxiety. We investigated the effects of variable drugs involved in nociception on mechanical hypersensitivity and anxiety-like behaviour following a sciatic nerve lesion in rats. Even there are some compounds, which did not show a strong anxiolytic effect in CCI injured animals, the model provides a tool for the assessment of novel therapeutic options and gives first insights into the analgesic or anxiolytic properties of a drug in a neuropathic pain model. It is important to mention that the model investigates similar domains of clinical trials, thereby providing potential validity and reliability of the pre-clinic results. However, the effects of a compound in the present CCI model have to be critically evaluated since the outcome is dependent on variable aspects such as sedation, binding profile of the compound and the affected pain component. In conclusion, the data demonstrate that the model offers a great opportunity to predict not only efficacy on sensory pain parameters but also a potential effect on anxiety-like behaviour. It is suggested that the model provides a refinement of the animal model and a more detailed characterisation of novel analgesics in pre-clinic tests.

We successfully established the model to measure pain-related symptoms in rats with neuropathic pain. Although the model was well characterised pharmacologically,

the underlying mechanisms of pain-induced anxiety are still not completely understood. Therefore, in Chapter III it was investigated, whether the neuropeptides vasopressin and oxytocin are involved in the pathophysiology of pain-induced anxiety in CCI rats. Indeed, we found an upregulation of pre-pro oxytocin and pre-pro vasopressin mRNA in the amygdala of rats with neuropathic pain and associated anxiety-like behaviour. This study gives first insights into the neuronal substrates which are involved in higher pain processes of the CCI model. Furthermore, we assessed the influence of oxytocin and vasopressin antagonist on pain-induced mechanical hypersensitivity and anxiety-like behaviour in rats with sciatic nerve lesion. Our findings indicate that oxytocin but not vasopressin is implicated in anxiety-like behaviour in rats with neuropathic pain, while both neuropeptide systems do not affect mechanical hypersensitivity in CCI injured animals. This study provides new insights in the pathophysiology of pain-induced anxiety-like behaviour.

Within this study we also examined whether the amygdala might be responsible for the high processing pain. Accumulating evidences suggest that this cerebral structure participates in processing sensory-discriminative and affective-motivational components of pain (39; 155). This is based on the fact that the amygdala exhibits the development of synaptic plasticity after pain stimuli as well as after stress exposure indicating that the amygdala is a very important structure for the dynamic processes caused by nociceptive as well as emotional information. Nishii and colleagues (157) for instance observed an upregulation of galanin mRNA in amygdaloid and hypothalamic nuclei in a model for visceral pain. Recent studies indicate that psychological stress represents a highly significant impact on the CRH mRNA levels in the central amygdala (132; 157). Together with our finding that pain-related anxiety triggers the upregulation of oxytocin and vasopressin mRNA, these data strengthen the idea that the amygdala is critically involved in the affective component of pain. Further studies exploring the relationship between pain and affective disorders would help to elucidate the neurobiology of these processes.

Pain is a complex experience that involves not only the transduction of noxious sensory stimuli, but also cognitive and emotional processing in the brain (103). We found that pain triggers anxiety-like behaviour in rats with sciatic nerve lesion resulting in changes of the neuropeptide levels of oxytocin and vasopressin in the amygdala. Since there are also multiple reports suggesting an implication of anxiety on pain sensation in humans (15; 43; 211), we were highly interested in examining the impact of different anxiety states on pain responses in rats. We addressed this issue by using a model for trait anxiety (Chapter IV) as well as for state anxiety (

Chapter V). In both models a change of pain response to mechanical stimuli was observed. In the trait anxiety model, the HAB rats, the PWT was significantly decreased two and three weeks after sciatic nerve injury in comparison to the LAB rats. We also observed an accelerated recovery in the HAB rat strain, which demonstrates, that anxiety can exert a biphasic effect on pain responses. In the second state anxiety model we found a decreased PWT after inducing anxiety pharmacologically with the anxiogenic drug PTZ.

It is obvious that anxiety is a type of stress. Stress exposure was also shown to result in long-term functional changes in the central nervous system. The expression patterns of Fos in immobilization and conditioned fear are similar to that induced by noxious stimulation (193; 194) indicating that these stimuli provoke similar cognitive and emotional processes in the brain. Thus, these data support our hypothesis that emotional stress may influence nociception. This is furthermore based on the fact that several animal studies reported a changed pain sensation when exposed to anxiety (107; 124; 211). Koegh and colleagues (106) confirmed in their experiment that anxiety is associated with increased susceptibility to negative pain experiences. We supported these results in our studies, since we could clearly induce mechanical hypersensitivity in the trait anxiety model (HAB) as well as in the state anxiety model (PTZ).

We investigated two different anxiety-related states, since it is often distinguished between personality-based temperament sustained trait anxiety and transient, fear-induced state anxiety (1; 105; 157). Trait anxiety may be modelled by a genetically-modified line or inherent differences in anxious behaviour between individual groups. Therefore, the selectively bred rat strain for high anxiety (HAB) offered a good model to mimic trait anxiety. State anxiety is a temporary and acute situation, which was entirely modelled by an injection of the anxiogenic compound PTZ. This pharmacologically-induced anxiety was reversible and dose dependent. Both kinds of anxiety were triggered by different mechanisms. While PTZ mediates its anxiogenic action via $GABA_A$ receptor inhibition, the exact anxiogenic target for HAB rats is still not completely understood. There is evidence that due to a polymorphism in the vasopressin gene an increased release of this neuropeptide might be responsible for the anxiogenic phenotype of the HAB rats (119). It needs to be discussed, whether the neuropeptide only plays a role in anxiety circuitry or might also be involved in

processing pain information. This question is justified, since we found an upregulation of AVP in the amygdala in CCI injured Wistar rats with strong mechanical hypersensitivity. One might hypothesise that the neuropeptide is strikingly involved in developing hypersensitivity in the Wistar CCI model as well as in HAB rats.

Intriguingly, an analgesic role of vasopressin is suggested by varied studies. It was shown that AVP is released in the paraventricular nucleus after pain stimulation (241). Moreover, microinjection studies provide evidences for an analgesic effect of AVP in the caudate nucleus and the raphe magnus (242; 243). These reports rather indicate an analgesic effect than a participation in increased nociception. Nevertheless there are some evidences supporting an implication of AVP in pain elevation. It is for instance proposed that AVP also produces thermal hyperalgesia in capsaicin-treated skin in human subjects (59). Moreover, nociceptive behaviour is induced in a model for chronic post-ischemia pain by vasopressin (239). This clearly indicates that the neuropeptide is implicated in analgesic as well as algesic effects. It supports our idea, that vasopressin may not only be important for the hypersensitivity but can also exert biphasic effects on pain responses. However, due to our results we can not certainly conclude that vasopressin might play a role in pain-related processes. Since we did not observe an increase or decrease after the treatment of the vasopressin antagonist in CCI injured animals, a definite behavioural relevance has to be demonstrated. Therefore, to fully understand the role of this neuropeptide in anxiety and nociception processes further studies have to be performed.

Although there is strong evidence for a link between anxiety and pain, it is still not clear why such a relationship exists and how anxiety can result in different pain experiences. Two studies have been conducted, which specifically examined the role of anxiety in pain perception: Weisenberg and colleagues found that anxiety, which is related to the pain stimulus, can increase subjective pain, whereas anxiety that is related to any other stimulus than pain, can lead to a reduction of pain experience (230). This means, when distracting attention away from pain, pain is less severe (attributional theory). A second hypothesis proposed by Cornwall and Donderi was not in agreement with the finding from Weisenberg. This group investigated that the relevant or irrelevant anxiety state is not responsible for different pain states (perceptual disruption theory). In contrast, they proposed that anxiety, whether relevant or irrelevant, ought to interfere with ones ability to discriminate between different levels of pain intensity (43). When transferred to our study it is difficult to

assess which theory can be applied for the HAB rats or the PTZ treated rats, since the relevance of the painful stimuli for the animal cannot exactly be assessed. It is possible that the unexpected first pain test resembles the pain irrelevant stimulus, and therefore is not inducing an increased hypersensitivity. As the animals get used to the test procedure, the applied stimulus becomes relevant to a painful sensation, and therefore even potentiates the pain response. This might explain the lack of pain behaviour in the acute phase, but the strong recovery in the HAB rats 4 weeks after surgery cannot be explained with this model. It is not clear, whether one of the proposed theories can be applied for any of the situations in both models. However, since a hyperalgesia was determined after pharmacological induction of anxiety as well as in the genetical model it is likely that trait as well as state anxiety in fact can influence pain sensation.

In the current study we attempted to investigate the relationship between pain and anxiety and vice versa. It turned out that due to anatomical and behavioural overlap, both phenomena are strongly connected. We found that animals with neuropathic pain are developing associated symptoms such as anxiety. On the other hand, we demonstrated in a model for state anxiety as well as in a model for trait anxiety that this behaviour also strikingly influences pain sensation reflected by a decreased PWT. Obviously, the present study demonstrated that pain itself increases anxiety-like behaviour, whereas anxiety also exacerbates pain sensation. This might lead to an unavoidable vicious circle with a continuing increase of anxiety and pain. This scenario is a big challenge for pharmacological and therapeutical approaches, since the underlying mechanisms are still not completely understood. However, the current study gave an insight in potential neuronal substrates which might be implicated in the link between pain and anxiety, and therefore offer a starting-point for drug research.

Taken together, we experimentally proved that neuropathic pain is associated with comorbid anxiety in an animal model for neuropathic pain, that can greatly affect quality of life and that anxiety itself can modulate pain experience. The study was performed in rats, but the results might be relevant for human situation. The goal will be, to optimize the efficacy of analgesic drugs, in the way that also psychological side effects are reduced, while minimizing the adverse events. Therefore, further research is needed to fully elucidate the dynamic relationship among neuropathic pain and

anxiety. This goal can be achieved not only by using animal models which are more predictive and include the affective component as an endpoint but also by investigating the molecular biological alterations of pain and anxiety. The exciting development of new models to assess cognitive, affective and sensory aspects of pain can be expected to greatly improve discovery of therapeutic agents and the development of successful remedies for chronic pain as well as affective disorders.

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LIST OF ABBREVIATIONS

18S rRNA	Ribosomal ribonucleic acid
a.m.	Ante meridiem
ACC	Anterior cingula cortex
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
AVP	Arginin-Vasopressin
BC	Brachium conjunctivum
CC	Corpus callosum
CCI	Chronic constriction injury
CCK	Cholecystokinin
cDNA	Complementary deoxyribonucleic acid
Ce	Central nucleus of the amygdala
CNS	Central nervous system
CRH	Corticotrophin releasing hormone
CRMP-2	Collapsin response mediator protein-2
CST	Complete sciatic transection
DRG	Dorsal root ganglia
EPM	Elevated plus maze
GABA	Gamma-aminobutyric acid
GAD	Generalized anxiety disorder
Gq	G-Protein (subunit q)
HAB	High anxiety behaviour
Hip	Hippocampus;
HIV	Human immunodeficiency virus
HPA	Hypothalamic-pituitary-adrenal axis
IASP	International Association for the Study of Pain
ic	Internal capsule
LAB	Low anxiety behaviour
LC	Locus coeruleus
LEW	Lewis rat strain
LTP	Long-term potentiation
MDZ	Midazolam
mRNA	Messenger Ribonucleic acid
NE	Norepinephrine
NNT	Number needed to be treated
NPY	Neuropeptide Y
OAT	Oxytocin-antagonist
OCD	Obsessive-compulsive disorder
OF	Open field
OXT	Oxytocin
p.m.	Post meridiem
PAG	Periaqueductal grey
PB	Parabrachial area

PD	Panic disorder
PNL	Partial nerve ligation
Po	Posterior group of thalamic nuclei
PTSD	Post-traumatic stress disorder
PTZ	Pentylentetrazol
PWT	Paw withdrawal threshold
Py	Pyramidal tract
RT- PCR	Real-time polymerase chain reaction
RVM	Rostroventral medulla
SEM	Standard error of the mean
SHR	Spontaneously Hypertensive Rats/Nico
SNL	Spinal nerve ligation
TCA	Tricyclic antidepressants
TST	Tibial and sural transcetion model
V	Ventricle
V1/V2	Vasopressin receptor
VMH	Ventral medial nucleus of the hypothalamus
VPL	Ventral posteriolateral nucleus of the thalamus
VPM	Ventral posteriomedial nucleus of the thalamus
WDR	Wide dynamic range

APPENDIX

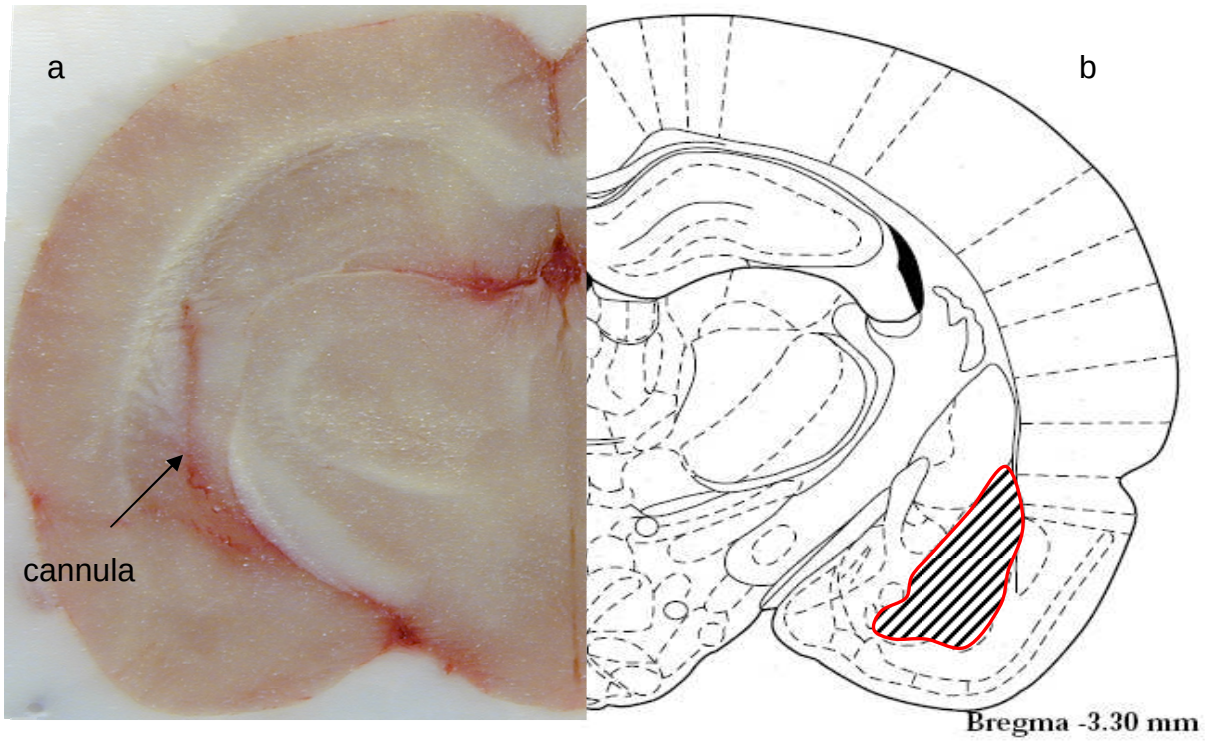

Figure 17 Histological confirmation of injection site in the left amygdala (a); location of the amygdala , taken from Paxinos & Watson (b)(161); (vide Chapter III)

CURRICULUM VITAE

Kerstin Röska

e-mail: kerstin.roeska@gmx.de

Akademische Ausbildung

Seit 02/2006 –02/2009	Promotion in der ZNS Abteilung von Boehringer Ingelheim Pharma GmbH und Co. KG, Biberach a. d. Riss *"Beziehung zwischen Schmerz und Angstverhalten in der Ratte"*
05/2005 – 01/2006	Diplomarbeit in der ZNS Abteilung von Boehringer Ingelheim Pharma GmbH und Co. KG; Biberach a. d. Riss *„Charakterisierung der Cannabinoid-Rezeptoren CB 1 und CB 2 und deren selektiver Liganden"*
10/2000 – 04/2005	Biologiestudium an der Maximilians Universität Würzburg Hauptfächer: Neurobiologie, Bioinformatik, Biochemie

Schulausbildung

09/1991 - 06/2000	Mozartgymnasium Würzburg, Abschluss: Allgemeine Hochschulreife
09/1987 – 07/1991	Grundschule Bütthard

Weiterbildung und praktische Tätigkeiten

06/2003 – 08/2003	Fortgeschrittenen-Praktikum in der neurologischen Abteilung der Universitätsklinik Würzburg *„Analyse der p75 Rezeptorsignalkaskade“*
08/2003 – 02/2004	Laborpraktikum in der genetischen Abteilung der Universität Pablo de Olavide, Sevilla, Spanien *„Arbeiten zur Klärung von Teilungsprozessen bei s.pombee“*
06/2004 – 08/2004	Praktikum in einem *in-vitro* Labor von Boehringer Ingelheim Pharma GmbH und Co. KG, Biberach a.d. Riss

Veröffentlichungen

Pain; 2008 Oct 15;139(2):349-57
“Anxiety-like behaviour in rats with mononeuropathy is reduced by the analgesic drugs morphine and gabapentin”
Roeska,K.; Doods,H.; Arndt,K.; Treede,R.D.; Ceci,A

Neuroscieince letters, 2009 Oct 30;464(3):160-4. Epub 2009 Aug 18:
“Effect of high trait anxiety on mechanical hypersensitivity in male rats”
Roeska K., Ceci A.; Treede RD.; Doods H.

Submitted at Neuroscience letters:
“Role of oxytocin and vasopressin in pain-induced anxiety of rats with sciatic nerve lesion”
Roeska K.; Bernloehr C; Treede RD.; Doods H.

Posterbeiträge

Second International Congress on Neuropathic Pain (June 07):
"Anxiety like behaviour is observed in two rat models of mononeuropathy"
K. Roeska, A. Kremer, H. Doods, B. Hu, A. Ceci

International Biology Meeting, Boehringer Ingelheim (Vienna, October 07):
„Interaction between neuropathic pain and anxiety like behaviour in rats"
Roeska K.; Kremer A.; Ceci A.; Doods H.

German Pain Congress, Berlin (October 07):
„Impact of analgesic drugs on anxiety-like behaviour in rats"
Roeska K.; Ceci A.; Treede R.D.; Doods H.

12th World Congress on Pain, Glasgow (August 08):
"Vasopressin and oxytocin are upregulated in the amygdala in rats with pain induced anxiety-like behaviour"
Roeska K.; Bernloehr C.; Ceci A.; Doods H.

German Pain Congress, Berlin (October 08)
Pharmacologically-induced anxiety triggers pain hypersensitivity in rats
Roeska K.; Treede R.D.; Ceci A.; Doods H.

Vorträge/Seminare

EFIC - European Pain School, Siena (06/2007):
Presentation : "*Can we measure pain induced anxiety?*"

Post-doc Forum - Boehringer Ingelheim *(09/2007)*
Presentation*: "Investigations concerning the relationship between chronic pain and anxiety in rodents"*

Baden Baden , 17. November 2009

AFFIRMATION

für das Gesuch um Zulassung zur Promotion in den Fachbereichen 17 – 22 der Johannes Gutenberg-Universität Mainz

Name: Kerstin Röska _____

Hiermit versichere ich gemäß § 11, Abs. 3d der Promotionsordnung vom 22.12.2003:

- Ich habe die heute als Dissertation vorgelegte Arbeit selbst angefertigt und alle benutzten Hilfsmittel (Literatur, Apparaturen, Material) in der Arbeit angegeben.
- Ich habe oder hatte die jetzt als Dissertation vorgelegte Arbeit nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht.
- Ich hatte die heute als Dissertation vorgelegte Arbeit als Prüfungsarbeit für folgende Prüfung eingereicht:

Bezeichnung der Prüfung: Mündliche Prüfung _____

Prüfungsstelle: Biologische Fakultät der Universität Mainz _____

- Ich hatte weder die jetzt als Dissertation vorgelegte Arbeit noch Teile einer Abhandlung bei einer anderen Fakultät bzw. einem anderen Fachbereich als Dissertation eingereicht.
- Ich hatte die folgende Abhandlung mit nachstehendem Ergebnis eingereicht:

Titel der Abhandlung:

Relationship between pain and anxiety in rats _____

Fakultät bzw. Fachbereich und Hochschule:

Biologische Fakultät _____

Mainz, den _____

_____

(Unterschrift)