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Faculty of Pharmacy in Hradec Kralove

Study Programme: Pharmacy
Department of Pharmaceutical Chemistry and Pharmaceutical Analysis

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Delineating Pain and Fear Engrams in the Prefrontal Cortex

Diploma Thesis

Kristýna Ludínová

Supervisor:
PharmDr. Jan Zitko, Ph.D.
External supervisors:
Dr. Manfred Oswald, Prof. Dr. Rohini Kuner

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Kristýna Ludínová

Školitel:
PharmDr. Jan Zitko, Ph.D.
Externí školitelé:
Dr. Manfred Oswald, Prof. Dr. Rohini Kuner

Hradec Králové, 2018

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Kristýna Ludínová

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Kristýna Ludínová

ABSTRAKT

Univerzita Karlova

Farmaceutická fakulta v Hradci Králové

Katedra farmaceutické chemie a farmaceutické analýzy

Kandidát: **Kristýna Ludínová**

Školitel: **PharmDr. Jan Zitko, Ph.D.**

Externí školitelé: **Dr. Manfred Oswald, Prof. Dr. Rohini Kuner**

Název diplomové práce: **Vymezení bolesti a paměťových stop strachu v prefrontální kůře**

Bolest je komplexní proces zahrnující aktivaci různých mozkových center. Dle dostupných výsledků ze zobrazovacích metod na lidech a hlodavcích, mediální prefrontální kůra , patří mezi oblast, která je konstitutivně aktivovaná během bolesti. Mediální prefrontální kůra zpracovává funkčně rozdílné procesy jako bolest, emoce, rozhodování, pozornost, avšak její přesná role a souvislost ve vnímání bolesti a jiných procesech není známa.

Naším cílem bylo vymezení, jak se strach (emoce) a bolest manifestují na buněčné úrovni. S použitím značení závislého na aktivitě jsme testovali, zda jsou soubory buněk v mediální prefrontální kůře aktivované bolestí odlišné od souborů buněk aktivovaných strachem. Zkoumali jsme potenciální využití DREADDs (konstruované receptory výhradně aktivované konstruovaným aktivátorem) pro testovaní funkční role prefrontální kůry v bolesti a ve strachu.

Naše výsledky umožňují orientačně nahlédnout na to, jak je prefrontální kůra aktivovaná v bolesti a ve strachu a srovnání jejich buněčné exprese pomocí imunohistochemických metod. Také přinášíme možné návrhy na studování překryvu neuronálních populací.

Náš navrhovaný postup s použitím DREADDs metody se neosvědčil, proto navrhujeme metodu dále optimalizovat zvýšením výkonosti značení a užitím vhodných kontrolních skupin. Avšak pokud by se metoda stále zdála jako nevhodná, jako další krok navrhujeme optogenetiku jako potenciální metodu.

ABSTRACT

Charles University
Faculty of Pharmacy in Hradec Králové
Department of Pharmaceutical Chemistry and Pharmaceutical Analysis
Candidate: **Kristýna Ludínova**
Supervisor: **PharmDr. Jan Zitko, Ph.D.**
External supervisors: **Dr. Manfred Oswald, Prof. Dr. Rohini Kuner**
Title of diploma thesis: **Delineating Pain and Fear Engrams in the Prefrontal Cortex**

Pain is a complex process associated with activation of various brain centres.
According to evidence of imaging studies in humans and rodents the medial prefrontal cortex (mPFC) ranks amongst the brain area consistently activated during painful perception.
The mPFC circuits underlies functionally-distinct processes, such as pain, emotional response, decision-making, attention amongst others. However, the precise contribution of mPFC in pain related function remains to be unknown.
The aim of the study was to delineate how pain and fear are manifested at the cellular level within the regions of PFC. By employing activity dependent neuronal labelling we tested if cellular ensembles activated in pain and fear behaviours within the mPFC are distinct. We investigated a potential use of activity-dependent DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) expression in order to test for the functional role of PFC ensembles in pain and fear behaviour.
Our findings provide the potential insight of the c-Fos expression within the prefrontal cortex (PFC) separately in pain, fear and their comparison. They also propose future experiments for studying ensemble overlap. Our DREADDs approach to test the functional role of PFC in pain and fear behaviour proved not to be effective, and we suggest further optimisation of this method by increasing labelling efficiency and by using appropriate controls. However, if the method appeared to be unsuitable for our experiments again, we would propose using optogenetics potential approach as a next step.

Table of Contents

1. Introduction....9
1.1 The goals of the thesis....9
Theoretical Part....10
2. Pain and Nociception....11
2.1 Classification of pain....11
2.1.1 Physiological pain....12
2.1.2 Pathological pain....12
2.1.3 Neuropathic pain....13
2.1.4 Inflammatory pain....13
2.2 Synaptic plasticity....14
2.2.1 Types of hypersensitivity....14
2.3 Transmission of pain....14
2.4 Nociception and pain....15
2.4.1 Nociceptors....15
2.4.2 Nociceptive transmission at the spinal cord....17
2.5 Transient receptor potential channels....19
2.5.1 TRP channels....19
2.5.2 The TRPV subfamily....20
2.5.3 TRPV1 channel and its role in nociceptive transmission....21
2.5.4 Activation of TRPV1....22
2.5.5 TRPV1 activation by capsaicin....23
3. Central modulation of pain....24
3.1 Psychosocial factors....24
3.2 Role of prefrontal cortex in pain and fear....25
3.2.1 Prefrontal cortex....25
3.2.2 Population of cells in PFC....26
3.2.3 Physiological functions of PFC....26
3.3 Fear memory and engram cells....27
3.3.1 Consolidation of fear memory....27
3.3.2 The role of anterior cingulate cortex in fear....28
3.3.3 Involvement of prelimbic cortex in fear....28
3.3.4 Involvement of infralimbic cortex in fear....29
3.4 Role of prefrontal cortex in pain....30
3.4.1 Dissociative roles of mPFC....30
3.4.2 Anterior cingulate cortex....30
3.4.3 The role of PL and IL mPFC....31
3.4.4 Lateral PFC/ Insular cortex....31
4. Principles of used methods....32
4.1 Tetracycline off system....32
4.2 Tetracycline on system....33
4.3 DREADDs – Designer Receptors Exclusively Activated by Designer Drugs....34
4.3.1 Biotechnology of DREADDS....34
4.3.2 Gq-DREADDS, CNO Analogues, and Basal Activity....36
4.3.3 Gi-DREADDs....36
4.4 Immunohistochemistry....37
4.4.1 DAPI staining....37
4.4.2 NeuN – Neuronal nuclei....37
4.4.3 Immediate-early gene product c-Fos....37
4.5 Fear conditioning....37
4.5.1 Contextual fear conditioning....38

4.5.2 Cued fear conditioning....38
Experimental Part....40
5. Materials....41
5.1 Animals....41
5.2 Equipment....41
5.3 Instruments....41
5.4 Chemicals....42
5.5 Antibiotics....42
5.6 Plasmid constructs....42
5.7 Recombinant viruses....43
5.8 Software....43
5.9 Antibodies....43
5.9.1 Primary....43
5.9.2 Secondary....43
6. Methods....43
6.1 Generation of recombinant AAV particles....44
6.2 Surgical procedures....44
6.3 Behavioral testing paradigms....45
6.4 Fixation....48
6.5 Brain slice preparation....49
6.6 Immunohistochemistry protocols....49
6.6.1 c-Fos and NeuN....49
6.7 Image analysis....49
6.7.1 Confocal microscopy....50
6.8 Data analysis....50
7. Results....51
7.1 Immediate-early gene (IEG) expression in pain....51
7.2 IEG expression in fear....53
7.3 Comparison of IEG expression within PFC in acute pain and conditioned fear....57
7.4 Ensemble overlap & Labelling efficiency....58
7.5 Behavioural effects of engram neuron manipulation with DREADDs....64
8. Discussion....67
8.1 IEG expression in pain....67
8.1.1 Comparison of different forms of capsaicin injections....68
8.2 IEG expression in fear....68
8.3 Comparison of IEG expression within PFC in acute pain and conditioned fear....69
8.3.1 Comparison of recent and remote fear....69
8.3.2 Comparison of acute pain and conditioned fear....69
8.4 Ensemble overlap....70
8.5 DREADDs and labelling efficiency....70
9. Conclusion....73

1. Introduction

Pain as early-warning is major part of physiological protective system, however under pathological conditions, pain loses its protective function and becomes excruciating and distressing feeling. Despite the several currently approved analgesics, the treatment of pain still remains to be challenge in clinical practice and basic science due to the fact, that the current analgesic therapy is often associated with different unwanted side effects or the available drugs do not exert sufficient efficacy in pain relief. Therefore, the investigation of brain mechanisms underlying pain remains to be in focus in many pharmacological studies.

1.1 The goals of the thesis

This thesis was conducted as a part of a larger project on **"The Role of the Prefrontal Cortex in Nociception and Pain and Underlying Circuitry".** The involvement of PFC circuits underlies functionally distinct processes, such as pain, attention, learning, and emotional responses. The main project addresses these aspects by studying the cellular contribution in distinct PFC regions in mice using in vivo techniques for neuronal activity mapping and manipulation. The extent to which specific cell populations within cortical circuits govern acute pain and pain chronicity in conditions of inflammatory and neuropathic pain is studied with genetic tools for reversible, minimally-invasive silencing or activation of specific cell populations.

The central aims of the thesis are:

a) To delineate how pain and fear are encoded at the cellular level within medial and lateral regions of the PFC.

b) To test if the cellular ensembles activated in pain and fear behaviours within the mPFC are distinct.

c) Characterize initial experiments with activity-dependent DREADD expression in the lateral and medial PFC in order to test for the functional role of PFC ensembles in pain and fear.

Theoretical Part

2. Pain and Nociception

2.1 Classification of pain

The International Association for the Study of Pain (IASP) [1] defines pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage. The sensation of pain produces a reflexive retraction from the painful stimulus, and tendency to protect the affected body part while it heals and avoids a painful situation in the future. Pain is a fundamental part of the body's protective system [2].

Pain can be in general classified according to its length of duration as acute pain, subchronic and chronic pain. Duration of acute pain lasts only until the noxious stimuli are removed and the intensity of acute pain may change rapidly over a short period of time. Acute pain presents itself as a sudden and sharp sensation in a response to noxious stimuli [2]. It fulfils a warning role, it is highly complex physiological and protective sensation triggered in the nervous system to alert to real or impending injury. In contrast to chronic pain, acute pain is often associated with response of autonomic nervous system (tachycardia, elevation of blood pressure). The presence of acute pain serves as indication of injury or acute disease and the intensity of pain often corresponds to severity of insult, also in clinical practise the severity of acute pain correlates with the pharmacological treatment that is needed for pain relief [3].
Subchronic pain lasts for hours up to days. Chronic pain is any painful condition persisting for three or more months (persists beyond tissue healing) or the painful sensation regularly recurring in time. It does not serve as any biological function and has no benefit or usefulness. The intensity of chronic pain very often does not correspond with proportion of disease or injury [2]. Chronic pain can be understood as a disease on its own and it has the deleterious impact on quality of life. The profound differences between acute and chronic pain emphasize the fact that pain is not generated by an immutable, hard-wired system, but rather results from the engagement of highly plastic molecules and circuits. In contrast to acute pain, the contributions of autonomic nervous system are absent or exhausted in chronic pain conditions. The causes of chronic pain are mainly associated with less curable and chronic diseases [2].

Another way of pain classification is based on mechanisms of its development. This classification separates pain into nociceptive pain, neuropathic and inflammatory pain. It is critical to distinguish between these, as the mechanisms and treatment are different [2].

2.1.1 Physiological pain

Under physiological conditions, acute pain is defined by its obvious cause and short duration. However, the definition is not absolute. Acute pain as physiological pain is a sensation, which we all experience frequently in our everyday life. Due to the nature of unpleasant sensations, we learn to avoid certain stimuli. Pain as a term is used in order to describe the sensation that is experienced in response to noxious stimuli. Physiological pain can be evoked only by stimuli that reach the pain threshold, such as mechanical, thermal or chemical stimuli [4]. The increase of intensity of noxious stimuli, further produces quantifiable-stimulus response until the pain exceeds a level of tolerance. Nociceptive pain results from noxious stimuli capable of tissue damage and is further subdivided into visceral pain and somatic pain. Visceral pain is an important part of the normal sensory repertoire of all human beings and a prominent symptom of many clinical conditions [5].

2.1.2 Pathological pain

Pathological pain can be subdivided in the inflammatory and neuropathic pain, which have important common features in comparison with physiological pain. Clinically common types of pathological pain are spontaneous pain, hyperpathia/ hyperalgesia, allodynia, referred pain, sympathetic dystrophy, sympathetically maintained pain [4]. However, pathological pain may also develop in response to damage or alterations in primary afferent neurons (stimulus-dependent). In pathological pain, there is no adequate response to the stimuli, the sensation of pain is in this case completely excessive and may occur even in absence of any apparent stimulation (stimulus independent) [6]. In pathophysiology of pain processes, it is very hard to describe the single mechanism. Whereas the physiological pain can be produced only by activation of high-threshold nociceptors, pathological pain such as neuropathic pain and inflammatory pain is caused by disturbances in somatosensory system and can be triggered in very wide variety of different conditions, such as clinical

manifestation of diverse disorders, infectious, toxic, metabolic disease or trauma. It can also be developed as a result of damage or alterations in primary afferent neurons [6]. Sensitization, as a mechanism involved in the development of pathological pain, may include less common low-thresholds in primary afferent nociceptor or plasticity induced central mechanism in the spinal cord or forebrain [7].

2.1.3 Neuropathic pain

Neuropathic pain was defined by IASP as a pain resulting from a primary lesion or dysfunction of the nervous system, usually involving an element of sensory dysfunction [8]. Whereas in nociceptive pain the nociceptors are activated by an adequate stimulus, neuropathic pain results from the activation of nervous system, even in absence of nociceptive input [9]. Typically neuropathic pain occurs due to a primary insult or dysfunction of peripheral or central nervous system. It might also occur independently of other tissue damage and in contrast to other kinds of pain it lacks a protective purpose [10]. It is often irreversible and persists even after the cause of nerve damage is eliminated. It may also occur as a secondary symptom of other diseases such as metabolic syndrome in diabetes, infection of herpes zoster and cancer [6]. Mechanism of pathophysiology underlying development of neuropathic pain encompasses both peripheral and central components [11].

2.1.4 Inflammatory pain

One of the main and unique features of an inflammatory state is that the evoked sensation of pain is produced by normally innocuous stimuli [12]. Transduction itself can be enhanced by inflammatory mediators which are released during inflammation, metabolic stress and tissue injury. In the group of inflammatory mediators belongs wide range of chemical substances such as prostaglandins, bradykinin, ATP, protons, and nerve growth factor (NGF) [10]. NGF belongs to a family of neutrophic factors and is an essential factor for development and maintenance of central and peripheral nervous system. The inflammatory mediators further initiated a second messenger cascade by sensitization and activation of membrane ion channels [5].

2.2 Synaptic plasticity

Neuronal plasticity is a term that refers to fundamental ability to learn and adapt neuronal functions in response to environmental or intrinsic changes [12]. Shifts in pain thresholds and responsiveness are an expression of neuronal plasticity. Dynamic changes can occur on the molecular, synaptic and cellular levels, which alter the strength of synaptic connections between neurons. Neuronal plasticity is determined by activation, modulation, and modification and for this reason in mature nervous system synaptic transmission can be potentiated, suppressed and modulated in many different ways [13]. Neuronal plasticity plays a major role in development of chronical pain and stands for wide range of deviations in clinical manifestation of pain [14]. Conversely, plasticity can decrease the body's own pain inhibitory systems, leading ultimately to increased pain. Neuronal plasticity also causes hypersensitivity, which is very important because it arises when the pain pathways increase their sensitivity while relaying pain messages [14].

2.2.1 Types of hypersensitivity

In terms of hypersensitivity, two forms are distinguished [13].

Allodynia is evoked by non-noxious stimuli. In case of allodynia high thresholds are lowered so pain can be triggered by stimuli which would not normally under physiological conditions produce pain. Allodynia can be experienced as a burning sensation in response to thermal or mechanical stimuli. It can often be caused by injury [15].

Hyperalgesia so-called "over pain" occurs when responsiveness to noxious stimuli is increased and the noxious stimuli induce exaggerated and prolonged pain. This can be caused by damage to peripheral nerves, nociceptor or even during changes in higher centres [15].

2.3 Transmission of pain

Transmission of pain is a very complex process that includes integration within the periphery, spinal cord, brainstem, and forebrain [16]. The transmission of information related to pain from periphery to CNS is processed through three levels within CNS. First

neuron (pseudounipolar) resides the dorsal root ganglia (DRG) or trigeminal ganglia. Second-order neurons are located in the dorsal horn, those neurons project through the ascendant spinothalamic tract. The third neuron resides in thalamus and is further projected to the primary somatosensory and cingulate cortices. The emotional, affective and motivational factors related to pain are transduced by distinct pathways, that involves structures such as parabrachial nucleus, amygdala, and intralaminar nucleus of the thalamus [15].
Emotional, affective and motivational aspects highly predominate during chronic pain and are rather transduced by wide dynamic range neurons (second-order nociceptive neurons) [8].

2.4 Nociception and pain

Nociception is critical survival mechanism in an organism and triggers appropriate protective responses. Under physiological conditions the nociceptive sensation of pain can be produced only as a reaction to noxious intense stimuli. These noxious stimuli are recognized and mediated by very specific threshold primary neurons in peripheral endings. There they generate signals that are subsequently transferred to higher CNS centre [17]. The threshold for recognizing and eliciting pain are not fixed and can be shifted either up or down. This ability may later occur by either as adaptive or maladaptive [8]. Shifts in pain threshold are enabled by changes in the nervous system and these changes can modify responses to any stimuli. This process is called neuronal plasticity mentioned earlier and has a key role in clinical pain syndromes. In clinical practice, patients can show similar neuroplastic changes in nociceptive transmission, however, the cortical processing in higher CNS of nociceptive signals are very individual and subjective. As a result, the level of pain which patients experience can vary greatly [7].

2.4.1 Nociceptors

Nociceptors are distinguished from the other sensory nerve fibres by their ability to be excited by a noxious stimuli that has sufficient energy to overcome the high-threshold. Nociceptors are highly specialized to respond only to noxious stimuli such as heat, intense pressure or irritant chemical, but on the other side to be completely unexcitable to an innocuous stimulus such as only warming or light touch [18].

Within nociceptive pathways can be found many different types of nociceptors responsible for various types of pain. Nociceptors are morphologicaly pseudo-unipolar cells, fibres that are responsible for innervating region of head and body. Their cell bodies reside in trigeminal and dorsal root ganglion (DRG). Nociceptor has four major functional components (Fig 2.1). According to Woolf and Ma the following parts of nociceptor are distinguished:

1. the peripheral terminal that transduces external stimuli from the target tissue and also initiates the action potentials,
2. the cell body, whose role is to secure the identity and integrity of the whole neuron,
3. the axon that conducts action potentials,

and the central terminal ending as the part of presynaptic unit of the first sensory pathway of nociception [17].

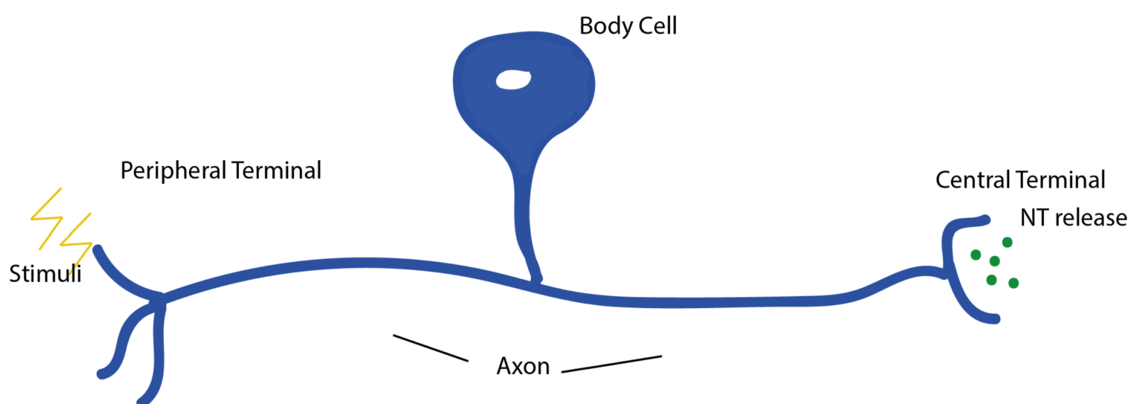

Figure 2.1: Schematic drawing of nociceptor showing the four regions of the cell [17].

Nociceptors can be categorized according to their anatomical and functional criteria. The natural stimuli of nociceptors might be difficult to identify due to their distinct characteristics [19] Aβ fibres are the primary sensory myelinated fibres with the largest diameters. Most of the Aβ fibres ensure the detection of non-noxious stimuli (applied to skin, joints, muscle). In the absence of tissue or nerve injury, they have a main role in proprioception and only respond to touch, vibration and to other modes of non-noxious, lower intensity mechanical stimuli [17]. Under physiological circumstances, they are considered as the fibres without any contribution to pain [20].

Fibres with a small- and medium-diameter cell bodies contribute to the transduction of noxious stimuli upon the exposure of the skin. Aδ fibres with medium diameter (2–6 μm) and two main classes are lightly myelinated with a velocity of conduction 12–30 $m.sec^{-1}$ [17]. Aδ fibres elicit the first phase pain rapid and sharp sensation [20]. One class of Aδ fibres belongs to rapidly-conducting, high-threshold mechanoreceptors the so-called „Type I". There are typically activated by mechanical and high-intensity noxious stimuli. Type II of Aδ fibres is very weakly responsive to high-intensity heat, cold and chemical stimuli [17]. C fibres are the thinnest (Ø = 0,4–1,2 μm) unmyelinated slowly conducting nociceptor with the conduction of action potentials in these fibres is the slowest (~0,5–2 m.sec−1). Most C fibres are also polymodal with ability to respond to noxious thermal, mechanical and also to chemical stimuli, such as substance capsaicin [17].

There is further one class of nociceptors called „silent nociceptor" or sleeping nociceptor. Those neurons are unresponsive to acute noxious stimuli, however, they may become sensitized in present of inflammatory process, chemical mediators or in case of tissue injury. Sleeping nociceptors are of an interest in the context of prolonged pain [20].

2.4.2 Nociceptive transmission at the spinal cord

Pain transduction is very complex process that employs multiple biological mechanisms, and peripheral and central levels of the nervous system. Nociception on its own involves a multiple innervations of the thalamus, midbrain, limbic system, cortex, reticular formation and many other cerebral structures via multiple ascending pathways (see Fig 2.2) [20].

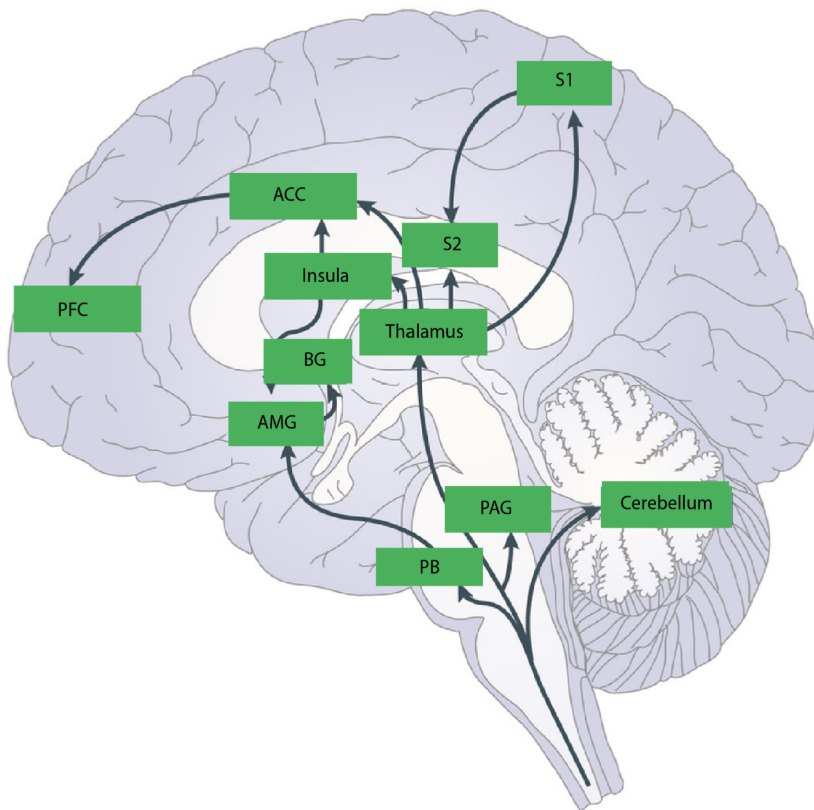

Figure 2.2: Afferent pain pathways include multiple brain regions. Afferent spinal pathways include the spinothalamic, spinoparabrachio–amygdaloid and spinoreticulo–thalamic pathways. The receiving information is projected from amygdala (AMY) to basal ganglia (BG) whereas thalamus projects the nociceptive information to the insula, anterior cingulate cortex (ACC), primary somatosensory cortex (S1) and secondary somatosensory cortex (S2). Figure adapted from [31].

First components of the nociceptive pain pathway are a peripheral terminals of nociceptor. Action potentials caused by noxious stimuli are transmitted by unmyelinated C-fibres, thinly myelinated Aδ fibres nociceptors, by specific receptors or ion channels which are sensitive to heat, cold, protons or mechanical stimuli. Those action potentials are driven along the afferent axons to the dorsal horns (DH) of the spinal cord. By means of nociceptor, the spinal neurons are activated and are evoked to send the electrical activity by axons across the region of spinal cord, medulla, midbrain, and thalamus. Terminal axons of thalamic neurons are further projected into cortex including somatosensory cortex [20].

2.5 Transient receptor potential channels

2.5.1 TRP channels

Transient receptor potential (TRP) ion channels are commonly understood as tetrameric transporters, located in plasmatic and intracellular space and their role is to regulate the flux across the plasma membrane as a result of various environmental or intracellular impulses [21]. TRP proteins belong among Ca^{2+}-permeable ion channels and are a superfamily (28 members in the mammal) with diverse physiological functions (chemosensation, mechanosensation), tissue distribution and subcellular localization. The ion channels facilitate the transmembrane flux of cations and trace metal ions along their electrochemical gradients. In mammals, the TRPs are expressed in almost each cell in both excitable and non-excitable tissue [22]. The so-called "sensory TRPs" are subsets of TRP channels in sensory cells, such as a somatosensory neuron. The "non-sensory TRPs" are channels expressed in non-sensory tissues [23].

TRPs participate in many physiological processes. They are present in cellular membranes where they have a significant contribution to motile function and sensory functions, such as taste transduction, nociception, and thermosensation, also in the maintenance of homeostasis by regulation of pH, osmolality and intra and extracellular caution of Ca^{2+} and Mg^{2+} levels [22].

When activated, most TRPs conduct Ca^{2+}, as well as Na^{+} and K^{+} ions. Most TRPs are considered as non-selective cation channels, only a few have the ability of high selective permeability for Ca^{2+}. TRP channels family possess diverse gating mechanisms such as ligand binding activation, temperature dependent activation, and voltage-gated activation.The activation of TRPs channels results in depolarization of the cellular membrane and accumulation of cations. The activity of TRP channel leads to an accumulation of cations and depolarization of the cellular membrane, which in terms activates voltage-dependent ion channels, resulting in changes of intracellular Ca^{2+} concentration. TRP channels serve as gatekeepers for transcellular transport of several cations especially for $Ca^{2+}$and Mg^{2+} and are the reason why TRP channels stay in the centre of many vital cellular processes [23].

Reports of several studies have shown that, TRP channels are sensitive to various multiple intracellular signals such as the increased concentration of cytoplasmic Ca^{2+}, phosphorylation, and phospholipids. In particular, many TRPs are highly sensitive to the most frequent acidic phospholipid in the plasma membrane, and to phosphatidylinositol 4,5-bisphosphate [24]. Ligands activating TRP channels are broadly divided into the groups of exogenous small organic molecules (capsaicin, icilin) and endogenous lipids or product of lipid metabolism (eicosanoids, diacylglycerols) [25].

In mammals, 28 channel subunit genes have been identified and subdivided into 7 subfamilies TRPA, TRPC, TRPM, TRPML, TRPN, TRPP, and TRPV, E [24].

2.5.2 The TRPV subfamily

The first mammalian TRPV was discovered during the research for channels activated by inflammatory vanilloid compound capsaicin [24]. TRPV family encompasses six members, possible to further classify on the basis of their structure and function into two groups TRPV1–V4 and TRPV5 and TRPV6. The so-called thermal TRPs comprise TRPV1-TRPV4 that are activated by heating in heterogeneous expression system. TRPV channels seem to be employed in thermo-sensation. **TRPV1** receptors are highlighted in the text below [24].

TRPV2 receptor can be activated by noxious stimuli (≥52°C) but not by capsaicin or homeostasis changes in pH. TRPV2 receptors are distributed in dorsal root ganglia, CNS neurons, gastrointestinal tract, spleen, mast cells, smooth, cardiac and skeletal muscle cell.
TRPV3 and **TRPV4** are activated at warm temperatures in the ranges of 33–39°C and 27–34°C [24]. TRPV3 receptors are mainly located in dorsal root and trigeminal ganglia for mediating thermo-sensation to moderate heat, nociception and wound healing.
TRPV5 and **TRPV6** differ from other TRPV sub-families in the way that they do not mediate a thermo-sensation because, they are not activated by heat. TRPV5 and TRPV6 belong to the most Ca^{2+} selective channels (P_{Ca}: P_{Na} > 100) of the mammalian TRPs. Although both channels are highly selective and permeable to $Ca2^{+}$, in the absence of extracellular $Ca2^{+}$ they might be permeable to monovalent cations [26].

2.5.3 TRPV1 channel and its role in nociceptive transmission

The TRPV1 channel stays in the centre of focus due to their ability to be activated only be painful stimuli. Topically used capsaicin binds to TRPV1s and desensitizes the nociceptive terminal to all modes of noxious stimuli by inhibiting C-fibres, this interaction can provide a pain relief [26]. Consistent with their essential role in nociception, the expression of TRPV1 is mainly detected in small to medium size neurons in the trigeminal ganglia (TG) and dorsal root ganglia (DRG). TRPV1s are non-selective cation channels, which mediate the response to a number of painful stimuli, such as noxious heat, extracellular acidosis, and they are also the receptor for capsaicin, which is the pungent agent from chilli peppers that elicits burning pain. TRPV1s can be modulated by inflammatory agents as well as by noxious heat. TRPV1s are arguably considered as best-characterized member of vertebrate TRP family. It was the first member of mammalian TRPV sub-family that was discovered in sensory neurons. Its wide expression and function of TRPV1s have become a subject of many types of research and have been studied most extensively. TRPV1s and other members of TRP family have become considered as fundamental targets for analgesic drugs and their development [23]. TRPVs are composed of six transmembrane spanning domains and establish the channel structure with a pore-loop region interdicted between transmembrane domains five and six (Fig 2.3) [27].

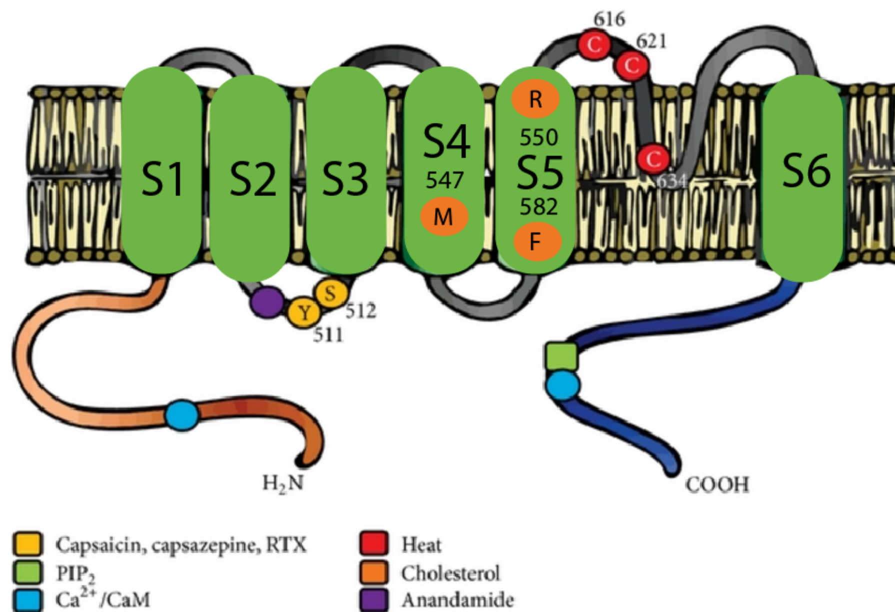

Figure 2.3: The TRPV1 primary structure involves six transmembrane segments (S1–S6) with a pore domain between the fifth (S5) and sixth (S6) segment, and both C and N termini are located intracellularly. Amino acid residues involved in the binding of chemical and physical activation/modulation of TRPV1 activity are indicated in a colour scheme. Figure adapted from [27].

2.5.4 Activation of TRPV1

TRPV1s are involved in many different signaling pathways in the cell and functions. Their role there is of an integrator for a variety of sensory inputs. They are a polymodal receptor that can function as a molecular stimulus integrator, especially in primary afferent fibres [20]. There is a wide range of extracellular stimuli capable of activating TRPV1s, including noxious heat (> 43-45 °C), plant derivatives (capsaicin), by low pH < 6 and other environmental irritants. TRPV1s are also activated by various endogenous lipids, such as anandamide,mN-arachidonoyl-dopamine, and various metabolic products of lipoxygenases. In the activation of TRPV1s can also participate extracellular events such as inflammation that may trigger signaling pathways leading to sensitization and activation. For instance, bradykinin, a potent pain-causing substance, indirectly activates TRPV1s via distinct associated receptor system (phospholipase C or protein kinase C pathway) [28].

2.5.5 TRPV1 activation by capsaicin

TRPV1 receptors can be activated by capsaicin, which belongs to vanilloids family (compounds which possess a vanillyl group). Capsaicin evokes the depolarization of cell membrane by an influx of sodium and calcium cations. It is suggested that to initiate a respond of TRPV1 the binding of at least two capsaicin molecules is required [23]. Capsaicin a member of group known as capsaicinoids cause the spicy flavor (pungency) of chili pepper fruit. Capsaicin is identified as a unique alkaloid primarily found in the fruit of the capsaicin genus due to its wide range of potential applications the interest of capsaicin has increased. The pungency of capsaicin has limited use in clinical trials to support its biological activity. Together with dihydrocapsaicin represent approximately 90% of capsaicinoids in chili pepper fruit. Capsaicin (trans-8-methyl-*N*-vanillyl-6-nonenamide) can by characterized as crystalline, colorless, lipophilic and oderless alkaloid (Fig 2.4) that is fat-, alcohol- and oil- soluble [29].

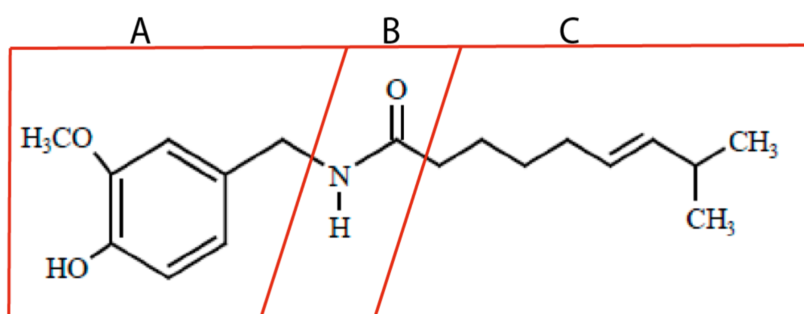

Figure 2.4: Regions of the molecule of capsaicin: (A) aromatic ring; (B) amide bond, and (C) hydrophobic side chain [29].

Structure-activity relationships (SAR) for capsaicin agonist can be divided into three regions. Aromatic ring, amide bond and hydrophobic side chain. It is known that the substituents in the positions 3 and 4 of the aromatic-ring are essential for potent agonist activity, and the phenolic 4-OH group in capsaicin analogues is of particular importance, H-bond donor/acceptor properties of the phenol group are key for agonist activity [29].

3. Central modulation of pain

3.1 Psychosocial factors

Despite the ubiquity of pain, its interaction with psychological and neurophysiological factors still remains controversial.

Psychosocial factors such as fear, depression and anxiety have powerful impact on the perception of pain however, the brain mechanisms underlying the connection between pain and emotions remain largely unknown [23].

The presence of mental disorder has negative impact on coping with chronic pain, whereas cognitive factors such as attention and emotional factors such as fear seem to be risk factors for acute pain and also influence acute pain intensity (Fig 3.1) [30]. Broad interaction between pain and emotions are given by the multiplicity of mechanisms of cerebral and cerebrospinal modulation of pain by emotions (Fig 3.1) [31]. One of the analysis examining the relationship between pain and emotions suggested an involvement of prefrontal, parahippocampal and brainstem regions as a modulation structures [31] .

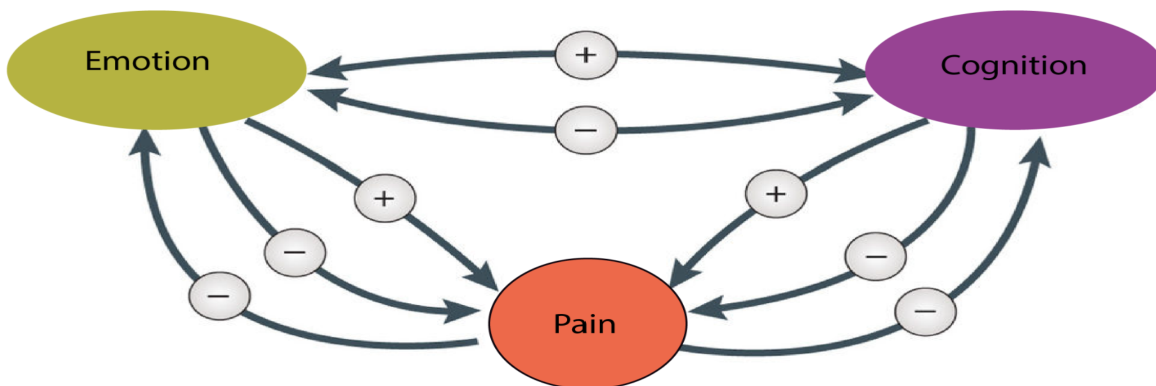

Figure 3.1: Emotional and cognitive components of pain. Feedback loops shows association between pain, emotion and cognition as and significant components of pain perception. Where cognitive states such as memory, attention but also negative or positive emotional state can cause significant changes in pain perception. The minus sign refers to a negative effect and the plus sign refers to a positive effect. Loop adapted from [31].

The correlation between the activation of sensory receptors (nociceptor), which are highly specialized to detect actual or potential tissue damage and the activation of brain regions that drive the experience of pain sensation is not always apparent. It has been known, that the experience of pain varies dramatically between individuals. Even in the similar cases of injuries, individual pain experience is highly variable. Another important part which can either enhance or diminish the pain experience are emotions. Emotional state, degree of anxiety, attention, memories and past experience may all have a large impact on the perception of pain [32]. Still today, how emotional responses to painful stimuli are generated in the brain remains one of the mysteries in the field of neuroscience [33]. Certain cortical structures such as somatosensory, cingulate and insular regions as well as subcortical structures such as the basal ganglia and amygdala significantly contribute to the perception of pain and are part of a larger pain processing network. It is believed that the forebrain plays an important role in emotional-affective and in cognitive dimensions of pain, however, this contribution to pain is little understood [34].

3.2 Role of prefrontal cortex in pain and fear

The prefrontal cortex (PFC), in general, is associated with high-order emotional and cognitive functions, including working memory, goal-directed behaviour [35], the ability to learn associations between contextual cues and behaviourally meaningful actions, decision making and emotional response [36].

3.2.1 Prefrontal cortex

The mammalian prefrontal cortex can be distinguished by anatomical criteria such as cytoarchtectonic features, connectivity patterns with the other brain regions, electrophysiological properties, protein expression and changes in behaviour following damage. There is a basic acceptance for a homology of PFC between rodents and higher-order species [37]. Rodent PFC like human PFC is fundamental to a broad array of behavioural functions [38].

The prefrontal cortex is part of the frontal lobe in rodents and is generally subdivided into three main sub-territories. The medial PFC (mPFC), the ventrally located orbital PFC, and

the laterally located cortical region termed the lateral (sulcal) PFC, which corresponds to the anterior insular cortex [39].
The medial and lateral PFC regions were the main focus of our experiments. The mPFC in rodents can be further subdivided on the basis of cytoarchitectonics into the dorsally located anterior cingulate cortex (ACC), the more ventrally located infralimbic area (IL), and the prelimbic area (PL), sandwiched in between the former two [40]. These mPFC regions are highly interconnected and have wide reciprocal connections to sensory, motor and limbic cortices. This feature makes the mPFC a central hub to integrate and synthesize information from large number of different sources [41].

In the lateral PFC, an accepted subdivision of the insular cortex (IC) is based again on cytoarchitectonic features. The three described subregions of the IC are agranular insular cortex (AI), dysgranular insular cortex (DI) and granular insular cortex (GI) [42, 43]. One of the main cytoarchitectonic patterns of rodent PFC are laminar structures organized into deep and superficial layers, which are distinguished between the neighbouring structures but can be less organized or defined compared to primates [44].

3.2.2 Population of cells in PFC

In order to process information in PFC, multiple interactions between distinct neuronal cell populations are required. As in other cortical areas there are two main neuronal cell categories: 80-90% of all neurons are principal cells which encompass subclasses of pyramidal cells that are well suited for long-distance projections and the remaining 10-20% are covered by several classes of interneurons exhibiting different firing patterns [45].

3.2.3 Physiological functions of PFC

Tight control of fear regulation encompass prefrontal cortex (especially mPFC) which seems to be a major part of top-down control driven over subcortical structures [40, 46] and it is in the centre for modulating goal-directed behaviour [47]. The medial prefrontal cortex has been associated with numerous diverse functions including decision making, memory, emotions, attentional processes, visceromotor activity, working memory, long-term memory, and in regard to our work fear memory aquisition, storage and retrieval as well as cognitive dimension of pain [40, 47]. Tight control of fear regulation encompassing

prefrontal cortex (especially the mPFC seems to be a major part of top-down control driven over subcortical structures [47].

3.3 Fear memory and engram cells

Fear memories allow an individual to avoid a dangerous situations and thus increasing the chances of survival. Fear learning is closely connected with time-dependent reorganization of neural circuits, whereas the innate fear behaviour appears to be constant with time. Fear memories can be retrieved and reorganized long after learning and one of the regions considered as a crucial for fear retrieval is the medial prefrontal cortex [48]. It is suggested that the information stored in this neocortical structure is necessary for retrieval of recent and remote fear memory. During learning and the process of memory consolidation the preferential co-activation of a specific subset of neurons is required, forming an ensemble of so-called engram cells [49].

Engram cells are defined as specific population of neurons that are activated by learning, exhibits enduring molecular changes, and possess the ability to be reactivated in memory recall by the original stimulus. By its definition engram cells are fundamental at any stage of a memory [50].

3.3.1 Consolidation of fear memory

After the activation of a mPFV engram cells by a painful stimulus, fear-inducing components are not directly stored in the PFC but require other brain areas to become functionally mature with time [51]. These authors propose that during consolidation of the fear memory the initial information (recent memory) is first stored within engram cells of the hippocampal-entorhinal cortex (HPC-EC) formation, encoding contextual and spatial information, and over time becomes consolidated within mPFC cells. This corresponds to standard model of memory consolidation, where the first information is rapidly stored in the hippocampus and over longer period of time the memory is transferred to engram cells within the mPFC [52].

The activity of neocortical areas, such as the rodent mPFC was found to be necessary for recent and remote fear recall [53]. However, more recent experiments by the Tonegawa lab found that mPFC engram cells were preferentially reactivated at remote time points [54].

In early studies of the role of PFC, it has been noted that dorso-ventral regions of PFC mediate different behavioural responses and autonomic outcomes, pointing to the possibility of studying overlapping and distinct functions of PFC subregions [55]. The prelimbic and infralimbic subregions of mPFC are functionally distinct, and exhibit different activity during fear behaviour.. They differentially mediate stimulus response and action-outcome learning [40].

PL and IL have reciprocal c-Fos expression patterns in response to a contextual fear stimulus. IL exhibits increase of IEG during presentation of extinguished CS in extinction context whereas PL is associated with a robust increase of c-Fos expression during fear renewal (contextual fear recall) [40]. However, these studies suggest that even though the PL and IL have opposed roles in the expression and suppression of fear, activity in both of these mPFC regions may underlie new forms of learning [56].

3.3.2 The role of anterior cingulate cortex in fear

The anterior cingulate cortex (ACC), one of the mPFC subregions, which is ideally positioned to integrate emotion and cognition signals between limbic and other cortical structures. It is a key region for emotion and learning processing but also a region with strong involvement in several processes such as anticipation, pain, attention, error monitoring and effortful recall and remote spatial memory.

Due to its location, ACC may somehow be involved in the control of amygdala dependent fear learning. There is strong evidence for anatomical ACC-amygdala connectivity [47].

ACC consistently emerges from inactivation, activation and neuroimaging studies an involvement in normal and abnormal fear processing. More in detail the previous evidence shows that the inactivation of ACC, notably impairs fear learning, whereas potentiation of activity within ACC mediates and enhances fear acquisition and expression [57].

3.3.3 Involvement of prelimbic cortex in fear

The vmPFC is composed of the infralimbic cortex (IL) ventrally and the prelimbic cortex (PL) dorsally. Recent electrophysiological findings suggest that IL and PL have opposite influences on fear expression [58].

Prelimbic cortex (PL) has been implicated in memory modulation, memory consolidation and fear retrieval [59]. It is one of the proposal brain regions that integrates inputs from

amygdala, hippocampus and other cortical sources involved in fear conditioning, in order to regulate the expression of fear memories.

Studies show involvement of PL in the selective expression of conditioned fear to a certain cue that is fully dependent upon contextual information [60]. Due to the broad range of inputs from auditory cortex, hippocampus and amygdala into PL, there is a proposal that PL may participate in signal processing from various structures [61]. The higher responsiveness and excitability of PL for those inputs might be caused by fear-induced release of neuromodulators such as noradrenaline and dopamine within PL [62]. The release of neuromodulators could significantly contribute in augmentation of sustained fear response. Broad connections of PL within various brain structures allows PL to have important role as a main site that integrates stress-related, contextual and auditory signals [63].

Together with recently given evidence from lesion and inactivation studies of mPFC pointed out PL as crucial region involved in the expression of fear memory but not in the fear extinction, which is more likely associated with IL, This fact can by supported by suggestion that cases of extinction failure in mice can be caused by excessive activity in PL, combined with lack of activity in IL [64].

3.3.4 Involvement of infralimbic cortex in fear

Infralimbic cortex (IL) as a part of vmPFC is closely associated with neural circuits modulating extinction of conditioned fear, upon the contextual and temporal factors, it is also one of the main region of PFC responsible for suppression of fear.

Memory extinction can be defined as regulatory mechanism, when the inhibitory circuits gradually decrease the expression of fear memory, after repeated presentation of conditioned stimulus (CS). As a result of extinction learning, the fear response is no longer induced by the repeated CS [65].

One of the proposal mechanism, is that the extinction does not erase fear memory but more likely generates new safety memory [47]. Memory extinction can be also considered as highly labile mechanism of fear suppression that can easily relapse when previous extinguished cues is performed outside the extinction context [47]. This argument gives a proof to the fact that memory exctinction is highly context-dependent. In general fear extinction involves mainly three structure such as amygdala, PFC and hippocampus. The triad of these region has been deeply studied in terms of fear memory [47].

3.4 Role of prefrontal cortex in pain

In order to alter the sensory experience of pain "top down" modulatory circuits which may diminish or enhance to the perception of painful stimuli are involved.
Neuroimaging techniques in humans have been used to define the origin of pain modulatory systems within cortical and subcortical regions [65].
A line of neuroimaging studies led to the suggestion of the "pain matrix" theory, which includes brain regions, that are consistently activated by noxious stimuli. The PFC belongs among the brain areas that are highly associated with pain. Brain regions that are part of the "pain matrix" are highly interconnected. These brain regions are involved also in other neurological functions such as cognition and emotion and collectively give rise to the complex experience of pain [66].

3.4.1 Dissociative roles of mPFC

In order to reveal and explain precise function of medial prefrontal cortex, it is necessary to distinguish between different subregion of mPFC to highlight the importance of individual anatomical regions. A robust dorsal-ventral dissociation within mPFC was observed. The dorsal ACC region is responsible in variety of stimuli and psychological states such as pain and cognitive control. The cognitive effects and conflict were overlapping more dorsally, whereas pain seems to be localized more ventrally [33].

3.4.2 Anterior cingulate cortex

The ACC mediates the affective component of pain , the placebo effect and together with ventral mPFC also anticipation of pain [61, 66, 67]. Numerous sources indicate that the ACC is a crucial part of a "neuromatrix" involved in affective-motivational dimension of pain and in pain processing in general [68].
Neurons of ACC encode a broad spectrum of functions and consistently exhibit activation in response to noxious stimuli but not to non-noxious stimuli, such as thermal, chemical and mechanical stimuli [33]. It is unlikely that the dorsal ACC selectively encodes pain as suggested by Lieberman and Eisenberger [69], as the dACC is activated also in anticipatory or empathic situations and participates in diverse cognitive functions such as memory, learning and decision-making. [61].

Accordingly another imaging study in humans suggested that the ACC together with insula are encoding the emotional and motivational aspect of pain [67].

3.4.3 The role of PL and IL mPFC

The PL and IL mPFC regions are involved in diverse emotional, cognitive and mnemonic processes. In addition the ventromedial region of the mPFC was found to make a significant contribution to processing the unpleasantness of pain [31]. Neuroimaging studies in humans revealed that spontaneous pain is associated with novel activity in mPFC [48] subregions that may be comparable with activity patterns occurring during acute pain stimuli [70, 71].
A modulatory function of prelimbic cortex on pain perception was demonstrated by neural imaging studies in humans [72, 69, 73] and the optogenetic manipulation of inhibitory interneurons in mice [74]. Zhang's team also point out a crucial role of the PL in spontaneous pain caused by peripheral nerve injury [48]. Generally these studies found that the neuronal activity of mPFC is enhanced during painful experience and reduced in chronic pain situations [48]. However, how the prelimbic subregion participates in pain processing and how reduced activity in the mPFC may affect pain sensation remains to be resolved [75].

3.4.4 Lateral PFC/ Insular cortex

An increasing evidence of neuroimaging and electrophysiological studies propose the insular cortex as a crucial site processing multimodal salient information to an affective event [48, 76]. Regarding pain there is a suggestion that the IC is involved in encoding both sensory-discriminative and affective-motivational aspects of pain. Due to its abundant connections with other brain areas, the IC likely seems to be an important interface where cross-modal shaping of pain occurs [77].
The subregions of IC have been associated with somatosensory features of pain as well as with modulation of affective aspect [76].
The IC can be viewed as an interface where attention, anticipation or belief shape pain perception by activating cognitive areas such as the dorsal and ventral mPFC. A number of studies on pain anticipation [78] propose that IC activity may be responsible for modulation and enhanced responsiveness within other cortico-cortical areas. Taken

together, it is proposed that the IC serves as an interface to synthesize multimodal information and integrate it with nociception [76].

4. Principles of used methods

4.1 Tetracycline off system

Tet-off technology, also known as the tTA-dependent system, was developed by Prof. Dr. H. Bujard and his team at the University of Heidelberg and was first published in 1992. Since then Tet-off (Fig 4.1) and Tet-on (Fig 4.2) systems are broadly used techniques in order to control gene expression. It allows precise, reversible and highly efficient regulation of gene expression in eukaryotic cells. Tet-off technology (Fig 4.1) allows to switch off the target gene selectively by administration of tetracycline (Tet) or doxycycline (Dox) whereas the Tet-on system is used to selectively activate gene expression by doxycycline [79, 80].

The Tet System consists of two critical components. The first element is a regulatory fusion protein consisting of the tetracycline repressor protein (TetR) normally found in gram negative E.coli, and the activator protein VP16 from the Herpes Simplex Virus. [81]. The fusion of these two subunits generates a hybrid protein known as tetracycline-controlled transactivator (tTA) and allows to convert the tetracycline repressor protein with inhibitory activity into a transcriptional activator in the absence of tetracycline The second critical component of Tet System is the response plasmid. This plasmid is carrying the gene of interest (Gene X) whose expression is under control of the tetracycline-response element (TRE). The tetracycline-response element is 7 repeats of a 19 nucleotide tetracycline operator (tetO) sequence placed upstream of a minimal promoter and it is recognized by the TetR domain of tTA. In the absence of tetracycline, tTA dimers will bind these tetO sequences and the VP16 activation domain promotes gene expression. In the presence of Tet or Dox, these effector molecules induce a conformational change in the TetR domain and this precludes tTA binding to the TRE, thus silencing gene expression [80].

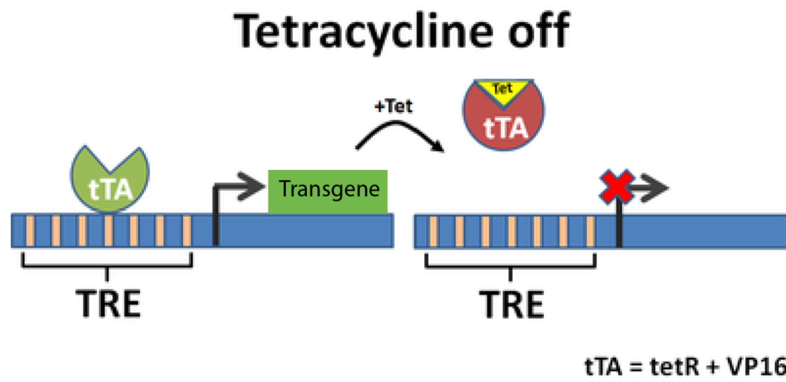

Figure 4.1: Scheme of Tet off system. In the presence of tetracycline, expression from the Tet-inducible promoter is inhibited. Adapted from [81].

4.2 Tetracycline on system

The system also known as the rtTA-dependent system has been discovered in the year 1995 when Gossen and his team randomly mutated amino acid residues of tetR, which are fundamental for tetracycline-dependent repression (Fig 4.2). They succeeded in developing a reverse Tet repressor or rTetR, which now binds the TRE only in the presence of tetracycline to induce gene expression [80].

In both Tet-On and Tet-Off Systems, transcription is turned on or off in response to Dox or Tet in a precise and dose-dependent manner. The main advantage of Tet and Dox is that they are inexpensive, well characterized, and yield highly reproducible results [80].

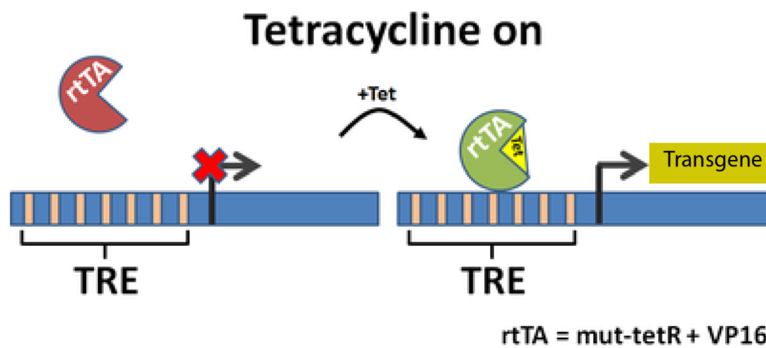

Figure 4.2: Scheme of the Tet on system in the presence of tetracycline, expression from the Tet-inducible promoter is initiated. To use tetracycline as a regulator of gene expression, a reverse tetracycline-controlled transactivator (rtTA) has to be present adapted from [81].

4.3 DREADDs – Designer Receptors Exclusively Activated by Designer Drugs

In the field of neuroscience a large number of chemogenetics method and technology have been made available for remote user-defiened control of neuronal activity [81]. DREADDS, the chemogenetically engineered proteins are to date to most widely used technique. This method exploits G-protein-coupled receptor signaling pathways and their high involvement in the control of physiological conditions to modulate cellular activity [82].

4.3.1 Biotechnology of DREADDS

Designer Receptors Exclusively Activated by Designer Drugs, DREADDs involve the use of receptor proteins derived from targeted mutagenesis of endogenous G-protein coupled receptor DNA to yield synthetic receptors [82].
DREADDs represent a biotechnology whereby designed G protein-coupled receptors (GPCRs) were synthetically created in order to be activated solely by an extrinsic ligand. These engineered GPCRs have become a widely used method, which aids neuroscientists to elucidate the circuits of perception, behaviour, emotions, innate drives and motor

functions [82]. DREADD development has been based on muscarinic acetylcholine receptors from which multiple teams of scientists developed a whole group of mutant muscarinic receptors for three G protein-dependent signaling pathways (Gi, Gq, and Gs). All three receptors should be solely activated by Clozapine N-oxide (CNO) (Fig 4.3) [83].

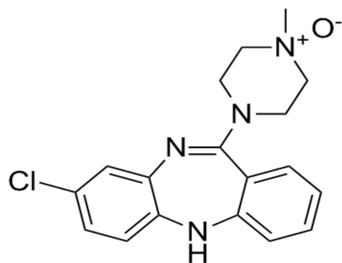

Figure 4.3: Structure of Clozapine N-oxide (CNO) [82]

In addition, DREADDS may be designed in many different pharmacological profiles. These highly specialized DREADDS are then expressed in target cell populations or disease-associated cells, aiding the search for pharmacological targets [83].

GPCRs with constitutive activity and with high levels of expression may lead to signaling even in the absence of ligand. Accordingly, DREADDs should be expressed at the lowest potential levels. However, for hM3Dq and hM4Di it was possible to achieve a life-long and extremely high levels of expression due to the use of the genetically encoded tetracycline-sensitive system, without inducing basal electrophysiological, behavioural, or anatomical abnormalities [80]. DREADDs as any other receptors may be desensitized and subsequently down-regulated due to the impact of repeated dosing of DREADD chemical actuator [83].
One of the most commonly used DREADDs for silencing and for activating neurons are hM3Dq and hM4Di (sections 4.3.2 and 4.3.3). An intracranial delivery of an expression vector via transgenic viruses is utilized most frequently to transfect brain cells. The viral construct typically carries the DREADD transgene, an ubiquitous or cell-specific promoter and a fluorescent reporter [82].

Despite the fact that DREADDs have been used in more than 800 studies this popular chemogenetic technique needs to be used cautiously when used for *in vivo* studies [82] [84].

4.3.2 Gq-DREADDS, CNO Analogues, and Basal Activity

hM1Dq, hM3Dq, and hM5Dq, belong to the group of excitatory Gq- DREADDS, and each of these receptors is based on different muscarinic receptor.h3MDq DREADD can be considered as one of the most frequently used excitatory DREADDs and is typically used in order to enhanceing neuronal excitability and firing activity [85]. Several options are available for expressing hM3Dq in genetically specified cells. Expression of hM3Dq in genetically modified mice can be controlled by the Tet- system and via Cre-mediated recombination [82].
The prototypical chemical actuator for Gi/Gq-DREADDs is Clozapine N-oxide (CNO). It has been long assumed that CNO, as an inert metabolite of the atypical antipsychotic drug clozapine, can cross the blood brain barrier and has very good pharmacokinetics properties [82]. However, recently it has been revealed that only clozapine, the reverse product of CNO crosses the blood brain barrier and possess a high affinity for DREADDs. This finding contradicts the long-term assumption that DREADDs are exclusively activated by the designer drug. Furthermore, it puts an emphasis on using appropriate controls such as admininstering CNO to animals that express only a fluorescent marker and do not express the designer receptor itself. In that way one can test for potential effects of CNO-derived clozapine, that potentially activates endogenous serotonin and dopamine receptors in addition to DREADDs [82].

4.3.3 Gi-DREADDs

Gi-DREADDs which allow the silencing of neuronal activity are hM2Di, hM4Di and the κ-opioid-derived DREADD (KORD). hM4Di is the most frequently use DREADDs with inhibitory activity.
hM4Di may be activated by clozapine [84] whereby KORD as another class of chemogenetic GPCR, is activated by salvinorin B, which does not exhibit any activity to other tested molecular targets (>350 GPCRs, ion channels, transporters, and enzymes

evaluated). According to reports from several labs, salvinorin B may by considered as pharmacologically inertand can serve as a KORD ligand to inhibit activity in neurons that express a complementary signaling cascade [84].

4.4 Immunohistochemistry

4.4.1 DAPI staining

4’, 6-diamidino-2-phenylindole, also called Dapi, is a blue fluorescent dye commonly used in immunohistochemistry methods for visualizing nucleic acids. Dapi has an ability to passes though the cell membrane and then bind to regions of dsDNA. It is excited by the violet (405 nm) laser line [85].

4.4.2 NeuN – Neuronal nuclei

Neuronal nuclei is a soluble neuron-specific nuclear protein recognized in a vertebrate nervous system. The expression of NeuN is observed in most neurontypes in the nervous system However in certain types of neurons, for instance in Purkinje cells, NeuN is not present. Immunohistochemical detection of NeuN is possible after the initial development serves as an excellent neuronal marker in the central and peripheral nervous system [86].

4.4.3 Immediate-early gene product c-Fos

c-Fos as a product of immediate-early gene (IEG) expression serves as a marker of neuronal activation in the central nervous system. Therefore c-Fos staining takes an important place among immunohistochemical methods and provides a powerful tool in experimental studies that aim to map cellular activation within brain areas [87].

4.5 Fear conditioning

In need to characterize phenotypes of mutant mice and the effects of genetic alterations fear conditioning (FC) has gained popularity over the last few decades. FC is a convenient tool to study associative learning processes. It is a subtset of classical conditioning that includes the association between conditioned stimulus (CS) and unconditioned stimulus

(US) and evokes freezing behaviours associated with fear. As unconditioned stimulus (US) an electric foot shock (in rats and mice) or an air puff to the eye are typically used [88].
A particular context and/or cue, for instance a tone typically serve as CS so that two types of FC, contextual or cued are distinguished (Fig 4.4). The freezing behaviour of an animal can be defined as "absence of movement except for respiration" and is usually quantified by measuring laser beam breaks or pixel changes in digital movie sequences [89].

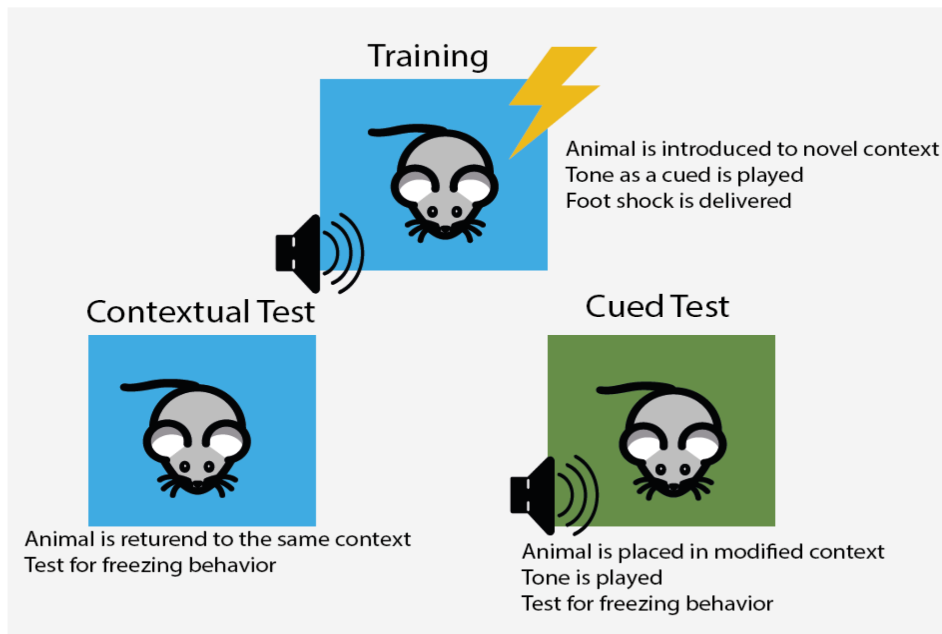

Figure 4.4: Fear conditioning is a form of associative learning in which an aversive fear-evoking stimulus (the unconditioned stimulus) is paired with exposure to a novel environment or a sensory cue such as a tone (the conditioned stimulus) [89].

4.5.1 Contextual fear conditioning

One form of conditioning involves placing an animal in a novel environment and providing an aversive stimulus. If the animal is later returned to the same environment, the freezing response related to fear can be assessed [90].

4.5.2 Cued fear conditioning

This form of conditioning differs from contextual conditioning by pairing a CS with the

US in a novel context (Figure 4.4). To be able to separate context from cue, the freezing behaviour of animals is then measured when re-exposed to the CS in the context that was used to assess baseline freezing behaviour [90].

Experimental Part

5. Materials

5.1 Animals

C57BL/6JRj mice (Janvier Labs, France),(25–30 g, eight weeks old) were housed in groups of 2–4 per cage and were maintained with food and water ad libitum on a 12 h light/12 h dark cycle. All experimental procedures were performed according to the German Animal Welfare Act and were approved by the local governing body (Governmental Council in Karlsruhe, Germany, approval numbers G115/14 and G205/14).

5.2 Equipment

Coverslips (Medite GmbH; Brugdorf, GE)
Falcon tubes 15 ml, 50 ml (Sarstedt AG & Co.; Nümbrecht, GE)
Microscope slides (Gerhard Menzel B.V&Co. KG. Braunschweig, GE)
Microscope slides (NeoLab Migge Laborbedarf-Vertriebs GmbH; Heidelberg, GE)
Microtube 1.5 ml (Sarstedt AG & Co.; Nümbrecht, GE)
Pipette (Gilson, Inc.; Middleton, WI, USA)
Pipette boy – neoAccupette (Wager & Munz GmbH; München, GE)
Pipette tips (Greiner bio-one GmbH; Frickenhausen, GE)
Serological pipette 5 ml, 10 ml, 25 ml (Sarstedt AG & Co.; Nümbrecht, GE)
Tissue culture dishes (Becton Dickinson Labware; Le Pon de Claix, France)

5.3 Instruments

Digital camera Nikon DS-Qi1Mc (Nikon Instruments Europe B.V, GE)
Digital camera Nikon DS-Ri1Mc (Nikon Instruments Europe B.V, GE)
Epifluorescence microscope Nikon Ni-5 (Nikon Instruments Europe B.V, GE)
Fear Conditioning System Ugo Basile (21036 GEMONIO - Varese – ITALY)
Leica Vibrotom VT 1000S (Leica Microsystems GmbH; Wetzlar, GE)
LSC microscope Leica TCS SP8 (Leica Microsystems GmbH; Wetzlar, GE)
Microscope Leica DM LS2 (Leica Microsystems GmbH; Wetzlar, GE)
Objectives 10x, 20x, 40x PL APO CS2 (Leica Microsystems GmbH; Wetzlar, GE)

5.4 Chemicals

All used chemicals were in the highest purity.
Capsaicin (Sigma-Aldrich Chemie GmbH; Steinheim, GE)
DAPI (Sigma-Aldrich Chemie GmbH; Steinheim, GE)
DMSO (Sigma-Aldrich Chemie GmbH; Steinheim, GE)
Fentanyl 0.05 mg/ml(Sigma-Aldrich Chemie GmbH; Steinheim, GE)
Formaldehyde, (Applichem, Darmstadt, GE)
Glycin (AppliChem GmbH; Darmstadt, GE)
Horse serum, liquid (Sigma-Aldrich Chemie GmbH; Steinheim, GE)
Isoflurane (Baxter Deutschland GmbH; Heidelberg, GE)
Lidocain 10%(Sigma-Aldrich Chemie GmbH; Steinheim, GE)
Medetomidine hydrochloride (1mg/ml) (Sigma-Aldrich Chemie GmbH; Steinheim, GE)
Midazolam 5mg/ml (Sigma-Aldrich Chemie GmbH; Steinheim, GE)
Mowiol 4-88 (Sigma-Aldrich Chemie GmbH; Steinheim, GE)
Paraformaldehyde (Applichem, Darmstadt, GE)
Pentobarbitalum 1mg/1ml (Sigma-Aldrich Chemie GmbH; Steinheim, GE)
Phosphate Buffered Saline (PBS)(Sigma-Aldrich Chemie GmbH; Steinheim, GE)
HEK 293 cell line(Sigma-Aldrich Chemie GmbH; Steinheim, GE)
Triton X-100 (Sigma-Aldrich Chemie GmbH; Steinheim, GE)
Tween 80 (Sigma-Aldrich Chemie GmbH; Steinheim, GE),

5.5 Antibiotics

Doxycycline Hyclate (Envigo, Huntingdon, UK)

5.6 Plasmid constructs

pAAV-cFos-tTA-pA
pAAV-PTRE-tight-hM3Dq:mCherry
pAAV-cfos-tTA2G
(all obtained from Addgene, deposited by W. Wisden, Imperial College, London, UK)
pAAV-6p-Cminibi.iCreCheery (provided by Rolf Sprengel, Max Planck Institute for Medical Research, Heidelberg, GE)

pAAV-PTRE-tighthM4Di:mCherry (Addgene, deposited by W.Wisden, Imperial College, London, UK)

5.7 Recombinant viruses

rAAV-cfos-tTA2G
rAAV-pTRE2G-M4:mCherry
rAAV-pTRE2G-M3:mCherry
rAAV-cfos-tTA-pA
rAAV-6p-Cminibi.iCreCheery

5.8 Software

ANY-maze behavioural tracking software (Stoelting Europe, Dublin 6, Ireland)
Fiji Is Just Image J (Image J, Curtis Rueder, UW-Madison LOCI, USA)
GraphPad, Prism 6 (GraphPad Software, USA)
Leica Microsystems CMS Software License (Leica Microsystems GmbH, Wetzlar, GE)
NIS-Elements Acquisition Software (Nikon GmbH, Düsseldorf, GE)

5.9 Antibodies

5.9.1 Primary

anti-c-Fos (rabbit; Millipore, Merck Milipore Burlingto, USA)
anti-NeuN (mouse, Millipore, Merck Milipore Burlingto, USA)

5.9.2 Secondary

donkey anti-rabbit Alexa 488 (Jackson Laboratory West Grove, PA, USA)
donkey anti-mouse Alexa 647 (Jackson Laboratory West Grove, PA, USA)

6. Methods

AAV TetTag-DREADD transgenes were generated as previously described [90].
Each of the Tet-Tag transgene components, cFos-tTA, TRE-mCherry, cFos-tTA2G, TRE-hM3Dq:mCherry and TRE-hM4Di:mCherry, were cloned in an AAV expression vector

and packaged separately in AAV capsids of mixed serotype (AAV1 and AAV2) as described by Zhang *et al.* (2015). The *pTRE-hM4Di:mCherry* construct was generated in the laboratory of W. Wisden by replacing the DREADD transgene of the PTRE-tight-hM3Dq-mCherry-WPRE-pA expression vector. These Tet-Tag transgene components contained the second generation (2G) tet-operator promoter ($P_{TRE\text{-}tight}$) [34].

6.1 Generation of recombinant AAV particles

All AAV transgenes were packaged into AAV capsids (mixed serotype 1 & 2, 1:1 LORR ratio of AAV1 and AAV2 capsid proteins using AAV2 ITRs) by a technician of the Pharmacological Institute at Heidelberg University according to established procedures [34]. As packaging cell line HEK293 cells were used.

6.2 Surgical procedures

Viral delivery: *In vivo* delivery of AAVs (Table 6.2) were performed by a qualified scientist of the Pharmacological Institute at Heidelberg University. Eight-week-old male C57BL/6JRj mice (Janvier Labs, France) were deeply anesthetized with an intraperitoneal injection of fentanyl (0.05 mg/ml), medetomidine hydrochloride (1 mg/ml) and midazolam (5 mg/ml) mixture (4:6:16, 0.7 µL per gram body weight). A thin layer of lidocaine (10%) was applied to the epidermis and a small craniotomy was made above the region of interest (Fig 6.1). The AAV mixture was slowly injected by applying of pneumatic pressure via a glass pipette using the stereotaxic coordinates listed in Table 6.1.

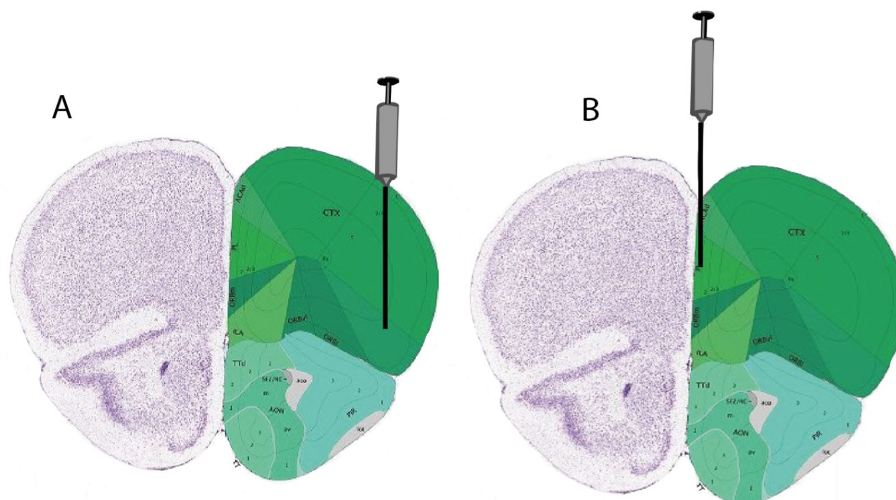

Figure 6.1: A) Injection site of Dox Delta animals. (B) Injection site of Funky and AlphaZ experimental group

Table 6.1: Coordinates of rAAV delivery to target the PL (Funky, AlphaZ) and the AIC (Delta) according to the mouse brain atlas [91]. ML, medio-lateral from the midline; AP, anterior-posterior relative to Bregma; DV, dorso-ventral from the pial surface membrane.

Dox Delta bilateral AAV injections, 6 X 0,5 µl over 10 min each	**Funky** bilateral AAV injections, 6 X 0,5 µl over 10 min each	**AlphaZ** bilateral AAV injections, 2 X 0,3 µl over 15 min each
ML(x)1,2= +/-2.5 AP(y)1,2= 1.8 DV(z)1,2= -1.5	ML(x)1,2 = +/- 0.25 AP(y)1,2 = +1.8 DV(z)1,2 = -1.5	ML(x) =+/- 0.25 AP(y)= 2,1 DV(z)= - 1.5
ML(x)3,4= +/-2.5 AP(y)3,4= 2.0 DV(z)3,4= -1.5	ML(x)3,4 = +/- 0.25 AP(y)3,4 = + 2.2 DV(z)3,4 = - 1.2	
ML(x)5,6= +/-2.5 AP(y)5,6= 2.2 DV(z)5,6= -1.1	ML(x)5,6 = +/- 0.25 AP(y)5,6 = + 2.6 DV(z)5,6 = -0.9	

Table 6.2: Recombinant AAVs used for individual experimental group

Animals	**rAAV construct**		**Ratio**
Delta 1	AAV-cFos-tTA2G	AAV-TRE2G-hM4Di:mCherry	1:1
Delta 2	AAV-cFos-tTA2G	AAV-TRE2G-M4:mCherry	1:1
Funky 1; 4; 14	AAV-cFos-tTA2G	AAV-TRE2G-M4:mCherry	1:1
Funky 2; 3	AAV-cFos-tTA2G	AAV-TRE2G-M3:mCherry	1:1
AlfaZ	AAV-cFos-tTA-pA	AAV-6p-Cminibi.iCreCheery	3:1

6.3 Behavioral testing paradigms

All behavioral tests were carried out during the light cycle of the animals and were conducted by M. Oswald (PhD) or MD student O. Retana. After an 11 day recovery period from surgery animals were acclimatized for 2 days to the behavioural testing environment and, after 90 s habituation in context A, **Baseline** spontaneous freezing levels measured over a 7 min period the day before fear conditioning. **Fear conditioning** (FC) took place in context B. Following 90 s habituation 5 tones (5 kHz, 75 dB, 30 s) were played over a 7 min period at semi-random intervals and paired to a mild electric foot shock (0.6 mA, 1 s) applied during the last second of each tone (time 29-30s). Except for the naïve group, the fear conditioning session was repeated twice at an interval of 3 h to obtain a robust fear memory lasting over the labeling and testing sessions. **Labelling** (Table 6.3) was conducted after placing animals for 48 h on normal chow without doxycycline (OFF Dox),

by subjecting them either to the first **fear retrieval** (FR) session (7 min period, 5 times 30 sec CS+ tone) or an acute pain stimulus. Animals were placed back on the doxycycline diet (ON Dox) 60 min later. Labelling of engram cells that were active during the conditioned fear response was again performed in context A, either 3-5 days after conditioning to test recent, or after 28-33 days to test remote fear memory (Table 6.3, see Fig 6.2, 6.3 for detailed timelines and behavioural paradigms).

To test for behavioural effects due to DREADD activation, injections of **Clozapine-N-oxide (CNO,** 2 mg/kg, i.p. [82]) or saline (100 µl) were administered.

Capsaicin injection: Mice were lightly anaesthetized in isoflurane and capsaicin (0.06%, 20 µl in 10% DMSO/saline; or 0.06%, 20 µl in 10% DMSO/7% Tween 80/saline) injected subcutaneously in the left hind paw. In functional tests involving DREADDs, capsaicin was administered 2-3 hours following the CNO or saline injection. Mice were tested for nocifensive behavior (paw lifting, paw licking, leg shaking, toe-splaying) within 5-20 minutes following capsaicin injection.. For capsaicin administration mice were lightly anaesthetized in isoflurane**.** The individual testing paradigms are described in detail for each experimental group in behavioural schemes (Fig 6.2, 6.3)

Context A : 20 x 20 cm plastic box placed on a smooth plastic surface inside a Faraday cage with the camera mounted above; a 70 % ethanol solution scented with 2 % benzyl alcohol was used for cleaning parts before the start of each session.

Context B: 18 x 18 cm Perspex box with raised metal grill floor placed inside a black 80 x 80 cm enclosure; the camera was mounted above; lemon-scented detergent was used for cleaning parts between each session.

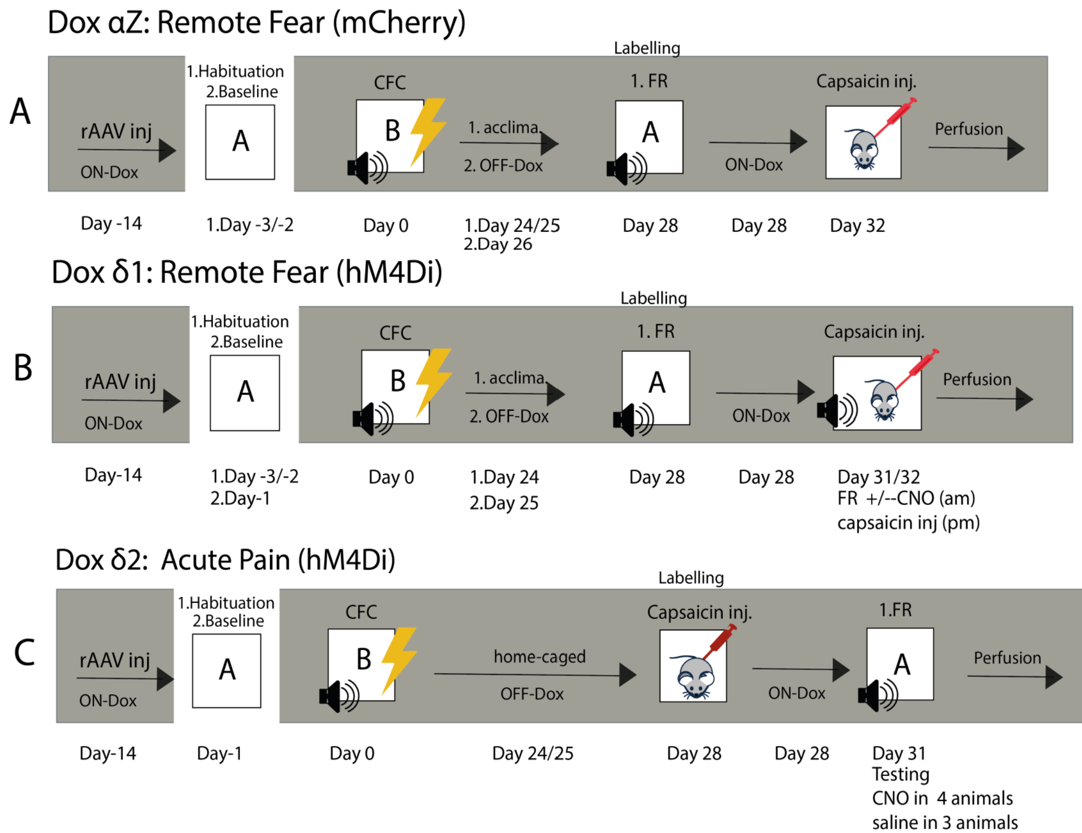

Figure 6.2: Schematic timelines and behavioural paradigm for experimental groups of mice TetTag-labelled for acute pain or conditioned fear. A) AplhaZ group animals transduced with activity-dependent fluorescent reporter vectors (mCherry) were subjected to CFC, engram cells labelled for remote FR and exposed to an acute nocifensive stimulus (0.06% capsaicin in 10% DMSO & 7% Tween 80) 60-90 min before perfusion. B) Dox Delta 1 group animals transduced with activity-dependent DREADD vectors (hM4Di) were subjected to CFC, engram cells labelled for remote FR and tested for acute pain behaviour (Capsaicin 0.06%, 20 µl in 10% DMSO/saline) while activating or not activating the DREADDs. C) Dox Delta 2 group animals transduced with activity-dependent DREADD vectors (hM4Di) were subjected to CFC, engram cells labelled in acute pain caused by capsaicin injection (0.06%, in 10% DMSO/ 7% Tween 80) and FR tested while activating or not activating. CFC= cued fear conditioning,, FR= fear retrieval.

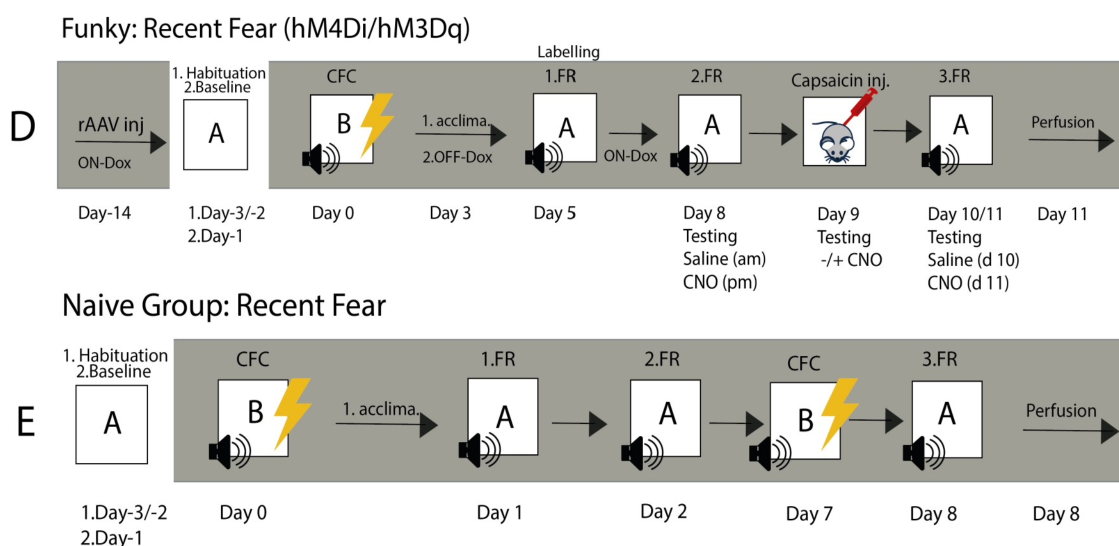

Figure 6.3: D) Funky group animals transduced with activity-dependent TetTag AAV vectors (hM4Di/ hM3Dq) were subjected to CFC, labelled in recent FR, and later tested for FR and acute pain (Capsaicin inj. 0.06%, 20 µl in 10% DMSO/saline) while activating (CNO) or not activating (saline) DREADDs. E) Naive mice were subjected to CFC twice and tested each time, once after the first CFC and once following the second FC. CFC= cued fear conditioning,, FR= fear retrieval.

Table 6.3: Behavioral tests used to label neuronal ensemble activity by the TetTag and endogenous c-Fos expression methods.

Experimental group	TetTag labelling stimulus	Endogenous c-Fos expression stimulus	Fear memory retrieval time
Dox Delta 1	Tone-cued Fear	Capsaicin (pain)	remote
Dox Delta 2	Acute pain (Capsaicin)	Tone-cued Fear	remote
Funky 1-4;14	Tone-cued Fear	Tone-cued Fear	recent
Alpha Z	Tone-cued Fear	Capsaicin (pain)	remote

6.4 Fixation

After the last behavioural test animals were returned to the homecage for 60 to 90 min before they were sacrificed with a pentobarbital overdose and immediately perfused transcardially with phosphate-buffered saline (PBS; pH 7.4) followed by 10% formalin-

fixative solution. Brains were removed and postfixed at 4 °C for a further 24 h in 10%formalin, and stored in PBS at 4 °C for up to 3 days until sectioning.

6.5 Brain slice preparation

The cerebellum was removed by a coronal cut at the level of the brainstem and the forebrain was glued via the coronal cut to the vibratome stage (Leica VT1000S, Germany). Coronal sections (50 µm) were collected and stored in 0.5% formalin at 4 °C until processed further with immunohistochemical procedures.

6.6 Immunohistochemistry protocols

6.6.1 c-Fos and NeuN

Brain sections (50m) were collected and washed in PBS containing 50 mM glycine for 10 min and rinsed in PBST (0.2% Triton in PBS). Section were subsequently blocked for 60 min in 10% horse serum and 0.2% Triton in PBS at room temperature under gentle agitation. Sections were then incubated in a primary antibody cocktail (rabbit anti-cFos 1:5000, mouse anti-NeuN 1:1000) in blocking solution overnight at 4 °C.

After incubation, primary antibodies were washed out with PBST three times, 15 min each, and the sections subsequently incubated with secondary antibodies (anti-rabbit Alexa 488, 1:700 and anti-mouse-Alexa 647, 1:700) in Ab-buffer (PBST/10%NHS) for 2 hours. After incubation, secondary antibodies were rinsed out with PBST twice for 10 min each. Sections were then incubated in DAPI/PBS (1:10000) for 15 min. This was followed by washing sections twice in PBS for 10 min and then 10 min in 10mM TRIS-HCl. The sections were mounted onto glass slides from TRIS-HCl and coverslipped with Mowiol. Immunostained sections were examined either with an epifluorescence microscope equipped with a digital camera or a laser-scanning confocal microscope and images were captured with a digital camera..

6.7 Image analysis

The experimenters were blinded to the identity of the mice during image analysis.

6.7.1 Confocal microscopy

Immunostained brain sections spanning the entire region of interest and covering the AAV injection site were visualized using a laser-scanning confocal microscope (Leica TCS SP8, Germany) with 10x, 20x or 40x magnification (Leica PL APO CS2 objectives). Consecutive confocal image stacks (10x, taken in interval 2,4µm) were acquired sequentially in a montage configuration to cover the entire region of interest. Each animal group image acquisition required separation into the two Sequences (405,594 nm), (633,488 nm), to reduce cross-talk (emission overlap) to a minimum.

Images and their maximum Z projections were captured and quantified in Leica software. From each animal, 3 to 5 sections were imaged and 3 to 4 hemispheres with the brightest expression levels around the injection site were used for quantification. For counting purposes, regions of interest (ROI) in Z projections of acquired image montages were traced for ACC, PL, IL, AID, AIV, and DI, based on the The Mouse Brain in stereotactic coordinates [91]. Neuronal counts were performed for each cortical region while blinded to the treatments of the animals. A cell was counted only if it displayed intensity values throughout the soma consistently above background intensity levels. A positive neuron-like signal was only included in the final analyses if it did possess simultaneously a nuclear (Dapi, blue) and (NeuN, pink) marker.

6.8 Data analysis

GraphPad Prism® 6.0 was used for all statistical analysis.
Data are presented as means ± S.E.M. (standard error of the mean) for n animals. One-way or two-way analysis of variance (ANOVA), was used to determine significant differences between the experimental groups followed by Tukey's or Sidak's multiple comparison tests. A paired t-test and an unpaired t-test was conducted to analyse behavioural differences for CNO and saline treatments. Values of * $p<0,05$ were considered significant, ** $p<0,01$ very significant and *** $p<0,001$ or **** $p< 0,0001$ extremely significant.

7. Results

7.1 Immediate-early gene (IEG) expression in pain

To evaluate the role of medial (ACC, PL & IL) and lateral (DI, AID & AIV) PFC regions in acute pain, a group of mice that had been transduced earlier with complementing activity- and tetracycline-dependent AAV vectors in the PL, received a capsaicin injection in the hind paw while on doxycycline. The patterns of endogenous IEG expression evoked by the acute pain stimulus were captured by perfusion fixation of the brains 60–90 min after the capsaicin injection. c-Fos immunolabeling of coronal brain sections suggested that PL-mPFC and anterior insular regions of the lateral PFC were mainly activated (Fig 7.1). Counting of individual c-Fos+ neurons in the six brain regions revealed significant differences with the highest expression observed in the PL and intermediate levels of activation in the ACC, AID and AIV (Fig 7.2A). Many c-Fos+ neurons were also apparent in the piriform cortex of treated animals and this number appeared to be increased compared to home cage controls but this brain region was not evaluated further in this study.

The magnitude of c-Fos expression evoked in the lateral PFC by the acute pain stimulus was further assessed by counting immunolabelled cells in brains from unstimulated home cage controls and animals that received injections of capsaicin prepared with two distinct solutions. Few PFC neurons expressed c-Fos in the home cage group in (Fig 7.2 B). If capsaicin was dissolved without the addition of detergent to the 10 % DMSO solvent a moderate increase in c-Fos expressing neurons was apparent in the anterior insular regions of the PFC (Fig 7.2B). Nocifensive behaviour lasted 1390 ± 85 s if the injected capsaicin solution also contained detergent and 138 ± 45 s when dissolved simply in 10 % DMSO. Accordingly, the potent detergent-solubilized capsaicin induced c-Fos expression in significantly more neurons in the AID compared to home-cage controls and mice that received the less potent solution of capsaicin (Fig 7.2B).

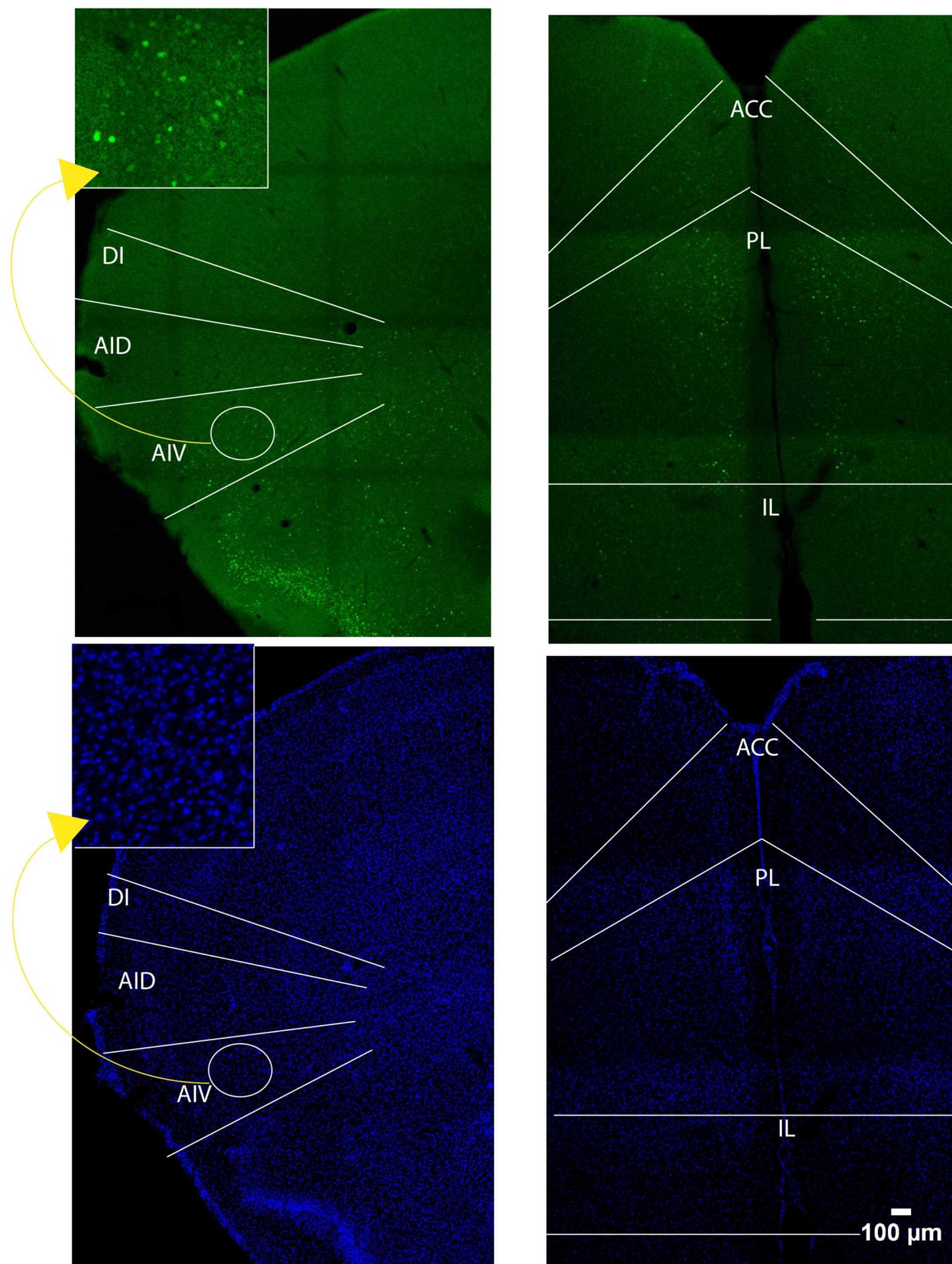

Figure 7.1: Example montages of confocal image projections of lateral and medial PFC (left and right panels, respectively) immunolabeled for c-Fos (top) and stained for nuclei with DAPI (bottom).White lines indicate borders of the assessed brain regions. Insets show zooomed detail of AIV. Abbreviations: ACC anterior cingulate cortex, PL prelimbic cortex, IL infralimbic, DI dysgranular insula, AID dorsal anterior insula, AIV ventral anterior insula., PIR piriform cortex.

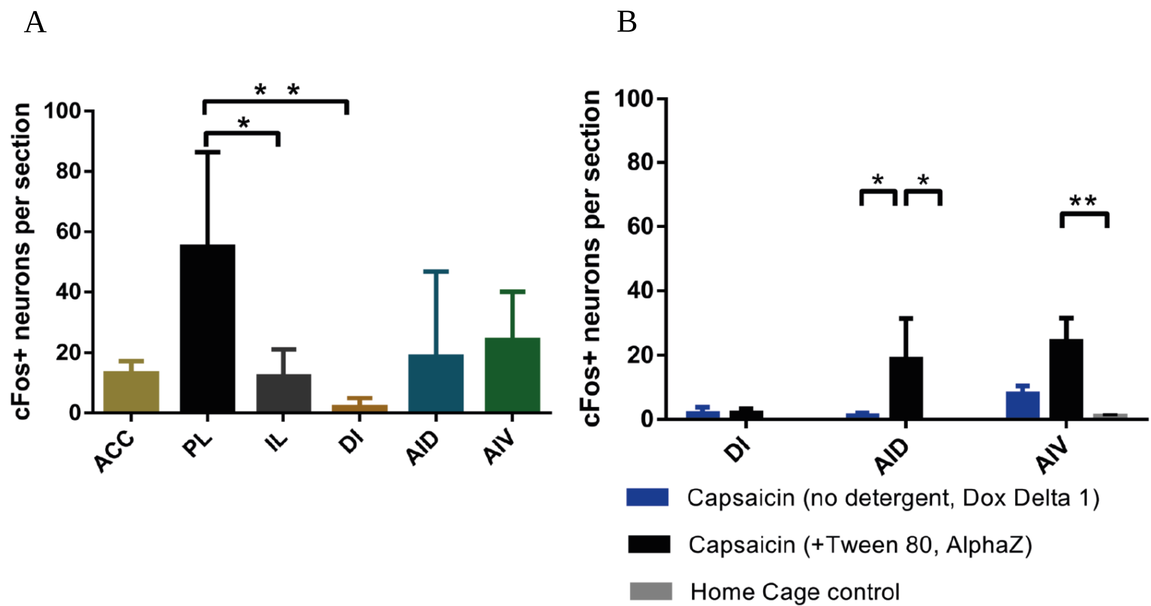

Figure 7.2: (A) Comparison of c-Fos expression in PFC regions induced by detergent-solubilized capsaicin injected in the left hind paw (AlphaZ experimental group, n=5, one-way ANOVA (p<0,05) and Tukey's post-hoc test). (B) Comparison of c-Fos expression in lateral PFC regions of home-cage controls (n = 6), and mice that received left hind paw injections of capsaicin solubilized either in 10 % DMSO and 7 % Tween-80 (AlphaZ, n=5) or in 10 % DMSO only (Dox Delta 1, n=6). Two-way ANOVA (p<0,05 for the treatment effect) and Tukey's post-hoc test for within brain region differences between the experimental groups. Abbreviations: ACC – anterior cingulate cortex, PL - prelimbic cortex, IL - infralimbic cortex, DI - dysgranular insula, AID - dorsal anterior insula, AIV - ventral anterior insula.

7.2 IEG expression in fear

The PFC has been tied to a wide range of functions such as attention, short-term memory, effort and motivation [91], and associative learning including conditioned fear behaviour [39]. We focused here on the activity of the PFC during fear retrieval as this should allow us to disentangle PFC activity encoding acute pain or an emotion such as fear evoked by a conditioned stimulus to a painful experience in the past. In order to evaluate the role of the PFC in tone-cued fear retrieval, two groups of mice (n=12 in total) received five pairings of a mild foot shock with an auditory tone and were re-exposed to the conditioned tone stimulus in a different context approximately 8 or 31 days later. The induced c-Fos expression was captured and analysed as described for the acute pain stimulus above. The pattern of IEG expression evoked by fear was somewhat similar to that observed in

response to the acute capsaicin pain (Fig 7.3) and detailed counting revealed notable differences between individual regions (Fig 7.4A). c-Fos expression was again highest in PL and lowest in DI, with intermediate numbers of c-Fos expressing neurons apparent in ACC, IL, AID & AIV.

To test if AAV mediated gene transduction and expression of the Gi:mCherry DREADD construct in the lateral PFC of the Dox Delta 2 mice had any impact on endogenous IEG expression, we compared c-Fos+ neuron numbers evoked by cued fear retrieval in Dox Delta 2 (n=7) with those evoked in naive (n=5) and untreated homecage control (n=6) mice. This analysis confirmed significant differences in c-Fos expression between homecage controls and both groups of fear conditioned mice (Fig 7.4B) and did not reveal significant differences in the fear retrieval-induced endogenous c-Fos expression between naive and DREADD expressing mice. Hence the endogenous c-Fos activation during cued fear retrieval of both fear conditioned groups was considered as comparable, justifying the combined analysis of c-Fos activity between the PFC regions in Figure 7.4A.

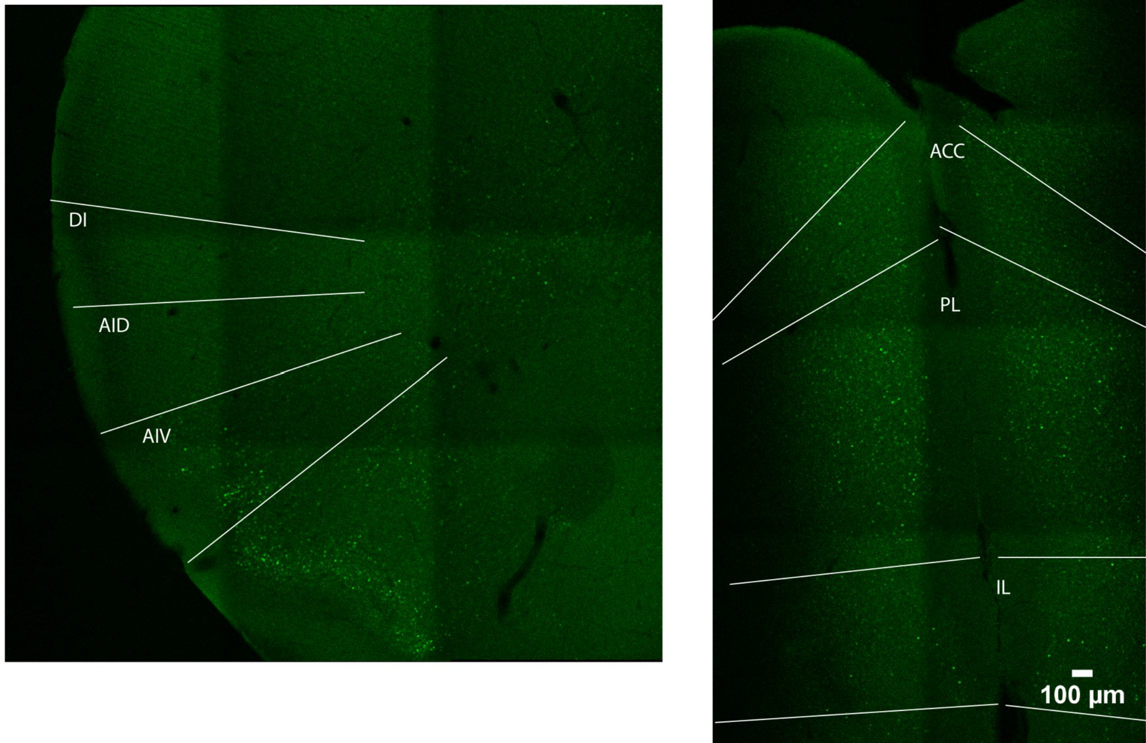

Figure 7.3: Example montages of confocal image projections of lateral (left) and medial (right) PFC regions showing representative low power images of c-Fos immunolabelled brain sections from an animal exposed to a cued fear retrieval stimulus. Borders of brain regions that were analysed by counting cFos+ neurons are shown as white lines..

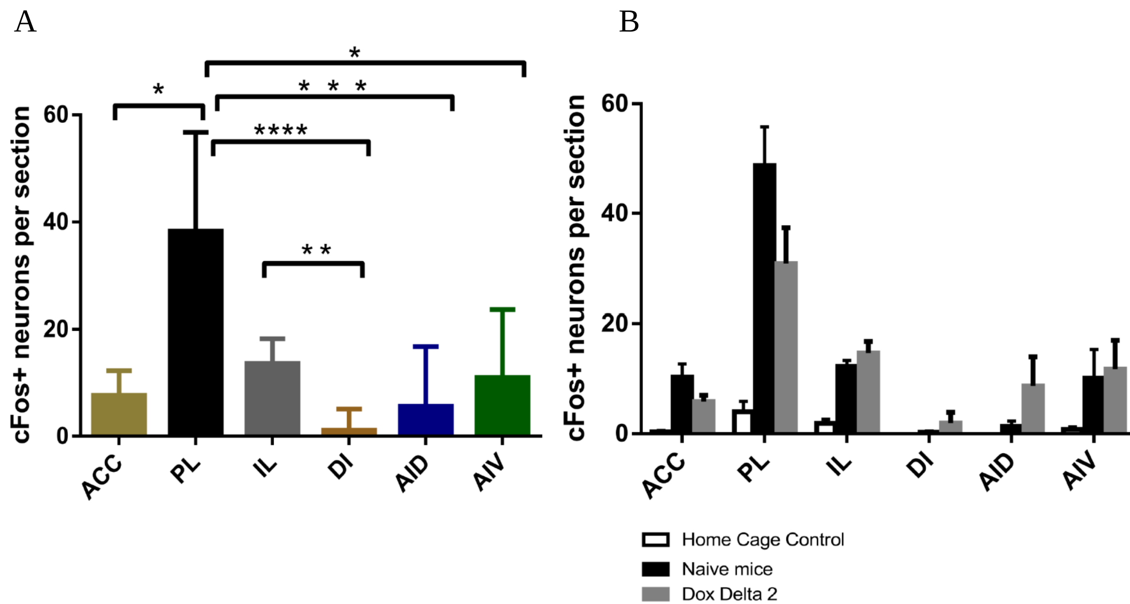

*Figure 7.4: (A) Comparison of c-Fos expression in PFC brain regions of fear-conditioned mice exposed to the CS+ to trigger fear memory 8-31 days after conditioning and 60 - 90 minutes before sacrifice (one-way ANOVA , n=12, p< 0,001, and Tukey´s multiple comparison test). (B) c-Fos expression in home cage controls (n=6) and in response to tone-cued fear retrieval 8 days (naïve mice, n=5) or 31 days (Dox Delta 2 group mice, n=7) after fear conditioning. Testing for main group effects in a two-way ANOVA confirmed significant differences in c-Fos expression between experimental groups and brain regions (both p<0,001) but the evoked c-Fos expression between the two fear conditioned groups was not different across all brain regions (Tukey's multiple comparison test for the main treatment effect). * p<0,05; ** p<0,01; *** p < 0,001; **** p< 0,0001.*

7.3 Comparison of IEG expression within PFC in acute pain and conditioned fear

In order to test for potential differences of IEG expression in PFC regions evoked by cued fear stimuli and detergent solubilized capsaicin, endogenous c-Fos expression patterns were compared between two distinct groups of mice. AlphaZ (n=5) mice that had been transduced earlier with the activity- and tetracycline-dependent AAV vectors in the PL, received a capsaicin injection in the hind paw while on doxycycline, and Dox Delta 2 (n=7) mice that were also transduced with activity- and tetracycline-dependent AAVs in the lateral PFC and subjected to tone cued fear retrieval. Both groups were exposed to the pain or fear stimulus 60-90 minutes before sacrificed while On-Dox (Fig 6.2)
The AlphaZ, Dox Delta 2 and untreated home cage mice were analysed for c-Fos+ neuron counts in the PFC regions (Fig 7.5A, two-way ANOVA, *$p<0,0001$ for the main treatment effect*). Multiple comparison testing showed that the c-Fos expression was evoked mainly in the PL, and significantly more so by the detergent-solubilized capsaicin compared to the cued fear stimulus (Fig 7.5A). While cued-fear retrieval did not induce a notable IEG response in any other region, the acute pain stimulus significantly increased c-Fos expression also in the ventral and dorsal anterior insula (Tukey´s multiple comparison test for the treatment effect within brain regions). * $p<0,05$; ** $p<0,01$; *** $p < 0,001$; **** $p< 0,0001$.

In addition we tested for differences in the number of endogenous c-Fos expressing neurons in PFC regions of homecage control, naive tone-cued fear, and AlphaZ acute pain mice. Two-way ANOVA confirmed significant differences between conducted treatments (Fig 7.5B, $p<0,001$). Again, PL was the only region where c-Fos was evoked by both, acute pain and conditioned fear stimuli above the untreated home cage controls. In this analysis a differential response to conditioned fear and acute pain was observed in the AID where very few c-Fos+ neurons were observed in response to conditioned fear. Acute pain evoked c-Fos IEG expression in both, AIV and AID in comparison to c-Fos+ neuron numbers in the untreated homecage controls (Fig 7.5B, Tukey´s multiple comparison test for the main treatment effect). Hence at the cellular level, IEG expression was induced in response to acute pain and conditioned fear stimuli in the PL- mPFC whereas in lateral AID and AIV PFC regions c-Fos expression was preferentially induced by acute pain.

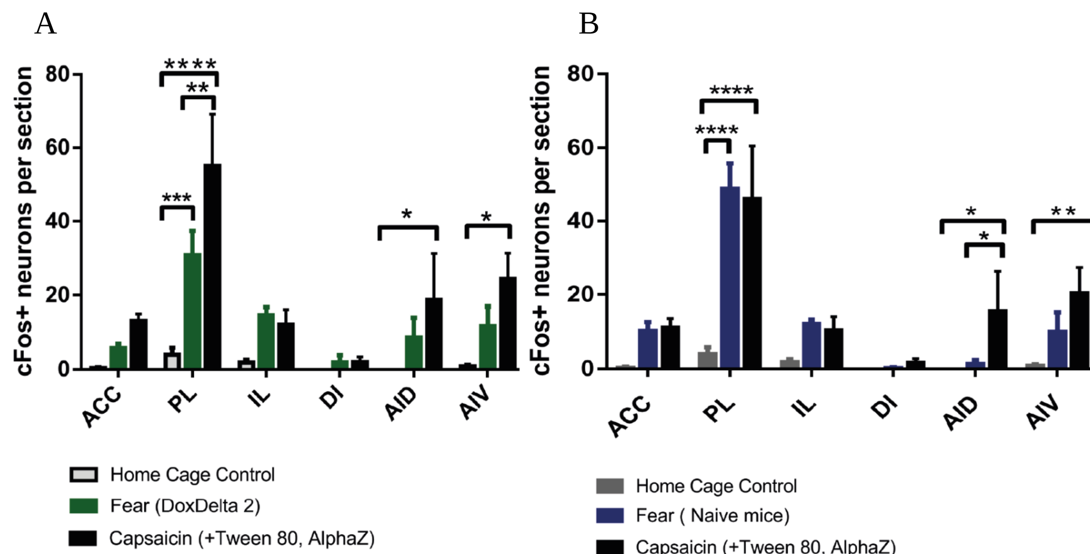

*Figure 7.5: (A) Comparison of endogenous c-Fos expression in PFC regions of home cage controls (n=6), and in response to capsaicin-induced pain (AlphaZ, n=5) or conditioned fear (Delta 2, n=7, two-way ANOVA (p< 0,0001), and Tukey's multiple comparison test). (B) Comparison of endogenous c-Fos expression in PFC regions of home cage controls (n=6), and in response to capsaicin-induced pain (AlphaZ, n=6) or conditioned fear (naïve mice, n=6, two-way ANOVA (p< 0,0001), and Tukey's multiple comparison test). * p<0,05; ** p<0,01; *** p < 0,001; **** p< 0,0001.*

7.4 Ensemble overlap & Labelling efficiency

We detected increased c-Fos+ neuron numbers in the PL in response to acute pain and conditioned fear stimuli but endogenous c-Fos expression alone is not sufficient to determine if the same or distinct neurons were activated in response to these two stimuli. To test for neuronal specificity encoding each stimulus, we next analysed the overlap of endogenous c-Fos expressing neurons with genetically labelled mCherry+ neurons in the mPFC (Fig 7.6). The Tet-Tag labelling approach provides a temporal activity-dependent marker for neurons that had their c-Fos promoter activity triggered either by capsaicin or tone-cued fear 3-7 days before the same mice were exposed to the alternate stimulus modality, the latter neuronal activity patterns are reflected by immunolabelling endogenous c-Fos protein levels. In AlphaZ mice activity-dependent mCherry expression was evoked by tone-cued fear stimulation 28 days after fear conditioning while animals were off Dox.

Endogenous c-Fos expression was then evoked by a capsaicin injection in the contralateral hind paw 4 days later when animals were back on Dox.

The percentage of mCherry+ neurons that were also c-Fos+ ranged from 4,94-16,7 % in the ACC and from 12,0-30,4% in the PL (Fig 7.7A). No mCherry+ neurons were detected in the IL mPFC of AlphaZ mice, the neighbouring area of the targeted PL mPFC region (Fig 7.7A). This indicates that the proportion of ensemble neurons contributing to both acute pain and conditioned fear modalities is reasonably low as less than 25 % of the mCherry+ acute pain ensemble neurons were also recruited into the conditioned fear ensemble.

To provide an unbiased estimate of genetic labelling efficiency in the PL and neighbouring ACC and IL areas we compared the number of mCherry+ fear neurons in AlphaZ mice with the number of endogenous c-Fos+ neurons obtained in a different group of mice that were exposed to the tone-cued fear retrieval session in the same manner as the AlphaZ group (Fig 7.7B). An influence on the endogenous c-Fos expression in response to the cued-fear stimulus in the Dox Delta 2 control group by the earlier capsaicin-induced Tet-Tag labelling of anterior insula neurons was unlikely as AAV injections were targeting exclusively lateral PFC regions in Dox Delta 2 mice. The activity-dependent cellular labelling in mPFC regions differed significantly between the two groups (Fig 7.7B). While the labelling efficiency was high in the ACC and no neurons were detected in the IL, the number of mCherry+ PL neurons only approached 50 % of c-Fos+ neurons in response to the same tone-cued fear stimulus. These findings confirm that the AAV injections in the AlphaZ group mainly targeted the dorsal mPFC regions and highlight the fact that the genetic Tet-Tag labelling efficiency in the PL could be improved.

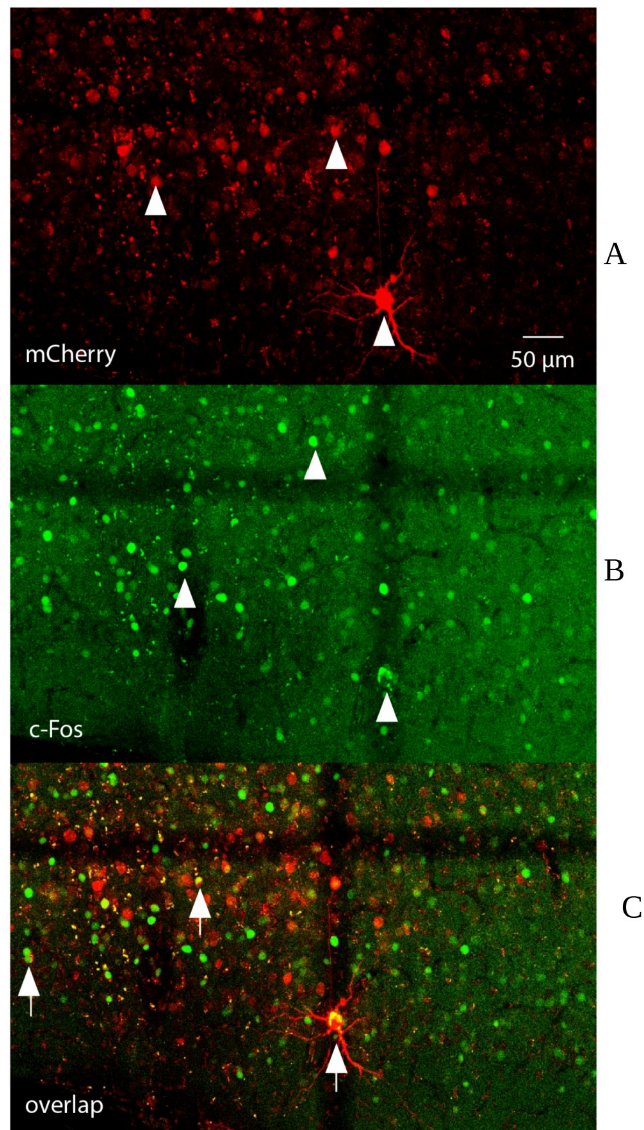

Figure 7.6: Representative example of cellular Tet-Tag expression in the PL mPFC evoked by conditioned fear (A, arrowheads mark mCherry+ neurons, red) and c-Fos immunolabelling following the acute pain stimulus in the same field of view (B, arrowheads mark endogenous c-Fos+ neurons, green). The images are overlayed in the bottom panel. (C: Arrowheads mark double-positive neurons) .

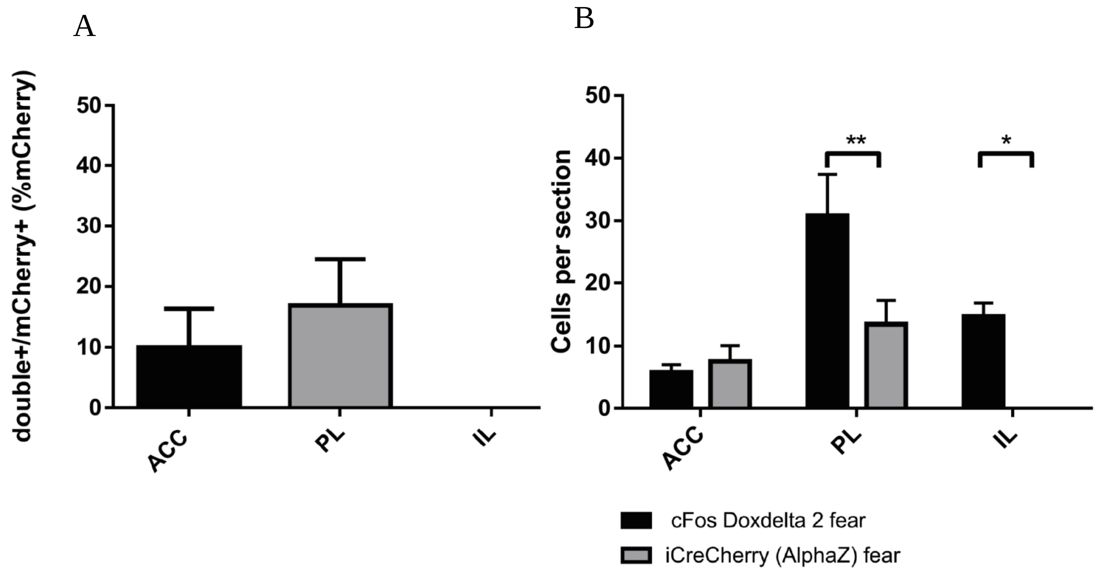

*Figure 7.7: (A) Estimate of the proportion of ensemble neurons activated by acute pain and conditioned fear stimuli (AlphaZ, n=5, the degree of ensemble overlap in PL and ACC was not different, paired t-test, p = 0,0896).). (B) Estimate of the genetic labelling efficiency in response to the conditoned fear stimulus in Alpha Z mice (mCherry+ neuron numbers) in comparison to the number of endogenous cFos+ neurons (Dox Delta 2, n=7) evoked by the same fear conditioned stimulus (two-way ANOVA, p<0,0001 for the main labelling effect between the two groups, and Sidak's multiple comparison test, * p*<0,05*; ** p*<0,01*).*

In comparison to the activity-dependent mCherry just described, the Tet-tag labelling efficiency of the activity-dependent DREADD vectors was found to be poor. Labelling efficiency of TRE-Gq:mCherry and TRE-Gi:mCherry constructs, with each of these injected at a 1:1 ratio with the cFos-tTA2G transactivator in the PL mPFC, was assessed in the group of Funky mice labelled with a tone-cued fear retrieval stimulus (Fig 6.3). In comparison to endogenous c-Fos+ neuron numbers observed in response to remote fear retrieval stimulation in Dox Delta 2 mice, mCherry+ neurons in Funky mice were significantly reduced in the mPFC, and in particular in the PL region at the centre of the injection site (Fig 7.8B, two-way ANOVA, *p<0,001,* and Tukey´s multiple comparison test).

A compromised Tet-Tag labelling efficiency was also apparent in the lateral PFC. In comparison to c-Fos+ neuron numbers observed in response to remote fear retrieval, the number of mCherry+ cells labelled by the same remote fear stimulation paradigm appeared reduced though in this case it did not reach significance (Fig 7.9A, two-way ANOVA, p=0,0539,). Tet-Tag labelling induced by acute pain stimulation was more convincing in the lateral PFC in Delta 2 mice where mCherry+ neuron numbers in the AID approached those for endogenous c-Fos+ neurons detected after the same acute pain stimulus applied to Alpha Z mice (Fig 7.8A). The improved efficiency in this situation may be explained by the fact that Dox Delta 2 mice were taken off Dox for 4-5 days before inducing Tet-Tag expression of the Gi:mCherry construct with the acute capsaicin pain stimulus (Fig 6.2). This would indicate that Tet-Tag labelling efficiency can be improved by optimising systemic Dox levels and the period of time that animals are taken off Dox before applying the Tet-Tag labelling stimulus.

Comparison of c-Fos expression as a response to fear stimuli in Dox Delta 2 control group and mCherry+ labelled cells in Funky group showed significant difference (two-way ANOVA, p(<0,001), The most significant difference was in PL and IL (Fig 7.8B) (Tukey´s multiple comparison test, * p<0,05; ** p<0,01; *** p < 0,001; **** p< 0,0001).

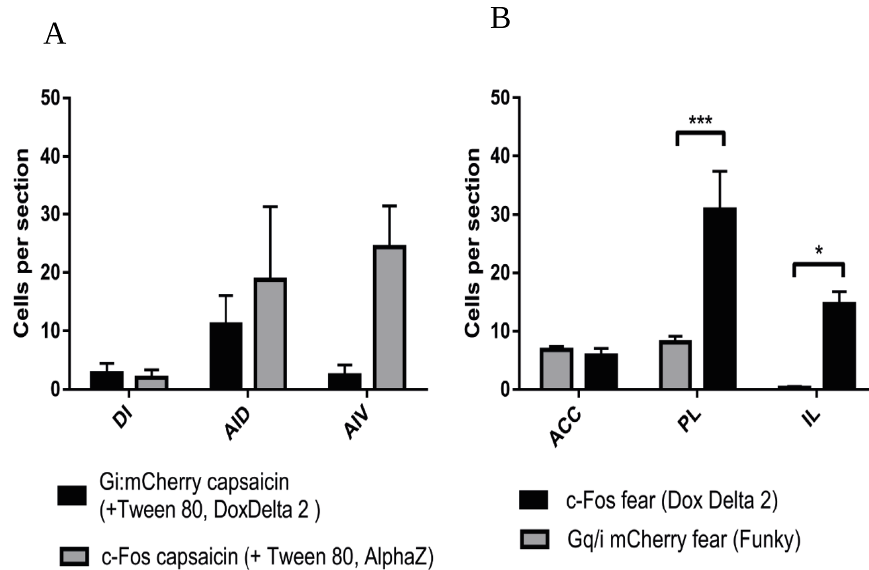

Figure 7.8: (A) Labelling efficiency in pain ,with potent capsaicin s.c. inj. Dox Delta 2, n=7, two-way ANOVA, p(>0,05) cFos-tTA, TRE-Gi:mCherry, c-Fos AlphaZ, n=5, two-way ANOVA p(< 0,001), Tukey´s test, (B) Labelling efficiency induced by fear retrieval stimulation in mPFC regions of Funky mice (n=5, mCherry+ neurons) compared to endogenous c-Fos+ neurons counted after similar remote fear retrieval stimulation in Dox Delta 2 mice (n=7, two-way ANOVA, p < 0,05 for the main experimental group effect, and Tukey´s multiple comparison test).

A

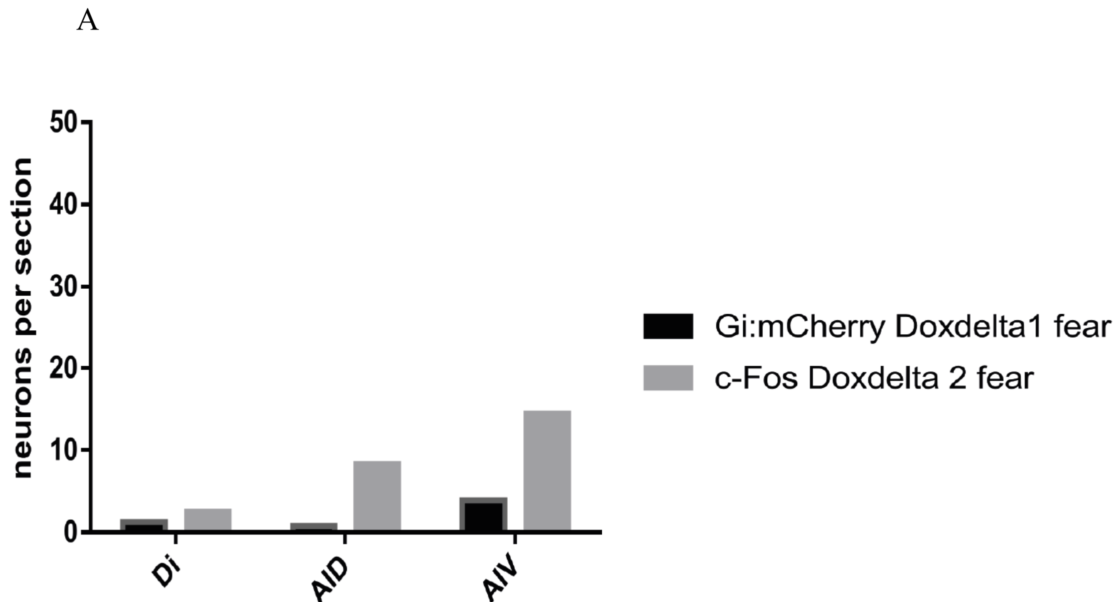

Figure 7.9: (A) Labelling efficiency induced by remote fear stimulation in Dox Delta 1 (mCherry+ neurons, n=5) was somewhat reduced compared to endogenous c-Fos+ neurons induced by the same stimulation paradigm in Dox Delta 2 mice (n=7, two-way ANOVA,p>0,05 for experimental group and brain region). (B) Labelling efficiency induced by acute pain stimulation with detergent-solubilised capsaicin in Dox Delta 2 mice (n=7, mCherry+ neurons) compared to endogenous c-Fos+ neuron numbers detected after the same acute pain stimulus in AlphaZ mice (n=5, two-way ANOVA, p>0,05 for experimental group and brain region).

7.5 Behavioural effects of engram neuron manipulation with DREADDs

Though the Tet-Tag labelling efficiency was sub-optimal we assessed pain and fear behaviour in animals that expressed DREADDs in Tet-Tag labelled engram neurons for one of these modalities. CNO (2mg/kg i.p.) or saline was administered at least 60 min before exposing animals to the acute pain or cued fear retrieval stimulus and assessing freezing or nocifensive behaviour, respectively.

Dox Delta 1 mice expressing the inhibitory Gi DREADD construct in fear-labelled engram neurons in the lateral PFC were re-exposed to the same fear retrieval stimulus on two consecutive days after having received alternating saline or CNO injections on either day. The proportion of time mice displayed freezing behaviour was similar in the CNO and saline conditions (Fig 7.10A) meaning the fear behaviour was not changed. On the second day of testing with CNO/saline (Fig 6.2, day 32), Dox Delta 1 mice were injected also with capsaicin (0,06% in 10% DMSO) in the left hind paw to assess nocifensive behaviour (paw

lifting or shaking, licking, toe-splaying). Again, we did not observe a treatment effect as mice having received either saline or CNO spent similar times displaying nocifensive actions (Fig 7.10B).

Similarly, fear freezing behaviour in Dox Delta 2 mice expressing the inhibitory Gi DREADD in acute pain engram neurons in the lateral PFC was not different in CNO compared to saline treated mice (Fig 7.10C).

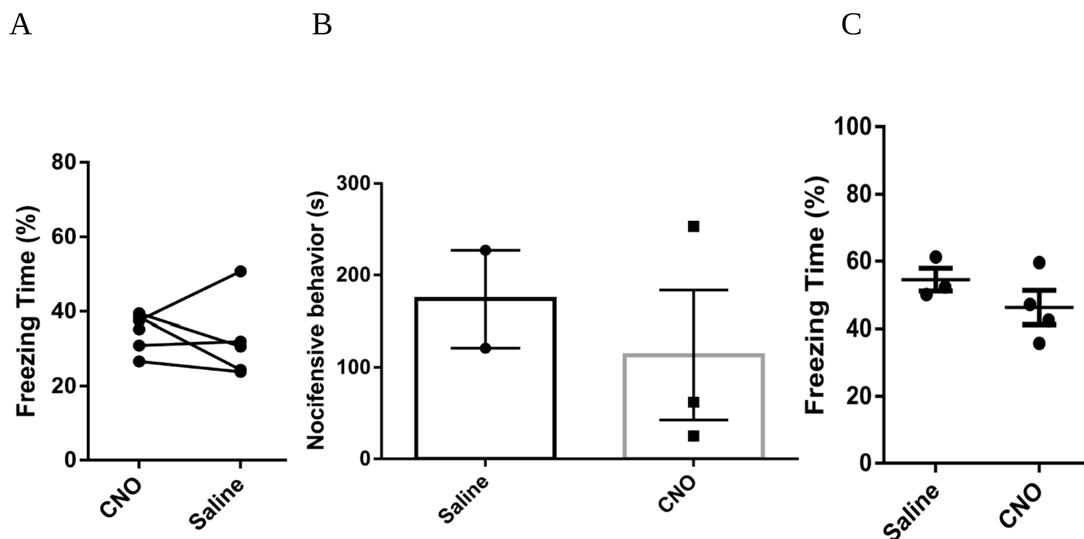

Figure 7.10: (A) Fear freezing behaviour of Dox Delta 1 mice with fear labelled engram neurons in the lateral PFC expressing Gi DREADD was similar in saline and CNO treatment conditions (paired t-test, p>0,05). (B) Nocifensive behaviour of Dox Delta 1 mice was tested on one day only with mice receiving saline (n=2) or CNO (n=3) treatment, (unpaired t-test, p>0,05). (C) Fear freezing behaviour of Dox Delta 2 mice with acute pain-labelled engram neurons in the lateral PFC expressing Gi DREADD was similar in saline (n=3) and CNO (n=4) treatment (unpaired t-test, p>0,05).

Since we did not observe any behavioural effects as a result of inhibiting engram neurons in the lateral PFC by activating the inhibitory DREADD with CNO, we counted cFos+ neurons in these regions (Fig 7.11). The number of endogenous cFos+ neurons was not different in CNO compared to saline-treated animals (p>0,05 for the main treatment effect for both, acute pain and fear retrieval stimuli (Fig 7.11) Although there was a tendency of reduced cFos+ neuron numbers in the AIV after inhibiting fear engram neurons and challenging with an acute pain stimulus (Fig 7.11A) the number of animals in these treatment groups was to low to draw any meaningful conclusions.

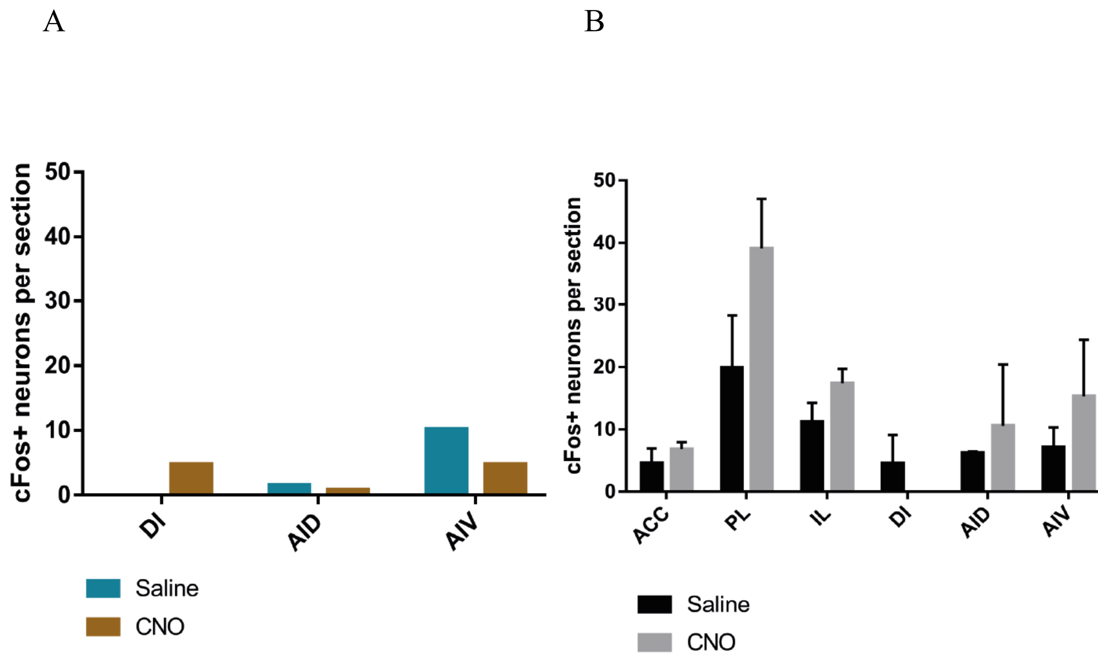

Figure 7.11: A) IEG expression in lateral PFC regions of Dox Delta 1 mice expressing inhibitory Gi DREADD in fear retrieval-labelled engram neurons after experiencing acute pain stimulation while on CNO (n=2) or saline (n=3) treatment (two-way ANOVA, p>0,05 for the main treatment effect). (B) IEG expression in PFC regions of Dox Delta 2 mice expressing inhibitory Gi DREADD in acute pain-labelled engram neurons after fear retrieval stimulation while on CNO (n=4) or saline (n=3) treatment (two-way ANOVA, p>0,05 for the treatment main effect).

8. Discussion

According to accumulating evidence, the prefrontal cortex is considered as one of the crucial brain regions involved in processing fear memory and pain [92, 47]. Particularly the medial prefrontal cortex (mPFC) comprising of the of anterior and cortex (ACC), the prelimbic cortex, (PL) and the infralimbic cortex (IL) are implicated in both pain and fear behaviours. Those regions rank high amongst regions that are consistently activated during painful perception [61]. It is not clear if mPFC circuits underlying functionally-distinct processes, such as pain and emotions are different, or if distinct subsets of mPFC neurons participate in distinct functions [61].

We have implicated the tetracycline-dependent tagging method (Tet-Tag) in combination with the activity-dependent cFos promoter. This Tet-Tag system provides temporal control to restrict labelling of neuronal activity within a defined time window, in the assumption that the behavioural stimulus used to label engram neurons is linked most prominently to neuronal activity patterns at the time. We also employed immunolabelling methods for immediate-early genes such as the activity-dependent marker c-Fos to delineate how pain and fear are encoded and manifested at the cellular level within PFC regions. These tools were used to test whether the cellular ensembles activated in pain and fear behaviours are distinct. We have also aimed to establish and explore activity-dependent DREADDs expression to in order test for the functional role of PFC ensembles in pain-related and non-related functions.

8.1 IEG expression in pain

We have examined and analysed c-Fos expression within medial and lateral PFC regions evoked by acute plantar heat following s.c administration of capsaicin (Fig 7.2) and fear (Fig 7.4). The results of IEG expression in pain suggested that PL- mPFC and anterior insular region of the lateral PFC were mainly activated (Fig 7.1). Our results are consistent with findings where mPFC [47, 61] and insular cortex as a part of lateral PFC are both involved in pain processing [93].

Consistent with the prediction of a regional dissociation within dorsal and ventral mPFC regions in pain processing [94], we had expected more c-Fos+ cells in ACC compared to

the PL, as pain processing was reported to be associated most prominently with the activity in the ACC [61]. However, a significant dissociation of c-Fos+ pain neurons in our analysis was apparent only between PL and IL mPFC (Fig 7.2A).

We also investigated IEG expression within regions of insular cortex in the lateral PFC evoked by capsaicin pain stimulation (Fig 7.2B) The lowest expression was observed in the dysgranular insular cortex, receiving the bulk of the gustatory afferents [67]. The agranular insular cortex (AIV, AID) is mainly responsible for the perception of the unpleasantness of pain which is later apparent in the actual behavioural reaction displayed [95]. The observed expression of c-Fos+ neurons within ventral and dorsal agranular insular cortex (AIV, AID) was much lower than we predicted, however we did test mainly at a certain anterior-posterior Bregma level (1,7-1,9) so it is possible, that insular neuronal activity at more anterior or posterior levels could be enhanced.
In general, to increase the statistical power of our findings, it will be essential to test more animals. The findings presented here suggest that the dorsal anterior insula of the lateral PFC is mainly active in acute pain, whereas pain and fear stimuli activate the prelimbic mPFC to a similar extent.

8.1.1 Comparison of different forms of capsaicin injections

The solvent used to dilute capsaicin makes a difference. In our experiments capsaicin was injected without and with the addition of detergent (Tween 80) to the 10% DMSO-based solvent. Including the detergent seems to be crucial in terms of evoking a cellular response. Capsaicin injected without detergent was not sufficient to increase c-Fos+ neuron counts above counts observed in the home cage control group (Fig 7.2B). Therefore for future experiments that rely on a robust IEG induction in PFC regions, Tween 80 detergent should be added to the capsaicin solvent.

8.2 IEG expression in fear

While a painful stimulus was employed as unconditioned stimulus (US) during fear conditioning, during recall of the fear memory was evoked exclusively by the conditioned stimulus (CS+). We avoided the use of the unconditioned electric foot shock to label fear engram neurons as the US by itself is highly painful. Using CS+ allows us later to make a comparison of the two distinct stimulus modalities, primarily inducing pain (capsaicin) or

an emotional response (fear).

As mentioned above we have activated fear engram cells within subregions of PFC. We observed a significant dissociation between mPFC and lateral PFC (Fig 7.4A), confirming that the insular cortex is highly associated with pain [94] PL – mPFC with both pain and fear memory [96].

This finding is consistent with the literature suggesting a high involvement of mPFC in fear memory recall [97, 40]. The observed high expression levels of c-Fos and associated cell counts suggest an essential role of the prelimbic mPFC in fear memory recall (Fig 7.4A/B) [98].

8.3 Comparison of IEG expression within PFC in acute pain and conditioned fear

8.3.1 Comparison of recent and remote fear

We compared c-Fos expression between recent fear memory (triggered 8 days after conditioning) and remote fear memory (triggered 31 days after conditioning) with the highest number of cFos+ neurons detected in the PL (Fig 7.4B). However, the number of c-Fos-expressing neurons in both these fear conditioned groups was not significantly different. This results support the fact that PL is involved in recent and remote memory [45] but is not entirely consistent with a recent report in Science by Kitamura *et al.*, who found that the PL mPFC has a functional role mainly encoding remote fear [47].

A role for the PL mPFC in encoding fear memory was also identified by De-Monte *et al.* [99], although the PL involvement in fear learning and retrieval became apparent within a few days of conditioning opposing to recent Science paper by Kitamura, where PFC is highly involved in remote fear [47]. Based on this contrast, testing more animals also at earlier timepoints following conditioning will be essential.

8.3.2 Comparison of acute pain and conditioned fear

We aimed to examine how pain and fear manifestation differs within mPFC and lateral insular cortex (Fig 7.5A/B). We have used s.c. capsaicin injection as an acute pain stimulus and exposure to conditioned CS+ tone to provoke fear memory. We expected a regional dissociation in cFos+ neuron counts between pain and fear, with the higher pain expression in ACC [100] and the opposed higher expression in fear in PL and IL, [67]. However, the

pain expression was significantly elevated only in PL and the anterior insular regions (Fig 7.5B) and cFos+ neuron numbers evoked by pain or fear stimuli differed only in the AID. In order to increase the statistical power and verify this finding, testing more animals would be essential.

8.4 Ensemble overlap

To test if the detected neuron ensembles recruited by the acute pain and cued fear retrieval stimuli are distinct we used the activity-dependent c-Fos promoter in the Tet-tag system to label neurons with the fluorescent mCherry marker in one modality and classical ICC for the endogenous c-Fos expression in the other modality. We calculated the percentage of mCherry+ cells that were also c-Fos+ (Fig 7.7A) to assess the extent of overlapping engram neurons. 16 % of fear engram neurons were also activated by the acute capsaicin pain stimulus. This percentage overlap is clearly below the 30% overlap reported for remote fear memory triggered by contextual cues and labelling performed during the fear conditioning session in the same context [53]. This comparison suggests that pain and fear encoding by engram neurons in the PL mPFC are mostly distinct, with a relatively small proportion participating in encoding both modalities. To achieve a better estimate of the neuronal overlap to pain and fear stimuli, it would be necessary to further improve the labelling efficiency (Fig 7.7B). As a control it would be very useful to include a group of animals labelled either with a cued fear or acute pain stimulus and tested later again for the same stimulus modality to directly compare the degree of overlap in same modality and distinct modality situations.

8.5 DREADDs and labelling efficiency

The PL mPFC is associated with pain [53] and fear processing [72]. Using the Tet-Tag labelling approach to express DREADDs in engram neurons we aimed to test if activating or inhibiting ensemble neurons has behavioural consequences.

We have labelled lateral PFC engram neurons with an inhibitory DREADD by providing a cued fear or an acute pain stimulus in two separate groups of mice, and later tested for behavioural effects while activating the DREADD with CNO and compared this to the performance in the saline control condition. We did not detect a change in the behaviour

when inhibiting engram neurons in the lateral PFC, not during fear when labelled with the same cued fear or a distinct acute painful stimulus, nor during acute pain when labelled with the distinct fear retrieval cue (Fig 7.10). Consistent with strong evidence for an involvement of the anterior insular cortex in pain perception [101]. Tet-Tag labelling and testing with DREADDs should be focused on an acute pain stimulus in order to test for behavioural functions of these engrams in the lateral PFC.

We have hypothesised that by using Gi/Gq DREADDs we could eventually inhibit or potentiate the activity of specific engram neurons within the prelimbic mPFC, a key PFC subregion implicated in pain and fear processing [76], 61]. As a first step we aimed to label neurons with a cued fear stimulus and later test fear behaviour again while activating DREADDs. Similarly, by labelling engram neurons with an acute pain stimulus and later testing for cued fear behaviour while activating DREADDs with CNO we hoped to disentangle functional roles of engram neuron activity in the mPFC. This intent to dissect fear and pain engram interactions was not possible, however as we did not achieve a high labelling efficiency.

By injecting the rAAVs for c-Fos-tTA and TRE-Gi/Gq:mCherry in a 1:1 ratio we have achieved a poor labelling efficiency in the PL (Fig 7.8) and neither behavioural testing with CNO/saline nor c-Fos to estimate engram neuron activity was suggestive of functional effect due to the DREADD engram manipulation (not shown) (Fig 7.10, 7.11). By adjusting the ratio of the two rAAV vector components it might be possible to optimize and improve the Tet-Tag labelling efficiency. Similarly, optimizing the doxycycline dosing regime and the period of time off Dox before stimulation should provide means to improve the labelling efficiency.

However, according to recent findings published by M. Michaelides and his team in Science [84] caution the specificity of the DREADD system is required. They demonstrated that CNO uptake in the CNS is extremely low and CNO is only activating DREADDs once converted to clozapine. The authors suggest that CNO is metabolized to clozapine which can cross the blood brain barrier and activate DREADDs, while CNO itself does not cross the BBB [84].
Hence it will be necessary in future to control for the non-specific effects of clozapine due to it activating endogenous receptors such as D2 dopamine or serotonin receptor types.

Using low doses of clozapine in absence of the designer receptors would be an appropriate control to be able to recognize clozapine-like behavioural effects not mediated by DREADDs themself. In parallel, treating a group of Tet-Tag labelled mice expressing a fluorescent marker with CNO, and testing for behavioural consequences will be an important control for the specific of the DREADD cellular manipulation approach [84].

Another concern that might be eventually problematic is the finding of Gold´s team that even in the absence of CNO, the expression of DREADDs had profound effects on the cellular physiology-disrupting normal signalling cascades simply by DREADDs being present [102]. DREADDs have been portrayed as a powerful chemogenetic tool to silence or potentiate neuronal activity very in a specific fashion. Despite the fact, that more than eight hundred studies employing DREADDs were published, many scientists are now warning to take great care in using DREADDs appropriately [84]. When used along with proper controls, and if the expression is optimized, they should still provide a powerful approach to manipulate the activity of a specific subset of neurons.

In case of using the proper control groups, and if the expression is optimized we could still draw conclusions in our experiments.

Optogenetic methodologies provide another possible approach to silence or excite neuronal ensembles specifically. This is achieved by expressing photosensitive ion channels in specific cell types and this approach has been used in Tet-Tag labelled engram neurons by the Tonegawa´ s lab [55]. Neurons are typically activated with blue light by expressing the cation channel, Channelrhodopsin-2 (ChR2) [101] and inhibited with green light by expressing the light-sensitive proton pump Archaerhodopsin (Arch) [103].

9. Conclusion

The prefrontal cortex is one of the most studied brain regions and its subregions, particularly in the mPFC, are highly associated with fear memory and pain processing.
We aimed to elucidate the contribution of lateral and medial PFC regions to pain-related functions. Our findings revealed acute pain-induced activation of mPFC regions, mainly the PL mPFC, as well as the dorsal and ventral anterior insular cortex regions of the lateral PFC. The results obtained by activating fear engram cells support the suggestions of a high involvement of the PL - mPFC in fear and point to a regional dissociation between pain and fear within the lateral PFC.
In order to test if cued fear memory encoding in the PL mPFC becomes more important over time, engram neuron activity detected at various time points for recent and remote fear memory need to be assessed in detail. Contrary to our expectation, c-Fos-based engram activity for pain and fear stimuli was elevated consistently only in the PL mPFC. cFos+ neuron counts were not significantly induced by the acute pain and cued fear stimuli in the ACC.
Further experiments are required to optimize the Tet-Tag and DREADD-based engram manipulation approach.
Here we aimed to conduct pilot experiments characterizing the activity-dependent DREADD expression in order to test for the functional role of different neuronal ensembles within the PFC. This approach so far proved to be ineffective and requires optimization. However, if this method proves to be unsuitable for this purpose we propose using an optogenetic approach as a potential alternative.

Abbreviations

AAV	Adeno-associated virus
ACC	Anterior cingulate cortex
Arch	Archaerhodopsin
AIC	Anterior insular cortex
AMY	Amygdala
ATP	Adenosine triphosphate
BG	Basal ganglia
BBB	Blood brain barrier
CFC	Cued fear conditioning
ChR2	Channelrhodopsin-2
CNO	Clozapine-N-oxide
CNS	Central nervous system
CS	Conditioned stimulus
DMSO	Dimethyl sulfoxide
Dox	Doxycycline
DREADD	Designer Receptors Exclusively Activated by Designer Drugs
DRG	Dorsal root ganglion
FC	Fear Conditioning
FR	Fear retrieval
GPCRs	G protein-coupled receptors
HEK 293	Human embryonic kidney 293 cell line
IASP	The International Association for the Study of Pain
IC	Insular cortex
i.p.	Intraperitoneal injection
IHC	Immunohistochemistry method
IEG	Immediate-early gene
IL	Infralimbic cortex
LSC	Laser-scanning confocal
mPFC	Medial prefrontal cortex
NeuN	Neuronal nuclei
NGF	Nerve growth factor

pAAV	Plasmid of adeno-associated virus
PFC	Prefrontal Cortex
PL	Prelimbic cortex
PtdIns(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
rTetR	Reverse tetracycline repressor
ROI	Region of interest
SAR	Structure-activity relationship
s.c.	Subcutaneous injection
S1	Primary somatosensory cortex
S2	Secondary somatosensory cortex
S.E.M	Standard error of the mean
TET	Tetracycline
Tet- off	Tetracycline off system
Tet- on	Tetracycline on system
tetO	Tetracycline operator
TetR	Tetracycline repressor protein
Tet-tag	Tetracycline-dependent tagging
TG	Trigeminal ganglia
TRE	Tetracycline-response element
TRP	Transient receptor potential
TRPV	Transient receptor potential vanilloid
tTA	Tetracycline-controlled transactivator
US	Unconditioned stimulus
vmPFC	Ventro-medial prefrontal cortex

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