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„Role of excitatory lateral parabrachial nucleus to central amygdala pathway in persistent anxiety in a rat neuropathic pain model“

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Abstract

Neuropathic pain is frequently associated with mood and anxiety disorders, affecting millions of people. The underlying cellular mechanisms are still not entirely clear, but previous studies have demonstrated the importance of synaptic potentiation of nociceptive pathways in the pathogenesis of neuropathic pain and related psychological disorders. As a part of the limbic system the highly plastic amygdala plays a key role in emotion and emotional memory. The central nucleus of the amygdala (CeA), specifically the laterocapsular division (CeC), integrates nociceptive input from the lateral parabrachial nucleus (LPB) and polymodal sensory input from the basolateral amygdala (BLA), attaching emotional significance to the experience of pain. For example, severe pain induced by peripheral nerve ligation strongly activates both inputs and causes persistent anxiety even after pain relief. Here, we tested a hypothesis that the excitatory LPB-CeC pathway, which showed long-term potentiation after neuropathic pain, is involved in the persistent anxiety. We chemogenetically inhibited/activated the pathway by using designer receptors exclusively activated by designer drugs (DREADDs) in a rat spinal nerve ligation release (SNLR) model. Male rats injected into the right LPB with AAV1-CamKIIa-hM4D(Gi)-mCherry underwent SNLR surgeries to induce states of anxiety. Clozapine N-oxide (CNO) was locally applied at LPB-CeC terminals expressing the inhibitory DREADD hM4D(Gi) in the right CeC and behavioral changes were examined in Open Field and Light/Dark-Transition tests. We found that hM4D(Gi)-mediated inhibition of LPB-CeC terminals was not sufficient to relieve anxiety in the SNLR model. We further tested the effect of chemogenetic activation of the LPB-CeC pathway by AAV-mediated expression of excitatory DREADD hM3D(Gq) and found this approach to be insufficient to induce anxious behavior. In addition, an immunohistochemical approach was used to identify target cell populations of incoming LPB fibers in the CeC and we found basket-like terminals of the LPB fibers on SOM+ and PKCδ+ cells.

Zusammenfassung

Neuropathischer Schmerz und damit verbundene Stimmungs- und Angststörungen betreffen Millionen von Menschen. Die zugrundeliegenden Zellmechanismen konnten bisher nicht völlig geklärt werden, aber synaptische Potenzierung nozizeptiver Nervenbahnen scheint eine wichtige Rolle in der Pathogenese von neuropathischen Schmerzen und den damit verbundenen psychologischen Störungen zu spielen. Als Teil des limbischen Systems spielt die hochplastische Amygdala eine Schlüsselrolle in Emotion und emotionalem Gedächtnis. Der zentrale Kern der Amygdala (CeA), speziell der laterokapsulare Teil (CeC), integriert nozizeptiven Input aus dem lateralen parabrachialen Kern (LPB) und polymodalen sensorischen Input aus dem basolateralen Kern der Amygdala (BLA) und versieht die Schmerzerfahrung mit einer emotionalen Komponente. Beide Inputs werden beispielsweise bei peripherer Nervenligation, die starke Schmerzen auslöst, aktiviert und verursachen persistente Angstzustände, die auch nach Abklingen der Schmerzen erhalten bleiben. In dieser Arbeit haben wir die Hypothese getestet, dass die exzitatorische LPB-CeC Nervenbahn, die eine Langzeitpotenzierung nach neuropathischem Schmerz aufweist, in diesen persistenten Angstzuständen involviert ist. Wir haben diese Nervenbahn in Ratten aus dem *spinal nerve ligation release* (SNLR) Modell für neuropathische Schmerzen mit Hilfe von *designer receptors exclusively activated by designer drugs* (DREADDs) chemogenetisch inhibiert bzw. aktiviert. Männliche Ratten, die mit AAV1-CamKIIa-hM4D(Gi)-mCherry in den rechten LPB injiziert wurden, wurden den SNLR Operationen unterzogen, um Angstzustände auszulösen. Clozapin N-Oxid (CNO) wurde lokal an den LPB-CeC Nervenendigungen, welche die inhibitorische DREADD-Variante exprimierten, im rechten CeC appliziert und in weiterer Folge wurden Verhaltensänderungen in *Open Field* und *Light/Dark-Transition* Tests untersucht. Wir haben herausgefunden, dass hM4D(Gi)-vermittelte Inhibition von LPB-CeC Nervenendigungen nicht ausreicht, um Angstzustände im SNLR Modell zu lösen. Weiters haben wir den Effekt von chemogenetischer Aktivierung der LPB-CeC Nervenbahn durch AAV-vermittelter Expression der exzitatorischen DREADD-Variante hM3D(Gq) getestet und herausgefunden, dass dieser Ansatz nicht ausreicht, um ängstliches Verhalten auszulösen. Zusätzlich wurde ein immunohistochemischer Ansatz verwendet, um Zielzellpopulationen von eingehenden LPB-Fasern im CeC zu identifizieren. Wir haben festgestellt, dass LPB-Fasern korbähnliche Strukturen an SOM+ und PKCδ+ Zellen bilden.

Contents

Introduction

Pain

Basics of pain, emotional component and complications

Pain is a multidimensional sensation usually caused by aversive and noxious stimuli. It is a subjectively unpleasant sensory experience with a strong negative emotional or affective component. Pain is thought to have evolved as an adaptive trait, protecting organisms from damage by withdrawal from and avoidance of potentially harmful situations (Bonavita and De Simone 2011). In most cases pain sensation is a transient experience that is triggered by a noxious stimulus and resolved once the stimulus is removed, but pain can also be experienced without a detectable external physical cause (Baron et al. 2010; Costigan et al. 2009). As a symptom in various physiological and psychological medical conditions and injuries it can also persist over longer periods of time, causing anxiety and discomfort and reducing quality of life of patients, in some cases permanently (McWilliams et al. 2003). Persistent or chronic pain can also lead to morphological changes of the underlying neural structures and pathways in the peripheral and/or central nervous system, which emphasize the negative emotional valence and establish states of anxiety and fear (Apkarian et al. 2009; Ikeda et al. 2007; McWilliams et al. 2003, Neugebauer et al. 2003, 2004). Since many such conditions are very resistant to therapy and affect millions of people (Apkarian et al. 2009, Zhuo 2007), pain is a complex and active topic of research.

Neuropathic pain and allodynia

Neuropathic pain is a form of pain that follows damage or injury to parts of the somatosensory nervous system, but it can also be mediated by diseases affecting the nervous system. Damage to the nervous system induces various changes on the cellular and molecular level that culminate in altered, pathological activity of peripheral and central neurons (Baron et al. 2010; Colloca et al. 2017). Such changes encompass altered gene expression and expression of ion channels, changes in neurotransmitters and neurotransmitter receptors, altered activity of ion channels and changes in cell morphology (Hains et al. 2004; Lai et al. 2003; Ultenius et al. 2006).

In general neurons become hyperexcitable and develop stronger outputs, i.e. they become sensitized (Baron et al. 2010).

As a consequence of pathological activity in the periphery central neurons become sensitized to afferent inputs. In this way previously innocuous and non-painful tactile or thermal stimuli can evoke pain responses in the brain. This pathological state is referred to as allodynia and is often associated with states of persistent neuropathic pain (Baron et al. 2010).

Neural circuitry of pain

The interplay of ascending and descending signal transmission provides ample opportunity for pain modulation on multiple levels. These include primary afferent fibers, the spinal cord, the brain stem as well as cortical regions. Pain is transmitted to the brain via multiple pathways, two of them being the spino-thalamo-cortical pathway, which deals with sensory-discriminative aspects of pain, and the spino-parabrachio-amygdaloid pathway, which has been implicated with emotional aspects of pain. They in turn modulate the subjective experience and descending signals (Bernard et al. 1996).

<u>The spino-thalamo-cortical and spino-parabrachio-amygdaloid pathways</u>

Noxious stimuli are transformed into electrical signals in specialized receptors called nociceptors. They are located in the free nerve endings of primary afferent fibers (lightly myelinated Aδ and unmyelinated C fibers for noxious polymodal stimuli, highly myelinated Aβ fibers for non-noxious light tactile stimuli) originating in the dorsal root ganglions. They project to laminae I, II, IV and V of the dorsal horn of the spinal cord, where they synapse with secondary afferent neurons. In the spinal cord a complex interplay between neurons of ascending and descending pathways and interneurons modulates and determines the activity of secondary afferent neurons (Baron et al. 2010; Bernard et al. 1996; Bourne et al. 2014; Dubin and Patapoutian 2010).

Afferent fibers of the second order decussate within one or two spinal nerve segments and ascend the contralateral spino-thalamic tract through the brainstem and synapse onto third

order neurons in the thalamus. From here, third order neurons project primarily to the primary somatosensory cortex (spino-thalamo-cortical pathway; Figure 1), where the information is processed and further transmitted to the lateral and basolateral amygdala (LA and BLA, respectively) among other targets (Bourne et al. 2014; Dubin and Patapoutian 2010).

Fibers of laminae I and II neurons, after crossing the midline and ascending the spinal cord, also project directly to the pontine lateral parabrachial nucleus (LPB) and synapse onto excitatory neurons. These in turn project to the central nucleus of the amygdala (CeA; spino-parabrachio-amygdaloid pathway; Figure 1) and to the hypothalamus, with the majority projecting to the laterocapsular division of the central amygdala (CeC; Bernard et al. 1993; Dong et al. 2010; Neugebauer et al. 2003; Sarhan et al. 2005). Since the CeC receives direct nociceptive input via the LPB it has been termed "the nociceptive amygdala" (Neugebauer et al. 2009).

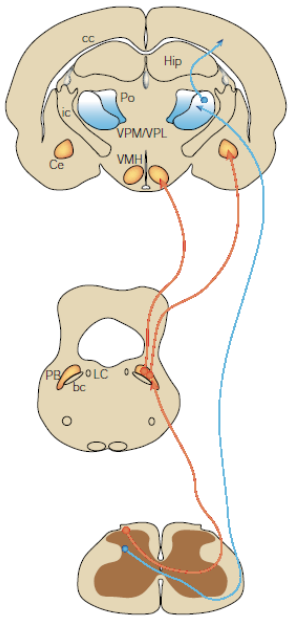

Figure 1: *Spino-thalamo-cortical (blue) and spino-parabrachio-amygdaloid (red) pathways (adapted from Hunt and Mantyh 2001).*

The lateral parabrachial nucleus and the central amygdala

The pontine parabrachial area (PB) is comprised of several nuclei and subnuclei, each associated with a distinct set of afferents and efferents. In general, the PB is a major integration and relay center for nociceptive information, cardiovascular, respiratory and other autonomic functions. The lateral parabrachial nucleus is primarily concerned with relaying nociceptive information from ascending second order neurons of the spinal dorsal horn to the central amygdala (Bernard et al. 1996). LPB output to the CeA is glutamatergic, and the vast majority of projecting fibers is CGRP (calcitonin gene-related peptide) positive (Dong et al. 2010; Yu et al. 2009).

The amygdaloid complex is similarly comprised of multiple distinct nuclei including the central amygdala, the lateral amygdala and the basolateral amygdala. In the central amygdala nociceptive input from the periphery via the LPB and polymodal sensory input from thalamic and cortical regions via the LA-BLA converge and are integrated (Figure 2, left) and emotional significance is attached to the sensory-discriminative aspect of pain (Neugebauer et al. 2004). The CeA serves as the major output region of the amygdaloid complex, regulating physiological and behavioral responses (Figure 2, right).

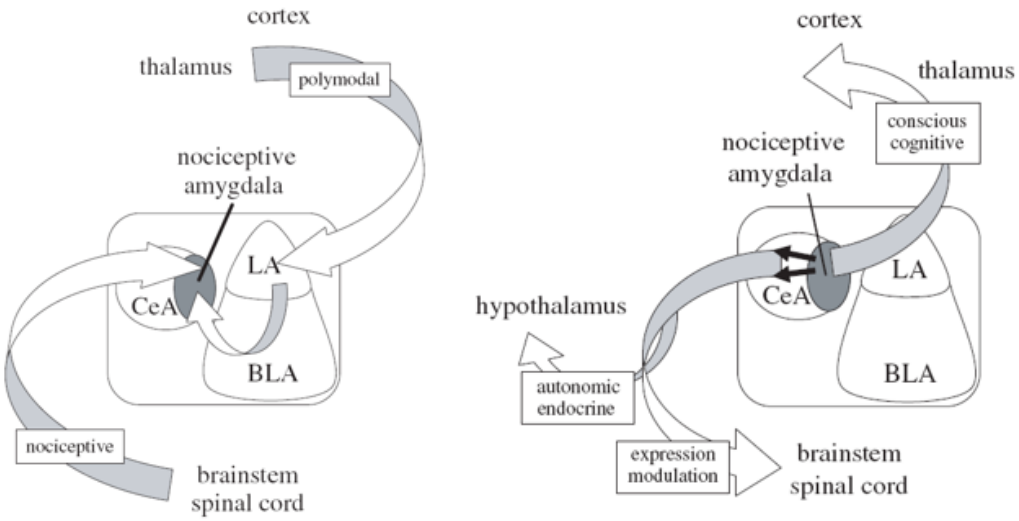

Figure 2: *Major inputs (left) and outputs (right) to the amygdala (adapted from Neugebauer et al. 2004).*

In detail, the CeA is comprised of a lateral (CeL) and a medial (CeM) part, whereby the CeL can be subdivided into a laterocapsular (CeC), an intermediate and a lateral division. The CeA is striatum-like and neurons in the CeA are mainly GABAergic, in contrast to the cortex-like architecture of the LA and BLA containing mostly glutamatergic neurons (Ehrlich et al. 2009), and subpopulations can be identified by the expression of cell markers including Somatostatin (SOM) and Protein kinase C delta (PKCδ; Han et al. 2015; Pomrenze et al. 2015). In the CeA the dense glutamatergic projections from LPB and BLA synapse onto local GABAergic interneurons that form a complex network of intranuclear feedback- and feedforward-loops spanning all subdivisions of CeL and CeM, but the projections are thought to generally follow a latero-medial direction. GABAergic CeM projection neurons target regions that produce autonomic, endocrinological and behavioral responses, including the brain stem, thalamus, hypothalamus and cortex (Ehrlich et al. 2009).

Neuropathic pain-induced psychological disorders

Anxiety and depression as a consequence of persistent (neuropathic) pain

Previous studies have consistently reported a significant correlation of patients diagnosed with chronic or persistent pain to mood disorders like depression (Dersh et al. 2002; Huyser and Parker 1999; Millan 1999; Wilson et al. 2001). Another study conducted by Mcwilliams et al. in 2003 reported an even stronger association between chronic pain and several anxiety disorders, including a variety of unspecified anxiety disorders, generalized anxiety disorder, panic disorders and various phobias.

Neuropathic pain is related to nerve injury and frequently associated with psychological mood and anxiety disorders, suggesting the existence of common pathogenic mechanisms. A recent review of the emotional aspects of neuropathic pain by Torta et al. (2017) discusses the need for further research into the association between chronic pain states with a neuropathic component and affective disturbances including memory, cognitive and social impairments, sleep disturbances, depression, and fear and anxiety disorders. In patients that suffer from

persistent pain caused by neuropathic injury to the nervous system, or disease thereof, the correlation of pain to emotional complications is statistically more significant than in patients that suffer from non-neuropathic chronic pain; neuropathic pain patients have a worse quality of life and experience greater psychological distress than the general population and non-neuropathic chronic pain patients. Furthermore the available clinical diagnostic tools lack precision and drug and behavioral therapies are in many cases inadequate, highlighting the need for further research (Torta et al. 2017).

Pathogenic mechanisms of neuropathic pain-induced anxiety

The underlying cellular mechanisms of anxiety caused by neuropathic pain are still not entirely clear. Spinal and cerebral neuro-inflammation following nerve injury have been suggested as potential mechanisms, classifying neuropathic pain-induced anxiety as a symptom of a neuro-immune disorder (Martini and Willison 2016). Further studies on a cellular and molecular level have demonstrated the importance of synaptic potentiation of nociceptive pathways in the pathogenesis of persistent neuropathic pain and related psychological disorders. As described above, the amygdala, specifically the laterocapsular division of the CeA, serves as a hub for integrating nociceptive and polymodal sensory input and attaches emotional significance to the experience of pain. The amygdala has also been shown to exhibit a high degree of plasticity in various behavioral, electrophysiological and pharmacological experiments (Adedoyin et al. 2010; Cheng et al. 2011; Ikeda et al. 2007; Neugebauer et al. 2003, 2004). For these reasons, a lot of recent research has gone into identifying the specific roles of the amygdala and the associated neural pathways in neuropathic pain.

Synaptic long-term potentiation (LTP) in the amygdala

Synaptic long term-potentiation has been extensively studied in the past and describes a phenomenon first discovered in the rabbit hippocampus (Bliss and Lomo 1973). It is thought to be one of the most important cellular mechanisms underlying learning and memory (Bliss and Collingridge 1993; Martin et al. 2000). In general LTP describes a long-lasting strengthening of

synaptic transmission between two neurons following recent activation patterns (Cooke and Bliss 2006), allowing for good experimental access via targeted electrophysiological or pharmacological stimulation. LTP has been observed in multiple brain regions, including the cerebellum, the cortex and limbic structures such as the amygdala (Cooke and Bliss 2006; Ikeda et al. 2007; Neugebauer et al. 2003, 2004; Zhuo 2007). Different neural structures exhibit different types of LTP, classified by the dependence on different receptors (N-methyl-D-aspartate [NMDA] receptor-dependent or -independent) and receptor subunits, or by the requirement for simultaneous or non-simultaneous pre- and postsynaptic activity (Urban and Barrionuevo 1996; Wigström and Gustafsson 1986).

In the amygdala, LTP has been reported to occur in various forms in multiple nuclei and subnuclei. In the LA LTP has been extensively studied and described in the context of fear-conditioning (Sigurdsson et al. 2007). Recent studies on animal models of chronic pain found LTP to also occur in the CeA, where glutamatergic LPB neurons conveying nociceptive information synapse onto GABAergic CeC neurons. NMDA receptor-dependent robust potentiation of LPB-CeC synapses within a few hours of the acute onset of pain has been reported in arthritic and visceral pain models of the rat (Neugebauer et al. 2003, 2004). Further studies have reported NMDA receptor-independent synaptic plasticity of LPB-CeC synapses in a spinal nerve L5 ligation (SNL) neuropathic pain model of the rat (Ikeda et al. 2006). LTP has also been shown to occur at BLA-CeC synapses in arthritic pain (Neugebauer et al. 2003) and SNL neuropathic pain models of the rat (Dong et al. unpublished).

Together these findings demonstrate the amygdala's high degree of plasticity and suggest a potential role of the amygdala, specifically the LPB-CeC pathway, in the establishment of neuropathic pain-induced anxiety.

Unilateral spinal nerve L5 ligation release (SNLR) model and designer receptors exclusively activated by designer drugs (DREADDs)

The introduction of animal models for neuropathic pain has greatly advanced knowledge about the underlying mechanisms and provides a useful opportunity for the search of new drugs. One widely used model is the spinal nerve ligation (SNL) model introduced by Kim and Chung in 1992. Here, the spinal nerve L5 is unilaterally ligated with a sterile thread, which results in long-lasting neuropathic pain behaviors, including signs of ongoing pain and thermal and tactile allodynia (Bennett et al. 2003), and also induces potentiation of LPB-CeC and BLA-CeC pathways (Ikeda et al. 2007; Dong et al. unpublished). Tactile allodynia can be quantitatively evaluated to estimate the success of the ligation procedure (Ikeda et al. 2007; Ji et al. 2017). In the present spinal nerve ligation release (SNLR) model the thread is removed after one day. Previous findings show that, while animals recover from pain and touch-induced allodynia in approximately 7 days, the synaptic potentiation of the LPB-CeC pathway persists (Dong et al. unpublished). In contrast, potentiation of the BLA-CeC pathway is reversed. Rats that underwent SNLR procedure showed no signs of tactile allodynia but remained anxious after 7 days (Dong et al. unpublished). Furthermore, in an experiment comparing SNLR rats with unilateral chemical LPB lesion to untreated SNLR rats, animals with LPB lesion were significantly less anxious. Together these findings suggest the potentiated LPB-CeC pathway as a likely candidate for inducing anxiety (Dong et al. unpublished).

Designer receptors exclusively activated by designer drugs (DREADDs) have been developed over the last few decades and are currently the most widely used chemogenetic tool to discern how cellular activity specifies animal and cell behavior, cell-cell-interactions and network consequences (Armbruster and Roth 2005; Armbruster et al. 2007). Essentially DREADDs are G protein-coupled receptors (GPCRs) engineered to be only activated by specifically designed ligands. DREADDs can be expressed and activated in a targeted manner to affect neuronal activity of cell populations by the introduction of DREADD-expressing genes by transgenics or delivery of an appropriate viral vector. Multiple DREADD variants have been developed to match the needs of different experimental designs, with hM3D(Gq) and hM4D(Gi) being the

most commonly used variants for facilitating and inhibiting neuronal activities, respectively (Alexander et al. 2009; Armbruster et al. 2007; Urban and Roth 2015). Both variants are activated by clozapine-N-oxide (CNO), a pharmacologically inert metabolite of the apsychotic drug clozapine (Armbruster et al. 2007; Roth et al. 1994). Ligands are usually injected intraperitoneal, but recent studies demonstrated that local micro-infusion to projection terminals is sufficient to activate DREADDs and modulate firing behavior of affected cells in a projection-specific manner (Stachniak et al. 2014; Mahler et al. 2014) as is shown in Figure 3.

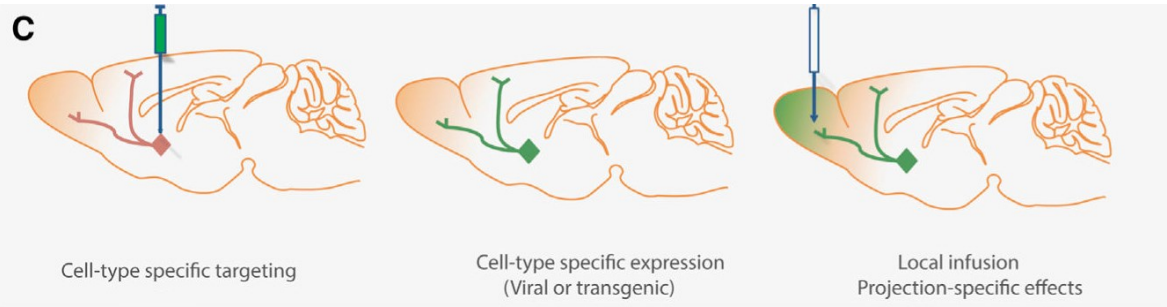

Figure 3: *Projection-specific modulation of neuronal activity using DREADDs (adapted from Roth 2016).*

Together these tools provide an elegant opportunity to chemogenetically manipulate synaptic transmission at the potentiated LPB-CeC synapses and study the effect on animal behavior.

Hypothesis

The present study aims to elucidate the role of the lateral parabrachial nucleus to central amygdala pathway in persistent anxiety in a rat neuropathic pain model.

Based on the following line of reasoning we hypothesize that chemogenetic inhibition of LPB-CeC synapses is sufficient to relieve anxiety in a rat SNLR neuropathic pain model:

(1) the amygdaloid complex plays a key role in emotional responses including fear and anxiety, (2) anxiety is significantly associated with neuropathic pain, (3) the central nucleus of the amygdala receives direct nociceptive input via the LPB and polymodal sensory input via the BLA, (4) the LPB-CeC pathway undergoes long-lasting long-term potentiation in chronic and

neuropathic pain models and (5) unilateral lesion of LPB in a rat SNLR model is sufficient to rescue animals from anxiety.

Materials and Methods

Animals

Male Sprague Dawley rats (obtained from Himberg, Austria) were used in all experiments. Animals were delivered at 8 weeks of age (weighing 250-350 g) and housed individually in stainless steel cages in a temperature- and light-controlled cabinet under a reversed 12 hour day/night cycle. Rats were allowed to get acclimated to the housing conditions for one week. After the acclimation period animals were handled by the experimenter for 10 minutes daily for 5 consecutive days and for 10 minutes every second day thereafter. Water and food was provided *ad libitum* and cages were cleaned in intervals of 3 weeks. All experimental procedures were conducted in accordance to European Union regulations and approved by the local Animal Care and Use Committee.

Timeline and procedure

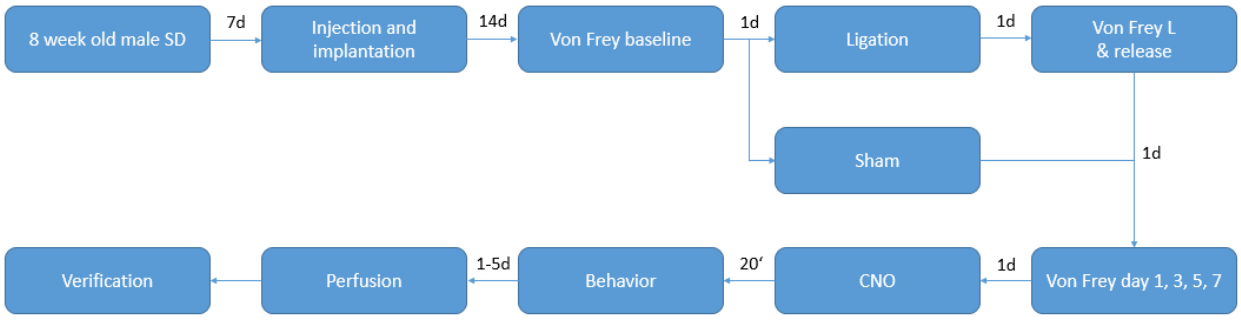

Figure 4: *Experimental design and timeline.*

For chemogenetic manipulation of terminals of LPB-CeC projecting neurons 9 week old male Sprague Dawley rats were injected with adeno-associated viral vectors carrying genes for DREADD expression and implanted with guide cannulas for intracranial CNO injection. Animals were given two weeks to recover afterwards. A Von Frey baseline value was obtained on the day before ligation or sham surgery. Left spinal nerve L5 was ligated to induce neuropathic pain and accompanying activity-dependent long-term potentiation (LTP) of the LPB-CeC pathway.

The ligation was released on the day after the ligation to let animals recover from the pain while maintaining LTP in the LPB-CeC pathway. In the control or sham-operated group surgery was performed but L5 was not ligated. On day 1, 3, 5 and 7 after ligation release or sham surgery Von Frey tests were performed to track the animals' recovery by calculating their 50% hindpaw withdrawal thresholds. On day 8 clozapine N-oxide (CNO) was intracranially injected through the guide cannula and after 20 minutes Light/Dark Transition and Open Field tests were conducted to examine anxiety-like behavior. In the following days the animals were perfused, the brains extracted and subsequently sectioned and examined via microscopy to verify the injection and implantation sites (Figure 4).

Stereotaxic surgery

Rats were shaved and anaesthetized in an induction chamber with 3% isoflurane in 100% O_2 for 5 minutes and then transferred and fixed to a stereotaxic setup (David Kopf Instruments) where anesthesia was maintained with 1.5% isoflurane in 100% O_2 for all subsequent surgical procedures. Core body temperature was maintained at 37° Celsius by use of a heating mat. Animals were injected s.c. with 0.1 mg/kg body weight Buprenorphine (Bupaq®) in 0.9% NaCl. Povidone-iodine solution (Betaisodona®) and 2% lidocaine hydrochloride gel (Xylocaine®) were applied to the scalp. Eye ointment (OLEOVital®) was applied. 30 minutes after Buprenorphine injection a longitudinal incision of approximately 20 mm length was made and the skull was exposed and cleaned with 5% H_2O_2 in H_2O. Bleeding was arrested either with 10% H_2O_2 in H_2O or by use of a thermal cautery unit. During surgery rats were injected s.c. with 1 ml 5% glucose solution hourly. One hour prior to the projected end of the surgery animals were injected s.c. with 1 mg/kg body weight Meloxicam (Metacam®). At the end of surgery the wound was stitched and closed with tissue adhesive and 2% lidocaine hydrochloride gel (Xylocaine®) and antibiotic ointment (Gentamicin) were applied to the wounded area. Anaesthesia was discontinued. Animals were allowed to recover from surgery for two weeks.

AAV injection into right LPB

For injection in the right lateral parabrachial nucleus the following stereotaxic coordinates were used: 6.15 mm caudal to bregma, 2.10 mm lateral to bregma, 7.35 mm deep from Dura mater. A craniotomy was performed with a 0.9 mm drill and a Hamilton microsyringe was lowered into the brain at an angle of 20°. Animals were injected with 1 µl of either 4.7x10^12 vg/µl AAV5-CamKIIa-hM3D(Gq)-mCherry, 2.8x10^9 vg/µl AAV1-CamKIIa-hM4D(Gi)-mCherry or 7.5x10^9 vg/µl AAV1-CamKIIa-mCherry (provided by Assoc. Prof. Kenta Kobayashi of the National Institute of Physiological Sciences, Japan) at a rate of 100 nl/min. The microsyringe was then kept at the injection site for 10 minutes to allow the virus to diffuse into the surrounding tissue before being retracted.

Cannula implantation into right CeA

Dummy cannulas were inserted into the guide cannulas (Plastics One) prior to being implanted into the animals. For implantation into the right central amygdala the following stereotaxic coordinates were used: 2.45 mm caudal to bregma, 4.50 mm lateral to bregma, 6.05 mm deep from Dura mater. 1 mm lateral to the sagittal suture two stainless steel screws were screwed into the skull and covered with dental cement. At the implantation site a craniotomy was performed with a 0.7 mm drill. The guide cannula was then lowered into the brain and its base was covered and connected to the screws with dental cement.

SNLR neuropathic pain model

Spinal nerve L5 ligation

For pain management animals were injected s.c. with Metamizole (100 mg/kg body weight; Novalgine®) 20 minutes prior to surgery. Immediately preceding surgery rats were injected i.p. with Ketamine/Xylazine solution (Ketamine 100 mg/kg body weight, Xylazine 10 mg/kg body weight) to maintain anaesthesia for approximately 60 minutes. Eye ointment (OLEOVital®) was applied. The animal's lower back was shaved and disinfected with 70% ethyl alcohol in H_2O and

povidone-iodine solution (Betaisodona®). 2% lidocaine hydrochloride gel (Xylocaine®) was applied to the shaved area. Surgeries were performed in a sterile environment and on a heating mat to maintain core body temperature at 37° Celsius.

A longitudinal incision of approximately 30 mm length was made on the left side close to the midline in the lumbar area. The underlying musculature was dissected to reveal the left L6 transverse process. The bone was carefully cut and removed to reveal the left L5 spinal nerve. Special care was taken to not damage any nerves. The left L5 nerve was isolated and ligated with a sterile silk thread and the wound was closed by stitching the muscles and skin. Again 2% lidocaine hydrochloride gel (Xylocaine®) was applied and animals were then placed into their home cages to recover.

Ligation release

The day after spinal nerve L5 ligation surgery animals were prepared in the same fashion as described above and the release surgery was conducted under the same conditions.

The wound from the previous day was reopened by removing the stitches and carefully dissecting skin and musculature without further damage. Again the left spinal nerve L5 was located and the silk loop removed. Muscle and skin were stitched close and tissue adhesive was applied. Before placing animals back into their home cages 2% lidocaine hydrochloride gel (Xylocaine®) was applied and animals were then left to recover.

Sham surgery

Animals of the sham-operated control group underwent the same surgical procedures as animals of the ligation group with the differences being the following: After cutting the left L6 transverse process the left L5 spinal nerve was located and isolated as described above, but not ligated. The wound was closed in the same fashion and post-surgical treatment was conducted the same way. On the next day, no sham-release surgery was performed to not expose animals to unnecessary distress.

Behavior

All behavioral experiments were conducted in the same light- and temperature-controlled room between 9 a.m. and 4 p.m.

Animal handling

To acclimate the subjects to the experimenter all animals were regularly and individually handled by the same experimenter. Prior to handling the home cages were removed from the housing cabinet and exposed to the environmental conditions in the experimental room for 10 minutes. Thereafter animals were removed from their home cages and placed on the experimenter's arms for 10 minutes. Additionally rats that were subjected to the Von Frey Filament test were placed in a wire mesh cage on an elevated position for 10 minutes after the regular handling procedure.

Von Frey Filament test

To estimate the effect of spinal nerve L5 ligation and accompanying changes in spinal reflexes expressed as changes in hindpaw withdrawal thresholds, rats were subjected to multiple Von Frey filament tests. Animals were placed in a wire mesh cage (25x25 cm) on an elevated position and von Frey monofilaments of varying force ranging from 0.4 to 15 g were applied to the planar surface of the hindpaws for 1-6 seconds each. Abrupt paw withdrawal or licking of the affected hindpaw were counted as positive responses. Starting with 2 g the force was increased after every negative response and decreased after every positive response until five responses were obtained counting from the first positive response, as was previously described in the up-and-down method by Chaplan et al. (1994). Responses were obtained for both the hindpaw ipsilateral as well as contralateral to the side of spinal nerve L5 ligation. The 50% hindpaw withdrawal thresholds were then calculated using the formula of Chaplan et al. (1994).

Rats were subjected to this test two days before spinal nerve L5 ligation or sham surgery to obtain baseline hindpaw withdrawal thresholds, on the day after ligation or sham surgery, on the day after ligation release surgery and every second day thereafter for 7 days.

Intracranial CNO injection

To induce DREADD activation before conducting Light/Dark Transition and Open Field tests rats were intracranially injected with clozapine N-oxide (CNO; Hello Bio) in artificial cerebrospinal fluid (ACSF) at a rate of 300 nl/min. For this purpose animals were manually restrained by the experimenter, the dummy cannula was removed and the internal cannula (10 mm length), connected to a Hamilton microsyringe loaded with 1 µl of 26 µM CNO in ACSF, was inserted into the guide cannula (9 mm length). 5 minutes after injecting CNO the internal cannula was removed and animals were placed back into their home cages for 20 minutes.

Light/Dark Transition test

The commonly used Light/Dark Transition test was used to examine anxiety-like behavior. The apparatus (61.5x31.5x30cm) was placed in an isolated chamber in the experimental room with separate and dimmer lighting. The apparatus was divided in half, where one half was open and illuminated by ambient light and one half was closed and dark. After being handled by the experimenter for 10 minutes, animals were placed into the light compartment facing the dark compartment. The subjects were recorded and tracked for 30 minutes by an automated software (Ethovision XT; Noldus) and the time spent in both light and dark compartments was measured and analyzed.

This test was performed on the day after the last von Frey Filament test.

Open Field test

The Open Field test was used to assess changes in general locomotion and anxiety-related behavior. The apparatus (96x96x61cm) was placed in an isolated chamber in the experimental room with separate and dimmer lighting. The apparatus itself was empty but digitally divided into borders (96x24cm) and a center (48x48cm). Animals were placed into the center and allowed to roam freely for 30 minutes. The subjects were recorded and tracked for 30 minutes by an automated software (Ethovision XT; Noldus) and the time spent moving and the time spent in the center were measured and analyzed.

This test was performed in succession to the Light/Dark Transition test.

Verification of injection and implantation sites

Perfusion

1-5 days after behavioral testing animals were transcardially perfused with 4% paraformaldehyde. 20 minutes prior to perfusion animals were injected i.p. with a Ketamine/Xylazine/Acepromazine cocktail (Ketamine 100 mg/kg body weight, Xylazine 20 mg/kg body weight, Acepromazine 2 mg/kg body weight). Anaesthetized animals were then fixed to a polystyrene board and the rib cage was opened. A small incision in the left ventricle was made and the perfusion needle, connected to a pump set to a flow of 18 ml/min, was inserted into the aorta. Animals were perfused with PBS for 5 minutes to wash out the blood, then with 4% paraformaldehyde in phosphate buffer (PB; 0.2 M, pH 7.35) for 15 minutes. Afterwards the needle was removed and the brain extracted and stored in 30% sucrose at 4° Celsius on a shaker overnight.

Brain sectioning

After infiltrating brain tissue with 30% sucrose to prevent frost damage the brain was cut into blocks with a razor and subsequently mounted on a SM2000R sliding microtome (Leica) with dry

ice. A series of 40 µm thick coronal slices was obtained from both the lateral parabrachial nucleus and the central amygdala and then stored in PBS until used for microscopy.

Microscopy

To verify the correct positioning of the injection and implantation and the expression of DREADD::mCherry fusion protein the coronal brain slices were mounted on glass slides and inspected in a BZ-9000 fluorescence microscope (Keyence) using a 4x magnification in both bright field and red fluorescent channels. Images of both channels were obtained and overlaid.

If red fluorescence of DREADD::mCherry fusion protein was detected in terminals of LPB-CeC projecting neurons the injection was considered successful. Off-site fluorescence was disregarded due to the small area of effect of targeted intracranial CNO injection.

The cannula implantation site was considered correct if the tip of the cannula was approximately 1mm distant to the central amygdala, since the internal cannula penetrated 1 mm from the tip of the guide cannula to prevent possible clogging.

Histology

Immunofluorescent labeling of CeC neurons

Coronal brain slices with a thickness of 40 µm were incubated in 20% NGS (normal goat serum) in 0.25% PBS-T (Triton X-100 in PBS) for 1h at room temperature and then washed three times in PBS for 10 minutes each. The sections were then incubated for 40-48 hours at 4° Celsius on a shaker in either of the following primary antibody solutions: mouse anti-SOM (Somatostatin; GeneTex, GTX71935) 1:100 in 2% NGS in 0.25% PBS-T or mouse anti-PKCδ (Protein kinase C delta; BD Biosciences, 610397) 1:500 in 2% NGS in 0.25% PBS-T. Brain sections were then washed three times in PBS for 10 minutes each and incubated for 1 hour at room temperature in either one of the corresponding secondary antibody solutions: Alexa Fluor 647 conjugated goat anti-mouse IgG1 (Thermo-Fisher Scientific, A-21240) 1:500 in PBS-T or Alexa Fluor 488 conjugated goat anti-mouse IgG2b (Thermo-Fisher Scientific, A-21141) 1:500 in PBS-T.

Afterwards the sections were again washed three times in PBS for 10 minutes. Then the sections were incubated for 10 minutes at room temperature in DAPI (4',6-diamidino-2-phenylindole) in 0.25% PBS-T and washed again three times in PBS for 10 minutes each. Finally the sections were mounted in antifade solution on SuperFrost Plus glass slides (Thermo-Fisher Scientific) and coverslipped and stored at 4° Celsius until use for microscopy.

Confocal image acquisition

All images were acquired with a 700 upright Laser Scanning Confocal microscope (Zeiss) with a 20x or 40x magnification. The images were then imported into Fiji imaging software where color contrast and intensity were adjusted.

Data analysis and statistics

All obtained data was handled with Microsoft Excel and imported into GraphPad (v. 6.0) for statistical analysis. Figures were created in Microsoft Excel and modified using Adobe Photoshop CS6.

Results

Tactile allodynia is induced and recovered in the SNLR model

Tactile allodynia of three different groups (Sham, n=4; hM4D, n=7; mCherry, n=4) was measured and calculated as 50% hindpaw withdrawal thresholds as described by Chaplan et al. (1994) using von Frey monofilaments with varying force (0.4 – 15 g). Von Frey tests were conducted on the day before ligation or sham surgery (Baseline), on the day after ligation (Ligation) and on days 1, 3, 5 and 7 after release or sham surgery.

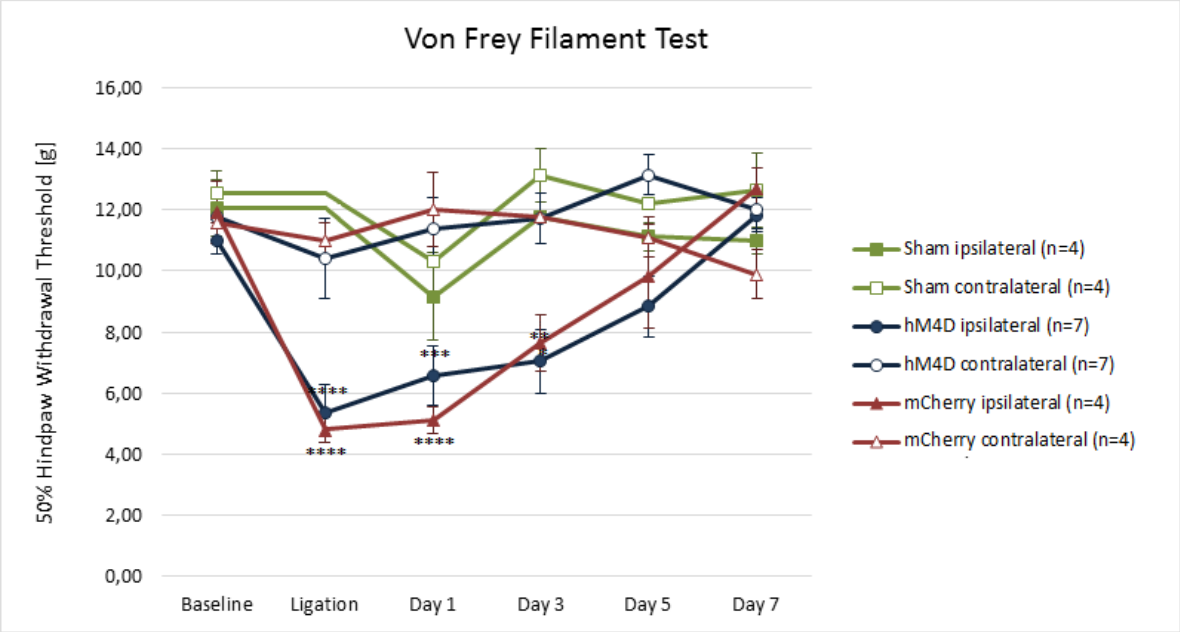

Figure 5: *Time-course of tactile allodynia estimated by 50% hindpaw withdrawal thresholds calculated from results of Von Frey filament tests. Open and filled symbols represent contralateral and ipsilateral sides to the ligation/sham surgery, respectively. Mean ± SEM, significance is indicated by *.*

Figure 5 shows that SNLR animals (groups hM4D and mCherry) had a significantly lower 50% paw withdrawal threshold on the ipsilateral side to the ligation on the day after ligation surgery (Ligation; $p<0.0001$, ANOVA with Tukey's post hoc test), on the day after release surgery (Day 1; $p<0.0001$ and $p<0.001$ for mCherry and hM4D, respectively, ANOVA with Tukey's post hoc test) and 3 days after release surgery (Day 3; $p<0.05$ and $p<0.01$ for mCherry and hM4D, respectively, ANOVA with Tukey's post hoc test) when compared to the baseline. On day 5 and 7 there was

no detectable decrease in hindpaw withdrawal thresholds in SNLR model animals. In contrast, sham-operated animals (group Sham) showed no change in mechanosensitivity on the ipsilateral side on the days after sham surgery (Day 1, 3, 5 and 7; $p>0.05$, ANOVA with Tukey's post hoc test). No differences were found on the contralateral side in any of the three groups ($p>0.05$, ANOVA with Tukey's post hoc test).

The data shows that unilateral ligation successfully induces unilateral tactile allodynia, while sham surgery does not result in decreased withdrawal thresholds. It further shows that animals recover from ligation-induced allodynia around 5 days after release surgery. The choice of viral vectors (AAV1-CamKIIa-hM4D(Gi)-mCherry in group hM4D and AAV1-CamKIIa-mCherry in groups Sham and mCherry) does not influence the severity and time-course of mechanical allodynia.

AAV injection and cannula implantation verification

To be able to chemogenetically modulate right-side LPB-CeC signaling animals were injected into the right LPB with either AAV5-CamKIIa-hM3D(Gq)-mCherry, AAV1-CamKIIa-hM4D(Gi)-mCherry or AAV1-CamKIIa-mCherry and a cannula was implanted into the right CeA. After behavioral tests animals were perfused and brain sections were obtained and mounted for microscopy to verify the injection and implantation sites.

Figure 6 and 7 show representative images of an injected and implanted animal. mCherry-expressing cell bodies were found to a large extent in the LPB and to a smaller extent in the medial parabrachial nucleus (MPB; Fig. 6, B and C). mCherry-expression was detected in the terminals of LPB-CeA projecting neurons, with the majority of labeled fibers in the CeC (Fig. 7, B and C). The guide cannula with a length of 9 mm was positioned apporixmately 1 mm dorsal to the CeA (Fig. 7) to allow the internal cannula with a length of 10 mm to inject CNO directly into the CeA without the risk of clogging. Animals that had no mCherry-expression in LPB-CeC terminals or where the cannula was implanted off-site were excluded from statistical analysis.

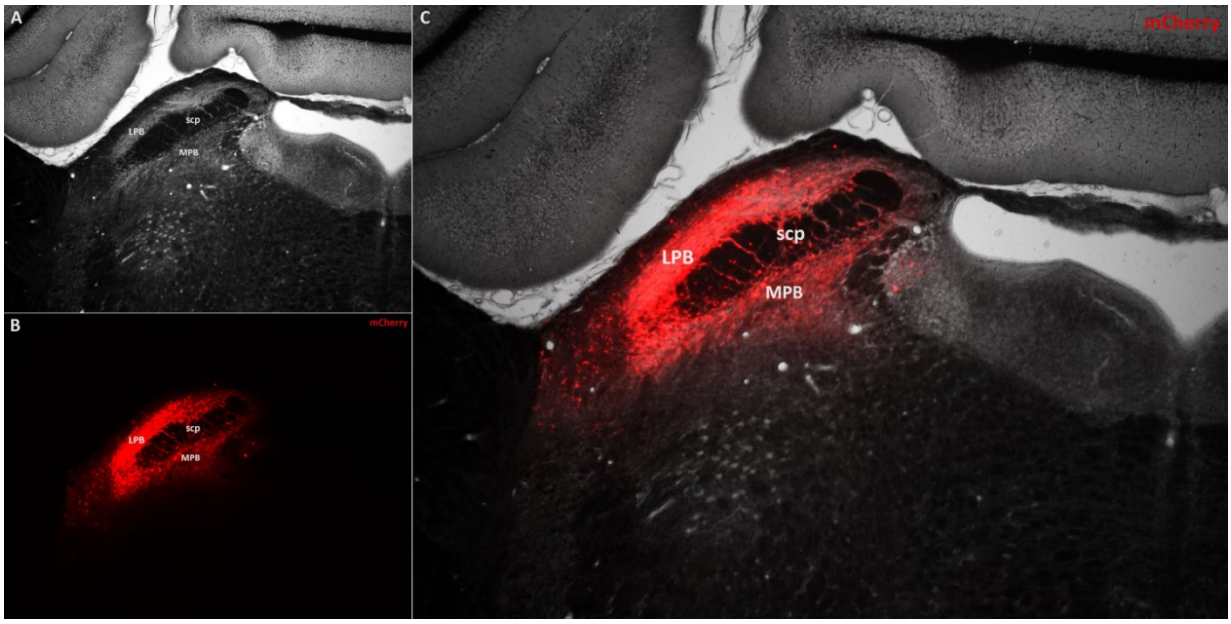

Figure 6: *Representative image of mCherry-labeled red fluorescent cells at the injection site in the LPB (4x magnification). (A) brightfield image, (B) red fluorescence, (C) overlay of A and B. LPB=lateral parabrachial nucleus, MPB=medial parabrachial nucleus, scp=superior cerebellar peduncle.*

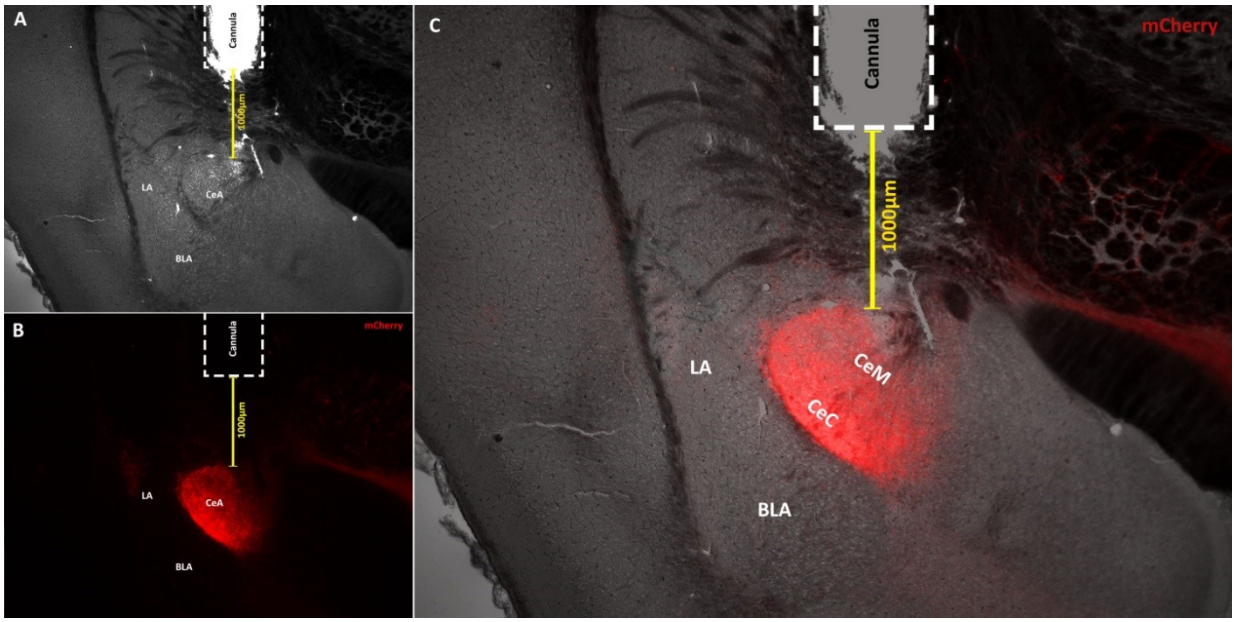

Figure 7: *Representative image of mCherry-labeled red fluorescent terminals in the CeA and positioning of the implanted cannula (4x magnification). (A) brightfield image, (B) red fluorescence, (C) overlay of A and B. CeC=laterocapsular division of central amygdala, CeM=medial division of central amygdala, LA=lateral amygdala, BLA=basolateral amygdala.*

hM4D(Gi)-mediated inhibition of LPB-CeC terminals is not sufficient to relieve anxiety in the SNLR model

To examine anxiety-related behavior in the SNLR model and the effect of inhibition of LPB-CeC terminals, the following experimental groups were tested and compared in Light/Dark Transition and Open Field tests: naive animals that were handled following the same protocol as the other groups but underwent no stereotaxic or ligation/sham-surgery (n=10), sham controls that underwent sham-surgery after stereotaxic delivery of AAV1-CamKIIa-mCherry into the right LPB (n=4), and ligated and released animals that were either injected with AAV1-CamKIIa-hM4D(Gi)-mCherry (hM4D, n=7) or AAV1-CamKIIa-mCherry (mCherry, n=4) into the right LPB. All animals except naive were intracranially injected into the right CeA with 1 µl of 26 µM CNO in ACSF 20 minutes prior to behavioral testing.

Light/Dark Transition test

On average naive animals spent 37.34% of the time in the light compartment. When compared to sham-operated animals (31.68%) no significant difference was found ($p>0.05$, ANOVA with Bonferroni correction). When comparing naive animals to ligated and released animals of the groups hM4D (14.50%) and mCherry (19.30%), a significant difference was found between naive and hM4D ($p<0.05$) but not between naive and mCherry ($p>0.05$, ANOVA with Bonferroni correction). In contrast, no significant differences were found when either hM4D or mCherry groups were compared to Sham ($p>0.05$, ANOVA with Bonferroni correction; Fig. 8).

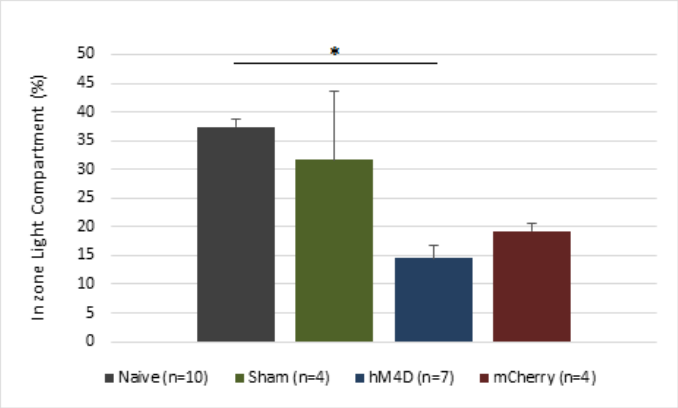

Figure 8: *Percentage of time spent in the light compartment in Light/Dark Transition test for naive, sham-operated (AAV1-CamKIIa-mCherry) and ligated (AAV1-CamKIIa-hM4D(Gi)-mCherry and AAV1-CamKIIa-mCherry for hM4D and mCherry, respectively) animals. 30 minute recordings, mean + SEM, significance is indicated by *.*

Open Field test

Three different parameters were used to assess anxiety-related behavior and changes in locomotion in the Open Field test: the percentage of overall time spent moving (Fig. 9A), the distance moved (Fig. 9B) and the percentage of overall time spent in the center (Fig. 9C).

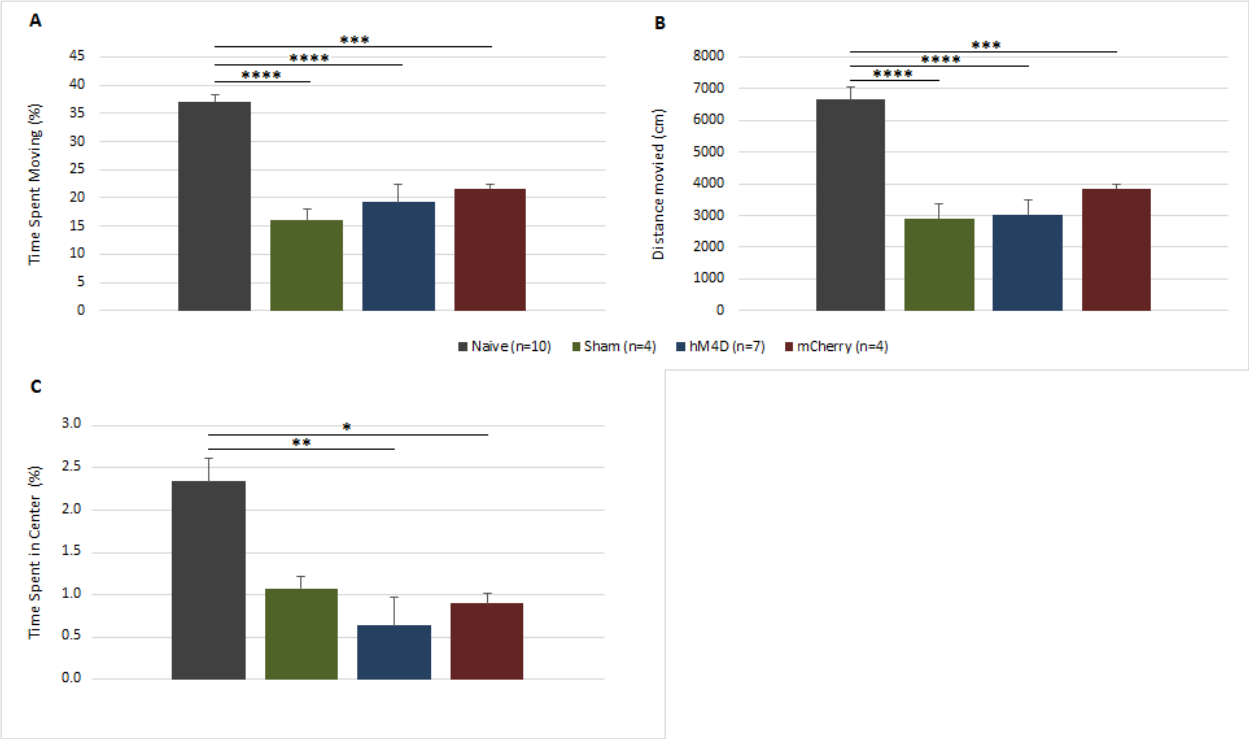

Figure 9: *Percentage of time spent moving (A), distance moved (B) and percentage of time spent in the center (C) in Open Field test for naive, sham-operated (AAV1-CamKIIa-mCherry) and ligated (AAV1-CamKIIa-hM4D(Gi)-mCherry and AAV1-CamKIIa-mCherry for hM4D and mCherry, respectively) animals. 30 minute recordings, mean + SEM, significance is indicated by *.*

Naive animals spent 37.05% of the time moving and crossed an average distance of 6666 cm. In all other groups both parameters were greatly reduced: on average, sham controls moved 16.17% of the time and moved a distance of 2893 cm, hM4D animals spent 19.24% of the time moving over a distance of 3025 cm and mCherry controls were in motion for 21.64% of the time and crossed a distance of 3844 cm (Fig. 9A and B). When compared to naive animals, the differences were significant for all groups ($p<000.1$ for Sham and hM4D, $p<0.001$ for mCherry, ANOVA with Bonferroni correction) but not significant between Sham, hM4D and mCherry ($p>0.05$, ANOVA with Bonferroni correction). Similarly, the time spent in the center of the Open Field box was reduced in Sham (1.07%), hM4D (0.64%) and mCherry (0.90%) compared to naive (2.34%, Fig. 9C). The reduction is significant in comparisons of either hM4D or mCherry to Naive

(p<0.01 and p<0.05, respectively, ANOVA with Bonferroni correction), but not significant between Sham, hM4D and mCherry (p>0.05, ANOVA with Bonferroni correction).

Taken together the data suggest an anxiogenic effect of experimental procedures, but the effect cannot be strictly attributed to the SNLR model since anxiety-related behavior was to some extent also evident in Open Field tests in sham-operated animals (Fig. 9), even though not as strong as in animals that underwent spinal nerve ligation and release (Fig. 8 and 9C). Sham animals did not behave significantly different from hM4D and mCherry animals (Fig. 8 and 9). Furthermore, the data indicate that activation of hM4D(Gi) via CNO injection into the right CeA is not sufficient to relieve anxiety-related behavior (Fig. 8 and 9).

hM3D(Gq)-mediated excitation of LPB-CeC terminals is not sufficient to induce anxiety

To examine the effect of chemogenetic activation of LPB-CeC terminals on anxiety-related behavior, animals that were stereotaxically injected into the right LPB with either AAV5-CamKIIa-hM3D(Gq)-mCherry (group hM3D, n=5) or AAV1-CamKIIa-mCherry (group mCherry, n=5) were tested in Light/Dark Transition and Open field tests. hM3D and mCherry animals were intracranially injected into the right CeA with 1 µl of 26 µM CNO in ACSF 20 minutes prior to behavioral testing. In addition, the data was compared to data obtained from naive animals that were handled following the same protocol as the other groups but underwent no stereotaxic surgery and CNO injection (n=10).

Light/Dark transition Test

On average hM3D and mCherry animals spent 21.04% and 15.05% of the time in the light compartment, respectively. Compared to naive animals (37.34%) the decrease of time spent in the light compartment is significant for mCherry (p<0.05, ANOVA with Bonferroni correction) but not hM3D (p>0.05, ANOVA with Bonferroni correction). No significant difference was found between hM3D and mCherry (p>0.05, ANOVA with Bonferroni correction; Fig. 10).

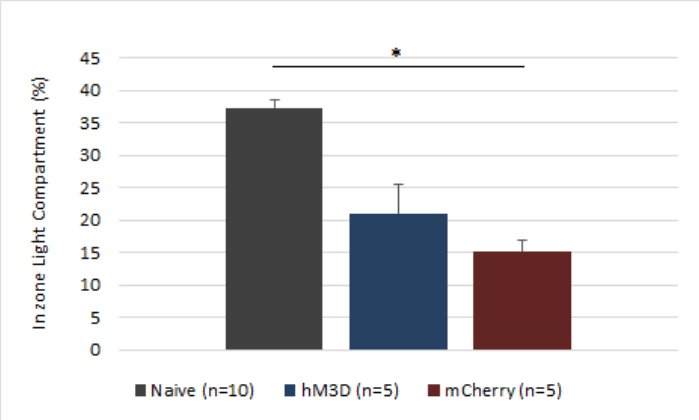

Figure 10: *Percentage of time spent in the light compartment in Light/Dark Transition test for naive, hM3D (AAV5-CamKIIa-hM3D(Gq)-mCherry) and mCherry (AAV1-CamKIIa-mCherry) animals. 30 minute recordings, mean + SEM, significance is indicated by *.*

Open Field test

The same three parameters as described above were used to assess anxiety-related behavior and changes in locomotion in the Open Field test: the percentage of overall time spent moving (Fig. 11A), the distance moved (Fig. 11B) and the percentage of overall time spent in the center (Fig. 11C).

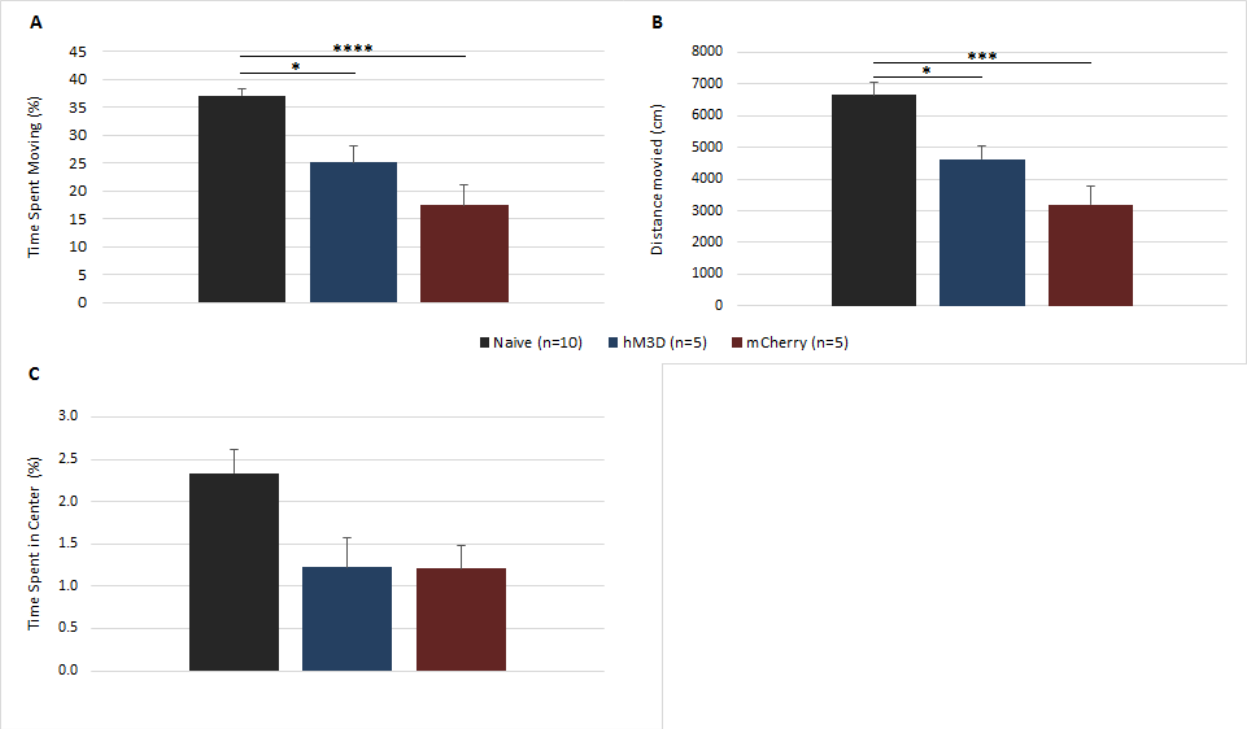

Figure 11: *Percentage of time spent moving (A), distance moved (B) and percentage of time spent in the center (C) in Open Field test for naive, hM3D (AAV5-CamKIIa-hM3D(Gq)-mCherry) and mCherry (AAV1-CamKIIa-mCherry) animals. 30 minute recordings, mean + SEM, significance is indicated by *.*

Naive animals spent more time moving (37.05%), moved over a greater distance (6666 cm) and spent more time in the center (2.34%) than animals of the groups hM3D (25.10%, 4625 cm, 1.23%) and mCherry (17.44%, 3200 cm, 1.21%; Fig. 11A, B and C). The differences in overall time spent moving to naive animals were significant for hM3D ($p<0.05$, ANOVA with Bonferroni correction) and mCherry ($p<0.0001$, ANOVA with Bonferroni correction) but not significant between hM3D and mCherry ($p>0.05$, ANOVA with Bonferroni correction). Similarly, the differences in the distance moved of hM3D and mCherry animals were significant when compared to naive animals ($p<0.05$ and $p<0.001$, respectively, ANOVA with Bonferroni correction) but not significant when compared with each other ($p>0.05$, ANOVA with Bonferroni correction). For the time spent in the center no significant differences were detected between any of the three groups ($p>0.05$, ANOVA with Bonferroni correction).

The data show that hM3D and mCherry animals exhibited anxiety-related behavior (Fig. 10 and 11), with anxiety being slightly more pronounced in mCherry animals (Fig. 10, 11A and B). This effect cannot be attributed to excitation of the LPB-CeC pathway by activation of hM3D-receptors of LPB-CeC terminals via injection of CNO into the right CeA, since mCherry animals lack hM3D-receptors.

LPB fibers form basket-like terminals on SOM+ and PKCδ+ cells in the CeC

Previous studies found that LPB fibers mainly make synapses on dendritic shafts and spines of GABAergic neurons in the CeC, but basket-like structures have also been observed to some extent. The identity of postsynaptic cells in basket-like terminals remained unknown. To identify them, coronal rat brain sections were obtained from animals stereotaxically injected into the right LPB with adeno-associated viruses expressing mCherry. Tissues were treated with mouse anti-Somatostatin or mouse anti-Protein kinase C delta and corresponding Alexa-conjugated secondary antibody solutions. Before mounting, tissues were stained with DAPI. All images were taken in a 700 upright Laser Scanning Confocal microscope (Zeiss) at a 40x magnification.

We found light-microscopic evidence that LPB fibers form basket-like terminals with SOM+ (Fig. 12) and PKCδ+ (Fig. 13) cells. Not all cells contacted by LPB fibers in this way were immunoreactive for SOM or PKCδ, indicating that other cell populations could also form basket-like terminals with LPB fibers (Fig. 12 and 13).

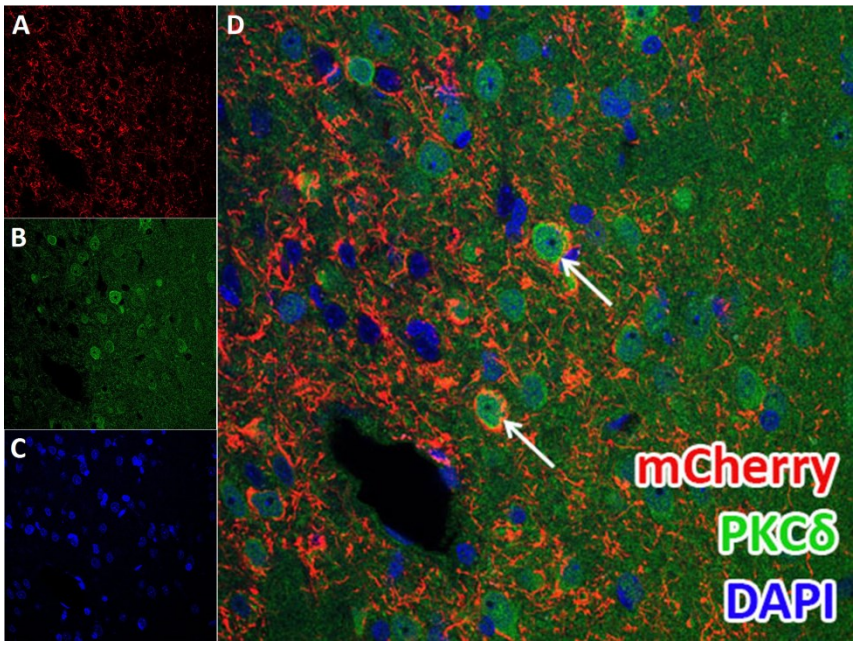

Figure 12: *Confocal image of right CeC with immunofluorescent labeling of PKCδ. (A) mCherry expression of LPB fibers, (B) PKCδ immunoreactivity, (C) DAPI staining, (D) merged channels. Some CeC neurons are immunoreactive for SOM and form basket-like terminals with mCherry-expressing LPB fibers, indicated by white arrows.*

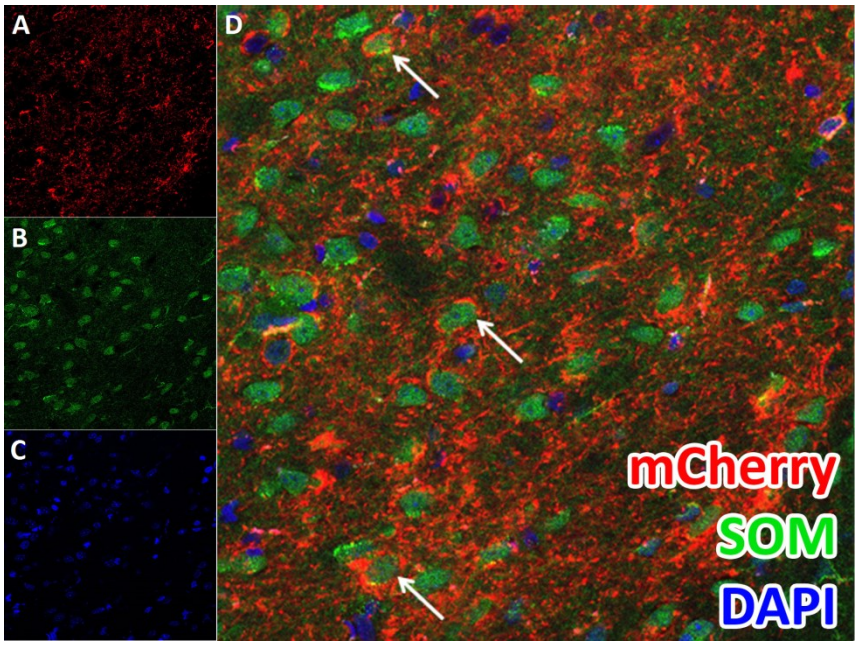

Figure 13: *Confocal image of right CeC with immunofluorescent labeling of SOM. (A) mCherry expression of LPB fibers, (B) SOM immunoreactivity, (C) DAPI staining, (D) merged channels. Some CeC neurons are immunoreactive for SOM and form basket-like terminals with mCherry-expressing LPB fibers, indicated by white arrows.*

Discussion

Neuropathic pain is frequently associated with states of mood disorders like depression and several anxiety disorders, affecting millions of people. The available clinical diagnostic tools lack precision and drug and behavioral therapies are in many cases inadequate, highlighting the importance of further research (McWilliams et al. 2003).

The present study aims to elucidate the role of the lateral parabrachial nucleus to central amygdala pathway in persistent anxiety in a rat neuropathic pain model. The LPB has been established as an important participant in the relaying of nociceptive information to higher brain areas including the CeA, specifically the CeC, and the hypothalamus (Bernard et al. 1993; Dong et al. 2010; Neugebauer et al. 2003; Sarhan et al. 2005). The CeA not only receives nociceptive information but also serves as a hub for integrating polymodal sensory input from the BLA (Neugebauer et al. 2004), and has been consistently implicated with emotional responses like fear and anxiety. It is therefore thought that a negative emotional component is attached to nociceptive information in the CeA.

In animal models of neuropathic pain, LPB-CeC and BLA-CeC pathways have been shown to undergo long-term potentiation. This potentiated state remained in the LPB-CeC, but not in the BLA-CeC, pathway after animals recovered from pain and mechanical allodynia in the presently used SNLR model (Dong et al. unpublished). It is conceivable that neuropathic pain-associated anxiety could be engrained in neuropathic pain-induced potentiation of the LPB-CeC pathway.

In this study we stereotaxically delivered AAVs into the right LPB to express hM4D(Gi) DREADDs. We then activated hM4D(Gi) in terminals of the LPB-CeC pathway by local microninjection of CNO into the right CeC, expecting to inhibit synaptic release and silence the potentiated pathway in a SNLR model. Anxiety-related behavior was then examined and the effect of chemogenetic inhibition of the pathway assessed.

We found that ligated and released animals recovered from mechanical allodynia after 5 days, which is in accord with previous findings. After chemogenetic inhibition of the LPB-CeC pathway anxiety-related behavior was still evident in the chosen behavioral paradigms, indicating that this approach is not sufficient to relieve anxiety in the SNLR model. We further tested the effect

of chemogenetic excitation of the LPB-CeC pathway in rats that had no ligation surgery. AAVs expressing hM3D(Gq) DREADDs were injected into the right LPB and CNO was transcranially injected into the CeC before behavioral testing. We found similar anxiety-related behavior as in the SNLR model, but were unable to attribute this effect to the chemogenetic excitation of LPB terminals in the CeC. Since animals of the mCherry control group displayed the same anxiety-related behavior, it is possible that anxious states had already been evoked by surgical procedures and/or housing conditions, as described in more detail below. In addition, an immunohistochemical approach to identify CeC neuron populations contacted by LPB fibers in basket-like structures revealed subpopulations to be immunoreactive for SOM and PKCδ.

Tactile allodynia is induced by spinal nerve ligation and recovered from 5 days after release

The spinal nerve ligation model for neuropathic pain is well established and frequently used (Bennett et al. 2003). It results in increased mechanosensitivity of the ipsilateral hindpaw, which can be monitored by regular Von Frey testing, and expression of anxiety-related behaviors. The present SNLR model where the ligation is released on the day after ligation surgery is a recent development and allows animals to recover from neuropathic pain while maintaining neuropathic pain-induced morphological changes in the LPB-CeC pathway and negative emotional behavior (Dong et al. unpublished). In this study we found the procedure to be generally successful, while some animals (5 out of 18) did not recover from tactile allodynia after one week.

Anxiogenic effect of SNLR

Previous findings demonstrated that ligated and released animals exhibited anxiety-related behavior, while sham-operated animals did not (Dong et al. unpublished). In this study we found locomotion – in terms of overall time spent moving and distance moved in Open Field test – to be reduced in ligated and released animals as well as in sham-operated animals, while anxiety-related behavior (overall time spent in the light compartment in Light/Dark Transition test and overall time spent in the center in Open Field test) was only evident in ligated and released

animals. Specifically, sham-operated animals did not differ significantly from naive animals, while the experimental groups hM4D and mCherry control spent significantly less time in the center of Open Field test compared to naive, and hM4D spent significantly less time in the light compartment of Light/Dark Transition test than naive. Our data indicate that the SNLR model is an effective approach to evoke anxious behavior, but also results in a significant decrease of general locomotion. The surgical procedures are very invasive and damage the musculature of the lower back, which could be irritating for animals even after one week of recovery and therefore explain this effect.

Effect of hM4D(Gi)-mediated inhibition of LPB-CeC terminals

Previous experiments where the LPB was unilaterally lesioned by local application of ibotenic acid concluded that SNLR animals were consequently relieved from anxiety-related behavior (Dong et al. unpublished). In the present study we chose a less invasive approach to inhibit LPB-CeC signaling by using a well-established variant of DREADDs. hM4D(Gi)-mediated synaptic silencing by local microinjection of CNO to fiber terminals has been successfully used in the past. In contrast to our expectations, in our study hM4D(Gi)-mediated inhibition was not sufficient to relieve SNLR animals from anxiety. The reason for this is difficult to discern, because multiple factors could come into play. Animals underwent stereotaxic injection and cannula implantation surgeries, ligation and release surgeries and were housed in individual cages in an environmentally isolated cabinet. Surgical procedures as well as single housing can cause symptoms of stress and anxiety in rats, possibly masking an anxiolytic effect of LPB-CeC silencing. Interestingly, sham-operated animals did not exhibit significant anxiety-related behavior even though they were housed under the same conditions, but they underwent no ligation and release surgeries. The stress caused by stereotaxic surgery and housing conditions could be amplified in states of neuropathic pain, explaining this difference; but extensive testing is needed to discern the reason why animals still displayed anxiety-related behavior after chemogenetic inhibition of the LPB-CeC pathway: behavioral testing should be performed after each individual surgical procedure and compared to control groups to examine the extent of possible anxiogenic effects; animals should be housed in environmentally enclosed cages to

reduce the influence of olfactory triggers from other animals (e.g. smell of blood, stress-induced cues) and, if possible, animals should be housed in groups to eliminate the stress induced by individual housing. Furthermore, electrophysiological data is needed to confirm synaptic silencing at the target site. It is also possible that other pathways in the brain may be involved in the establishment of neuropathic pain-induced anxiety. The LPB also transmits nociceptive information to other brain areas such as the thalamus and the paraventricular thalamic nucleus (PVT), which integrates and modulates visceral information and negative emotions. A recent study has shown that LPB-CeA projecting neurons also send collaterals to the PVT, and that this pathway is activated in parallel to the LPB-CeA pathway in the spared nerve injury (SNI) neuropathic pain model (Liang et al. 2016). The authors hypothesize that the LPB-PVT-CeA pathway forms a local neural circuit influencing the effect of neuropathic pain. Further research is needed to decipher the role of the PVT in neuropathic pain-induced anxiety-related behavior.

Effect of hM3D(Gq)-mediated excitation of LPB-CeC terminals

To elucidate the effect of DREADD-mediated excitation of the LPB-CeC pathway, animals were stereotaxically injected into the right LPB with AAVs expressing hM3D(Gq) and CNO was applied at the terminals in the right CeC. Both the experimental group hM3D and the mCherry control group exhibited anxiety-related behavior similar to SNLR animals, while the decrease in locomotion was not as strong. The display of anxious behavior could not be attributed to hM3D(Gq)-mediated excitation of the LPB-CeC pathway, because the control group lacking DREADD expression showed a similar result. The surgical procedure and housing conditions as described above might have evoked states of anxiety, thereby masking a possible anxiogenic effect of hM3D(Gq) activation at the terminals in already anxious animals. In addition, to our knowledge no studies so far have electrophysiologically confirmed hM3D(Gq)-mediated presynaptic release with local CNO application at the terminal site. hM3D(Gq) acts by inducing intracellular calcium release and increasing excitability (Roth 2016), but intracellular calcium stores are mainly located at the endoplasmatic reticulum and mitochondria in the soma (Verkhratsky 1998). Therefor local infusion of CNO at the terminals may not mobilize a sufficient amount of calcium for robust stimulation of presynaptic neurotransmitter release.

Basket-like structures at LPB-CeC terminals

Electronmicroscopical data has shown that LPB fibers mainly form synapses on dendritic shafts and spines of GABAergic neurons in the CeC (Dong et al. 2010), but some lightmicroscopical observations have found basket-like structures formed onto CeC soma (Dong et al. 2010; Lu et al. 2015). CeA populations have previously been characterized by the expression of markers such as Dynorphin, Somatostatin, Enkephalin and Protein kinase C delta (Pomrenze et al. 2015). Here, immunohistochemistry and confocal microscopy revealed that some of the CeC neurons enveloped by basket-like structures express the neuronal markers SOM and PKCδ. SOM+ neurons in the CeL have been demonstrated to play an active role in conditioned fear in mice (Li et al. 2013; Penzo et al. 2015), while PKCδ+ neurons in the CeL suppress fear conditioning (Ciocchi et al. 2010; Haubensak et al. 2010). The role of these populations in the CeC remains unknown. Whether these basket-like structures form synapses with the soma of CeC neurons has to be confirmed by electronmicroscopy.

Outlook

In the present study we were not able to relieve SNLR rats from anxiety by chemogenetic inhibition or to elicit anxiety by chemogenetic excitation of the LPB-CeC pathway. Experimental procedures and conditions can be improved in future experiments: Rats were housed in individual cages because group housing after surgeries usually results in reopening of wounds and/or damage to the cannula implant, but rats are very social animals and isolation over longer periods of time can lead to states of depression and anxiety; a major drawback for experiments assessing anxiety-related behavior. If wounds and implants would be protected from manipulation by cage mates this problem could be solved. In addition, recovered animals were sometimes housed in the same cabinet as animals that just underwent surgery, resulting in a continuous smell of blood in the cabinet, which can be irritating for rats. It would also be beneficial to use another system for excitation of LPB-CeC signaling. For example, since most CeC-projecting LPB-fibers are CGRP+, a virus could be created that expresses hM3D(Gi) with CGRP promotor sequence, allowing application of CNO at the soma to specifically activate CGRP+ neurons. Another, although very costly, approach would be to create transgenic rat lines

where the Cre/loxP-system is applied, further improving precision of chemogenetic manipulation. Still, the LPB-CeC pathway presents a very promising target for future research into neuropathic pain-induced anxiety.

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