What's the role of the amygdala in chronic pain? Evidence from magnetic resonance imaging studies in humans

Thesis for Doctor of Philosophy at The University of Nottingham, 2020

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Contents

Acknowledgements

Personal acknowledgements

Before I started my undergrad studies, I got a postcard for my birthday from my best friend which shows a peaceful boat in the harbour at sunset with a sentence that would translate into

“If you wish to want to find new shores, you have to abandon the [safe] harbour”. (The postcard does not state a source for this phrase so don’t blame me)

Ever since this postcard was on my desk and served as a source of comfort; consciously deciding for a PhD topic that I believe to be important but which I knew was overlooked (or even avoided) in the field meant it would not yield quick output. I’m grateful for the people who helped me throughout.

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It is gratefully acknowledged that data collection was collective effort. To the best of my knowledge, contributions of every individual for every data set are listed below (in no particular order).

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Study was designed by Professor Auer, data collected by Hamza Alshuft, with the help of Maggie Wheeler, Laura Condon and later William Cottam and Diane Reckziegel. Data entry was also done by these individuals, M.D. helped to recover some entries on STAI questionnaire. Radiographer: Anita French.

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Data on medication usage was entered by Marianne and William, checked and classified by Arman and Yasmine Zedan.

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Parkinson MR Imaging Repository (PaMIR)

This is another local study designed by Professor Auer and managed by Yue Xing on the subject of Parkinson; a small subset of this study's healthy volunteers data was considered for the current chapter.

Venous stasis and permeability assessment in multiple sclerosis (VeSPA) and Imaging markers of brain network disruption in multiple sclerosis (BraNDy)

A small subset of structural images from healthy controls was taken from data sets of these two studies, designed by Rob Dineen and managed by Tom Welton and Afaf Elsarrai.

The above studies were acquired with identical hardware at the same site and nearly identical scanning protocols. Any differences are described below in the methods sections.

SUBACUTE

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Magnetic Resonance Imaging specific acronyms:

MRI = Magnetic Resonance Imaging

fMRI = functional Magnetic Resonance Imaging

GMD = gray matter density

FC = functional connectivity

VBM= Voxel-based morphometry

Conditioning studies specific acronyms:

CS+ = conditioned stimulus preceding the unconditioned stimulus

CS- = conditioned stimulus not being followed by the unconditioned stimulus, usually neutral

General acronyms:

OA= Osteoarthritis

PTSD = posttraumatic stress disorder

Abstract

The amygdala is most known for its role in fear, threat, relevance processing but also aversive conditioning, is part of nociceptive pathways and has extensive reciprocal connections to many pain-implicated brain areas yet less than 5% of human pain imaging studies report the amygdala. The aim of this thesis is therefore to evaluate the amygdala's role in pain and pain progression mechanisms. Thus chapter 1 describes this relationship from conceptual and preclinical perspectives and elaborates on possible reasons for its infrequent appearance in human pain literature whilst chapters 4 and 5 evaluate it empirically and try to mitigate some of the knowledge gaps.

Specifically, chapter 4 examined amygdala morphometry in chronic knee Osteoarthritis pain patients with voxel-based morphometry, hypothesizing increased amygdala gray matter density in pain patients relative to controls in line with some preclinical reports on stress-induced amygdala hypertrophy.

This hypothesis could not be confirmed or refuted because extracting gray matter probabilities from quality-controlled gray matter segmentations with an accurate amygdala mask revealed practically no variance in either patients or controls, suggesting that this parameter reflected segmentation quality rather than neurobiological qualities. It is therefore advised not to use this parameter for clinically motivated questions regarding the amygdala unless gray matter atrophy is expected.

Chapter 5 investigated pain-related alterations to amygdalae functional networks in the same patient and control population. It was hypothesized that chronic pain would be linked with decreased amygdala-vmPFC connectivity because of proposed inhibitory mechanisms from animal pain work but increased amygdala-dmPFC connectivity because of human imaging studies linking this connection to aversive amplification. Furthermore, sex-effects were hypothesized. The first hypothesis could not be tested. Interestingly, findings supported the second hypothesis but in the opposite direction and future work is advised to investigate the discrepancy of this finding further as it has clinical relevance for serotonergic drugs. Unexpectedly, connectivity between the amygdala and the postcentral gyrus was also altered in chronic

pain patients relative to controls; this results should be investigated further as the postcentral gyrus is often part of imaging results in human pain but hardly discussed yet it was recently linked to genetic predisposition for anxiety disorders. Results are also suggestive of sex-effects in that the amygdala-dmPFC connectivity alteration was not found in post-hoc subsample analysis in males whilst subanalyses in females did not show alterations in amygdala-postcentral gyrus connectivity. This should be followed up as it may help to understand why females are more prone to develop chronic pain.

The last chapter reviews findings of this thesis and suggests further empirical avenues.

Chapter 1: Introduction

1.1 A definition of (acute) pain

One of the most cited definitions for pain was proposed by the International Association of the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey, 1994). This definition covers an evolutionary sensible reason for its existence and even if most rudimentary this definition hints at biological mechanisms when someone gets hurt: nociceptors in the tissue sense potential or actual damage to the body and send a respective signal to the spinal cord which in turn sends signal back that issues histamine release to initiate a cascade of different transmitters (see figure 1 from (Woodcock et al., 2007)) and induce swelling and other temporary physical changes that make the affected area more sensitive to painful stimuli ("hyperalgesia") and to non-noxious stimuli ("allodynia") such that the individual is easy with the affected area until it had sufficient time to recover from the incident (Sandkuhler, 2009). At the same time the spinal cord sends this information on to the brain where it is first processed by brainstem, thalamus, sensory motor areas before relayed to other cortical areas and then translated into an "unpleasant" sensation.

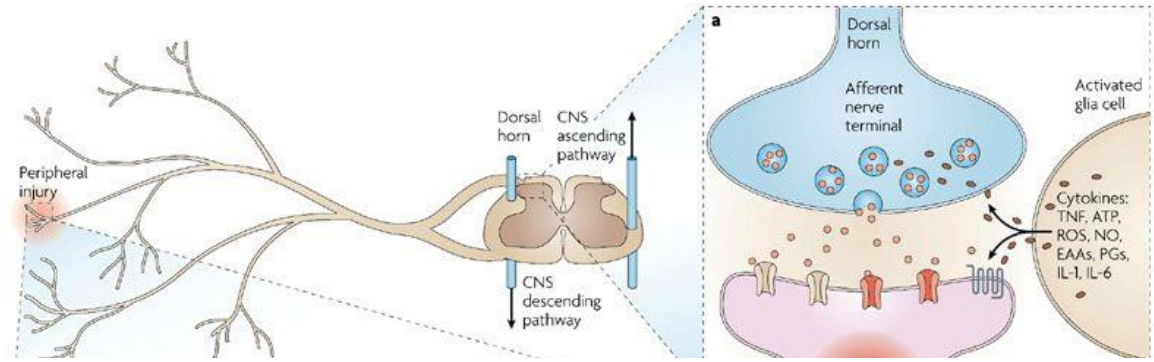


Figure 1: Molecular reactions to injury

Figure adapted from (Woodcock, Witter, & Dionne, 2007). The signal from nerve fibers in immediate viscinity of the injury is relayed to the dorsal horn and transferred through the spinal cord before it is relayed to the brainstem and other brain areas.

The described neurobiological process without an “unpleasant […] and emotional experience” is referred to as “nociception”- an automatic warning signal which can be suppressed by anaesthetics and some other drugs. A definition of pain should make clear that pain is a psychological construct which is usually the conscious and subjective interpretation of nociceptive input but it is possible to have pain without nociception and vice versa.

This is evident during surgeries under anaesthesia but anecdotal evidence includes congenital disorders, injured athletes and warriors who fail to notice severe injuries during performance or battle. Conversely, there are chronic pain disorders where pain is felt without apparent nociceptive input (e.g., phantom limb pain, fibromyalgia, or psychogenic pain, but also some osteoarthritis patients). Beyond these extremes, it is intriguing that for instance two Osteoarthritis pain patients may have comparable joint lesions (i.e. the source of nociceptive input) but a range of perceived pain levels which often persists after successful joint replacement (Wylde, Hewlett, Learmonth, & Dieppe, 2011).

Thus, subjectivity is an integral component of pain that is only partially covered by the term “emotional experience” because it depends on life experience and learning, personal upbringing, context, genetic and epigenetic makeup.

Relatedly, an individual perceives pain differently throughout life (e.g., as toddler, child, and adult), in parts because past experience enables a learning process to put the sensation into perception and judge the relative risk for the body. Accordingly, an infant’s reaction to the smallest injury is usually intense while most adults feel that a paper cut is a brief annoying experience that does not receive much attention. In that case, is a paper cut enough to be called pain? This may be part of a philosophical discussion on where to define onset of pain but literature on ‘aversion’ usually describes the sensation to be ‘of biological relevance’ for the individual (Delgado, Olsson, & Phelps, 2006; VanElzakker, Dahlgren, Davis, Dubois, & Shin, 2014) which may be a useful addition to the definition of pain to avoid ambiguities. Apkarian’s reasoning about refinements on the definition of pain is similar when he claims that pain

is the cue to make “urgent decisions” to protect the body (Apkarian, 2019); this definition, however, is slightly problematic as the individual often simply has to await healing, and not all pain is reflective of a life-threatening state to demand immediate action as is best evidenced by the example on small injuries in babies and children; thus ‘biological relevance for the individual’ is a more suitable addition to the definition on pain as it implies subjectivity. More importantly, pain’s evolutionary purpose is being a signal to motivate individuals to avoid further damage to the body (i.e., stop self-destructive behaviour immediately) and thus it is foremost a learning cue to avoid such damage in the future; this is evidenced in individuals who are unable to feel pain and therefore suffer from a shorter life expectancy as a result of reckless behaviour and self-harming, as they have not acquired the motivation to be careful.

With these additions, the given definition is adequate for acute pain but its coverage does not extend to chronic pain and known limitations should be mentioned as part of the definition.

1.2 A definition of chronic pain?

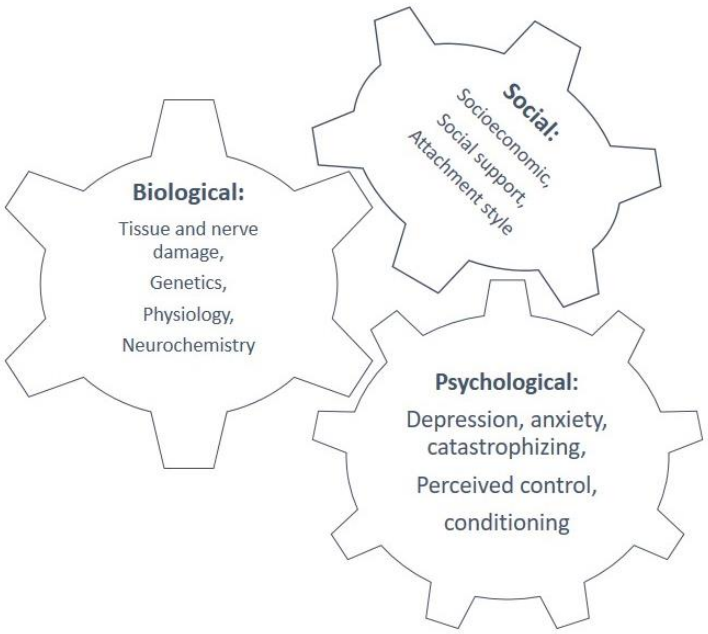
Indeed, an important distinction is that pain that persists beyond tissue recovery *or* is persistent but not linked to any (persisting) tissue damage is called ‘chronic pain’ and serves no evolutionary purpose like pain does. To better distinguish these two, Lippe’s introduced the terms *Eudynia* (“good pain”) and *Maldynia* (“bad pain” as a chronic disorder) (Lippe cited in Aronoff, 2016). The most recent consensus paper by the International Association for the Study of Pain (IASP) concludes to define chronic pain when it persists for more than three months (Treede, 2019).

This duration threshold, as well as the “serves no purpose”-aspect are the only aspects in a definition on chronic pain that have been agreed on because on the one hand any addition to the definition may be accurate for some chronic pain conditions and challenged by others and thus further definitions for specific pain conditions have been suggested (Treede, 2019); on the other

hand, our understanding of chronic pain and in particular its progression is limited. Often, chronic pain develops from acute pain and what drives the transition is not well understood (Pozek, Beausang, Baratta, & Viscusi, 2016). Consequently, many pain patients undergo a real odyssey starting with the diagnosis which, depending on country, resources, national guidelines and referral implementations, takes weeks, months or even up to five years as in the case of Canada (Lynch et al., 2007). This indicates that there is considerable waiting time in developed countries - even if no initial psychiatric or other mis-diagnosis or false accusation of malingering is given which is often the case (Katz, Rosenbloom, & Fashler, 2015).

1.3 A status quo on human chronic pain research: the biopsychosocial model

To improve the situation, over the past decades there has been a lot of work to refine our definition and understanding on chronic pain, identifying a myriad of interacting factors that can modulate pain and contribute to its progression. Together, this has been put forward as the "biopsychosocial model of pain" which is a summary of all known factors contributing to pain progression (see figure 2) that has been elaborated since the 1970s (reviewed in Bevers, 2016).



As much praise as the biopsychosocial model received since then its main criticism is that it is vague (see for instance Jull, 2017) and offers a tick-list rather than complementary mechanistic explanations.

Figure 2: Visualizations of the biopsychosocial model of pain,

This figure is inspired by (Adams, 2018).This figure gives examples of biological, psychological, and social factors believed to contribute to pain chronification.

Nevertheless, this model encourages to consider comorbidities such as depression quicker which is laudable in that patients are approached with a holistic view. On the other hand, the biopsychosocial model may lead to misdiagnoses if overlapping symptoms are not screened properly and hasty conclusions are made. Aronoff for instance remarks that many patients are inappropriately treated with psychoactive substances because of depressive symptoms or reduced concentration which can be simply due to pain-related sleep deprivation rather than depression (Aronoff, 2016); such an error may happen because depression is considered a frequent comorbidity and thus some clinicians may skip proper assessment of depression. This is not an argument to abandon the model but to caution against over-interpretations.

Until now discussions have always been about *the* biopsychosocial model of pain but as Jull points out the future is to individualize biopsychosocial models (Jull, 2017) by establishing what the relative contribution of factors (i.e., 'weight' or 'rank') for each patient is in order to decide on *prioritisation* of treatment approaches to maximise patient benefit (see also figure 3). Jull rightfully points out that there are as many biopsychosocial models of pain as there are patients otherwise the model assumes that the same mechanisms contribute to pain equally across patients.

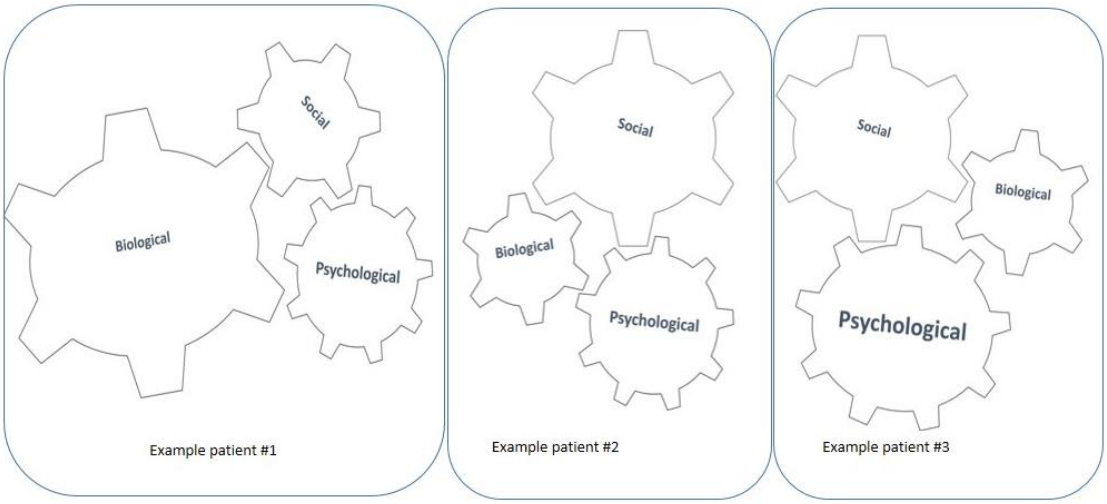


Figure 3: Three exemplary individual biopsychosocial models of chronic pain, adapted from (Jull, 2017). This figure illustrates how the relative proportion of each meta-factor that contributes to pain development can differ between patients.

This is not to be interpreted as an intention to separate components and re-ignite discussions on dualism but illustrates that the biopsychosocial model can serve as roadmap to precision medicine.

Jull's amendment to the biopsychosocial model should be regarded as a futuristic outlook as it assumes that all these components and their relative weight are known; in this way it is an important reminder that research should strive towards a holistic approach. This reminder is needed because research has focused mainly on the periphery or spinal cord for sources of pain progression for a long time, which evidently do not make the jigsaw puzzle complete.

Only for less than 30 years has human pain research taken an interest in the brain as another puzzle piece. Are there 'dysfunctional components' in brains of pain patients that contribute to their chronification? Are there obvious targets within the brain towards this question?

1.4 The amygdala – an intuitive choice for understanding pain processing and chronification?

There are several reasons why the amygdala may be such an intuitive target to understand chronic pain better. The following intends to give an overview on mechanistic arguments why the amygdala may be relevant to pain processing and finishes with a critical evaluation of its role in pain literature.

1.4.1 Mechanism #1 Associative learning and extinction deficits

It is not widely known that the amygdala is 'indispensable' in associative learning and extinction of such learned responses (Stockhorst & Antov, 2015) but it is fitting to start this discussion with this mechanism as most clinicians in a large survey felt the latter to be facilitating the transition from acute to chronic pain (Madden & Moseley, 2016).

Operant (or instrumental) conditioning links conscious reactions to unconditioned stimuli through associative learning; in case of aversive stimuli this is usually avoidance behaviour. Even the phenomenon that repeated empathic social responses from family members can worsen pain, which largely constitutes the social aspect of the biopsychosocial pain model, is based on operant conditioning which was already described as a pain progression model by Fordyce (1976). Also, many studies on nocebo hyperalgesia and placebo analgesia are described with the term conditioning because they are based on acquired expectations (also often referred to as 'contingency awareness') and thus fall under associative learning; this includes studies where conditioning is implemented already before the study visits by life-long learned expectation of pain killers to yield pain relief. Thus, there is a lot of empirical data to support a role of operant conditioning in pain progression even if there may be some room for discussion for some studies as to the definition on where operant conditioning begins (i.e., if a cream is used for placebo analgesia in a one-off exposure study and the participant has never experienced analgesic creams before, is this already operant conditioning? To what degree do pain killers still elicit conditioned responses when faith in their effectiveness is hampered by the patient's experience?). Beyond this, there is more evidence for a link between associative learning and pain.

Associative learning in the form of classical conditioning as described by Pavlov (1927) is the process of linking stimuli that repeatedly occur at the same time or follow closely in time such that an involuntary and automatic response that is normal only to one (or a subset) of the stimuli (unconditional stimulus) becomes a normal response for the stimulus that was neutral (conditioned stimulus).

It is widely known that aversive classical conditioning is a reliable way to make participants develop stress responses to previously neutral cues (a process in conditioning that is called 'acquisition'); this is not to be confused with conditioned pain modulation. Classical conditioning is almost unavoidable in settings involving stimuli that reliably follow closely in time in several

repetitions and thus this process would apply to chronic pain: for instance stairs at home may condition with knee pain such that climbing stairs in itself is tied with the anticipation of pain and thus may increase stress levels which likely worsen any present pain. This example would incorporate both classical and operant conditioning as the outcome is usually seeking to avoid climbing stairs as part of pain-related fear avoidance which in turn contributes to pain progression (Vlaeyen & Linton, 2000).

Repetitions of painful stimuli preceded by warning cues are also bases for many experimental pain studies to be inadvertently conditioning. Some experimental studies in healthy volunteers have shown that this can increase pain as evidenced in a recent meta-analysis (Madden, Harvie, et al., 2016). It should be pointed out, though, that many experimental human pain studies probably do not use sufficiently strong stimulus intensities to induce this effect since habituation to the unconditioned (i.e. painful) stimulus is common; this may in parts explain heterogeneity in the meta-analysis that prevents more comprehensive conclusions. Chronic pain on the other hand is so aversive that it is usually hard to ignore, repetitively occurs in places that the patient lives in, and is therefore likely to be subject to a conditioning effect that makes pain worse.

Beyond worsening pain, though, most clinicians believe that pain can also develop as a classically conditioned reaction and thus exist without nociceptive input (Madden & Moseley, 2016). This is plausible based on what we know from aversive classical conditioning: Pavlov's observation that dogs would salivate to the sound of a bell, even when the bell was no longer followed by food. Using the previous example, this would mean that simply climbing stairs could at some point elicit pain without nociceptive input (e.g., despite successful joint replacement).

Normally, once the unconditioned stimulus ceased to coincide with the conditioned stimulus, a learning process commonly called 'extinction' (see box 1) would take place whereby conditioned responses are being abolished over subsequent encounters with the conditioned stimulus; literature often also refers to this process as safety learning.

Depending on the circumstances during acquisition of the association, however, such reactions may be most challenging to eradicate, especially when strong stress reactions have established.

The latter problem is summarized by the term 'extinction deficits' which is another plausible pain progression model that integrates stress hormones and sympathetic arousal into mechanistic explanations of chronic pain and complements several components of the biopsychosocial model.

The idea that classical conditioning contributes to pain progression is entertained by several researchers, recently Apkarian even proposed to integrate it into a definition on chronic pain (Apkarian, 2008). Although the link between conditioning and pain has been studied since 1970s, this appears premature because focus of such studies has mostly been fear avoidance but also on placebo and nocebo effects. There is also the aspect that it is challenging to design human studies that prove this link as it is impossible to 'measure' nociceptive input and account for it when assessing pain; accordingly, there is relatively little direct empirical evidence on extinction deficits maintaining pain.

One such study recently showed that conditioning makes pain more likely in healthy volunteers for conditioning stimuli that are at the threshold of being painful compared to these stimuli being paired with non-noxious unconditioned stimuli (Madden, Bellan, et al., 2016). This finding is tentatively supportive of a pain model driven by classical conditioning but because stimuli at pain-threshold level were used it only proves that conditioning biases an individual towards perceiving pain. Another study applied motor imagery tasks in conditioning paradigms which showed that the sheer thought of painful movements increased pain and physical swelling of joints (Moseley et al., 2008). This finding makes a pain progression conditioning model credible, especially in combination with some observational studies showing impaired extinction (safety learning) in fibromyalgia patients (Meulders, Meulders, Stouten, De Bie, & Vlaeyen, 2017). Interestingly, cortical responses from chronic back pain patients are indicative of classically conditioned extinction deficits even though the study was mostly about operant conditioning effects

(Flor, Knost, & Birbaumer, 2002) which makes clear that in reality pain progression models on operant and classical conditioning would blend together.

Overall, it is still unclear how much extinction deficits contribute to pain progression. However, clinicians' hands-on observations on pain patients should not be dismissed either and thus their statement add some support to a not fully proven but plausible model, as rightfully argued in (Madden & Moseley, 2016). It should also be pointed out that there is consensus that extinction deficits is the base for posttraumatic stress syndrome (Zuj, Palmer, Lommen, & Felmingham, 2016) which has several communalities with chronic pain (as outlined in box 2). Conversely this adds to the likelihood that this mechanism may also exist to some degree in chronic pain.

It is therefore suggested here that the pressing question is probably not '*whether*' but '*to what extent*' pain progression and extinction deficits are linked and which pain patient groups would benefit most from interventions targeting the latter mechanism.

Once this is established, pain research benefits from a head-start for treatment optimization on extinction deficits seeing as other areas have thoroughly studied behavioural training but also many pharmacological avenues to tackle and even prevent extinction deficits. The challenge is then to identify interactions from medication, optimize treatments accordingly and identify key vulnerability factors. The added promise of such efforts is that it could help to understand pain killer addiction as literature beyond pain links dependence with associative learning problems (Di Chiara, 1999) and the amygdala is an emerging hub in addiction literature (Koob, 2009).

Box 1: Nomenclature of conditioning studies

In classic studies the conditioned stimulus is usually a neutral cue that is followed by an aversive stimulus (most commonly: electric shock) as the unconditioned stimulus. The usual outcome of such studies is that after a few trials participants get similarly stressed by the aversive cue (CS+) as they do for the unconditioned stimulus but they do not develop this reaction to a neutral

cue (CS-). Most studies on this phenomenon have focused on aversive stimuli for this matter and thus this process is commonly referred to as "fear conditioning" although this label is not adequate seeing as this process applies to aversive and appetitive stimuli alike (Parkinson, Robbins, & Everitt, 2000) and hence the term associative learning is used.

It is commonly found that this conditioned response to the CS+ can cease when the CS+ is repeatedly presented without the unconditioned stimulus and thus this "unlearning" process was called 'extinction' (VanElzakker et al., 2014). Both terms are misnomers as it turned out that the corresponding memory trace is not truly 'extinct' evidenced by spontaneous recovery of the learned association as well as facilitated learning of the same association upon re-exposure however in specific circumstances a true extinction is possible (i.e., interfering with the memory trace while it is not re-/consolidated will likely yield 'erasure') (VanElzakker et al., 2014). In turn this means that 'unlearning' is rather new learning (VanElzakker et al., 2014) and thus this term is increasingly being replaced.

Due to the majority of reports using neutral stimuli in the conditioning of aversive stimulus, extinction can be regarded as 'safety learning'.

It may seem odd that the first sentence on the discussion of this mechanism mentioned the amygdala as an integral brain area for associative learning and extinction whilst the large part that followed after did not mention it because most of the human studies linking pain and extinction deficits did not incorporate brain imaging data. An exception is Apkarian who implicates the amygdala in this process (Apkarian, 2008) however limits this discussion to operant conditioning by describing a mechanism based on social- and environmental cues without providing further data. However, in light of the last sentence of the previous paragraph, emphasizing the need to identify biomarkers for prophylaxis, it becomes important to specifically address the amygdala. There are claims that extinction-related plasticity in the amygdala is a "cause" rather than a consequence from impaired extinction (Maroun et al., 2013). Although recent data is indicative, a "direct demonstration" is pending

(see review by Farrell, Sengelaub, & Wellman, 2013). There is however more indirect data to support this mechanism as well as complementary mechanisms on amygdala plasticity and pain progression which will be elaborated in the next section.

Box 2: Similarities between chronic pain and PTSD?

Studies report between 10% of chronic pain patients with combat-related injuries (Benedikt & Kolb, 1986) and 35% of chronic pain patients with work-related injuries (Asmundson, Norton, Allerdings, Norton, & Larsen, 1998), or 45% of patients treating pain from burns a year after injury (Perry, Cella, Falkenberg, Heidrich, & Goodwin, 1987) also exhibit PTSD. Conversely as many as 80% of PTSD patients also suffer from chronic pain (Beckham et al., 1997). These rates vary considerably depending on the nature of the traumatizing event and the sustained injury but illustrate that overlap between these two aetiologies can be substantial. Studies also found that PTSD patients rate pain more intensely than healthy controls (Defrin et al., 2008; Morasco et al., 2013). These commonalities might not seem surprising when the nature of traumatizing events is often accompanied by severe injuries (e.g., combat-related, car accidents, fire) but considering the fact that situations or injuries that have the potential to elicit chronic pain or PTSD are encountered by most people at least once in their lives yet only about 5% of the population (n=1824) develop the disorder (although risk for women is double)(Frans, Rimmo, Aberg, & Fredrikson, 2005), the high comorbidity rate suggests the possibility of shared vulnerability and disorder progression factors between PTSD and chronic pain. Indeed, several hypotheses have been put forward; some of these have been summarized in the mutual maintenance model that focuses on disorder progression through heightened anxiety levels and -sensitivity, depression (Sharp & Harvey, 2001) and increased attention to threat cues and avoidance behaviour (Sharp & Harvey, 2001), which is the equivalent of the fear avoidance model of pain (Vlaeyen, Crombez, & Linton, 2016). This is expanded on by the Triple Vulnerability model on communalities in chronic pain and PTSD which describes that having at least two of three vulnerability factors make development of PTSD and chronic pain likely (Otis, Keane, & Kerns, 2003). These factors are:

-a biological vulnerability factor: genetic predisposition to anxiety disorder or chronic pain, physical pathology for pain

-a psychological vulnerability factor: anxiety sensitivity/heightened arousal, negative affect

-a specialized psychological vulnerability factor: perceived lack of control/unpredictability

Separate to these models, it has also been suggested that there is overlap in the brain networks involved in chronic pain and PTSD development (Zaman, Vlaeyen, Van Oudenhove, Wiech, & Van Diest, 2015).

Beyond these parallels, the mutual maintenance model also suggests that chronic pain as a result of trauma with injuries contributes to PTSD manifestation through operant conditioning whereby chronic pain itself serves as trauma reminders (Sharp & Harvey, 2001). This mechanistic link has received limited attention in this model compared to available literature on conditioning in chronic pain and PTSD. In short, there is consensus that PTSD is largely based on extinction deficits (Zuj et al., 2016) and as discussed earlier, it is very plausible that extinction deficits at least partially contribute to chronic pain progression in some aetiologies. It is therefore likely that extinction deficits are a common progression mechanism between PTSD and chronic pain which needs further investigation; although parallels between PTSD and chronic pain are frequently referred to, mechanistic overlap between these two disabilities have been hardly investigated.

1.4.2 Mechanism #2: Amygdala hyperactivity, chronic stress and extinction deficits

Neugebauer claims that insufficient control of the medial prefrontal cortex (mPFC) over the amygdala, specifically through the inhibitory intercalated cell mass within the amygdala as relay station, enables hyperactivity of the latter which in turn is responsible for chronic pain development (Neugebauer, 2015). This model is in line with an observational study that inhibition of the amygdala (central nucleus) has been found to be impaired in an arthritis model (Ren & Neugebauer, 2010).

Beyond modulating pain perception itself the amygdala is in prime position to influence other brain areas that are well established as core of pain processing. Animal work showed for example that the amygdala is part of the spinothalamic and spinopontoamygdaloid tract pain pathways and has strong mutual connections with pain processing networks (Strobel, Hunt, Sullivan, Sun, & Sah, 2014). Interestingly, a human pain study has shown that amygdala activity predicted activity in the insula, thalamus and striatum (Roy et al., 2009) even before the actual pain experience (Qin et al., 2008) which suggests that the amygdala may be in a prime position to modulate activity of other pain-processing brain regions and by this possibly pain itself.

Although not direct proof for this, it is interesting to add that Simons et al. showed a change of amygdala's activity after successful chronic pain treatment (as compared to before treatment) in a study on complex regional pain syndrome (Simons, Pielech, et al., 2014). Similarly, Lapate et al. found that the extent of altered amygdala activity was predictive of the success of pain regulation after an emotion regulation paradigm in healthy volunteers (Lapate et al., 2012).

Whilst a model on amygdala activity and - connectivity does not mention extinction deficits or stress, both can be combined into this theory. Psychological pro- and antinociceptive properties of stress are undebatable but only recently observational studies outlined that stress hormones have a substantial neurobiological impact on pain. In line with this, corticotrophin

releasing factor has been recently proposed to be part of an amygdala pain progression model (Rouwette, Vanelderen, Roubos, Kozicz, & Vissers, 2012).

This may possibly link to Averill's hypothesis that stress hormones trigger amygdala over-activity which results in excessive glutamate levels that impair inhibition over the HPA axis' stress hormones as part of a feed-forward loop and result in excitotoxicity and neurotoxicity (Averill et al., 2017).

It would be particularly detrimental if the inhibitory interneurons in the intercalated cell mass within the amygdala that connect to the vmPFC decay due to neurotoxic glutamate levels during prolonged stress. This intercalated cell mass has however been shown to be essential to fear extinction (Likhtik, Popa, Apergis-Schoute, Fidacaro, & Pare, 2008) which suggests that such detrimental neuroplastic changes may establish through prolonged stress and foster extinction deficits as well as amygdala hyperactivity to facilitate pain progression. Stress-induced extinction deficits are a common finding (Abiri et al., 2014; Maren & Holmes, 2016; Maroun et al., 2013). It is therefore likely that extinction deficits are also accompanied by prolonged pain. In turn, these can contribute to pain chronification as discussed in the previous section.

Although not linked to specific mechanisms, pain-related structural alterations in the amygdala have been found in animal pain models: some neurons within the anterior central part of the amygdala develop sensitivity only to specific noxious stimuli that bear close resemblance to the applied pain model but not noxious stimuli in general (i.e. applied arthritis model, result: a small number of neurons developed sensitivity to mechanical pain stimuli but not heat pain stimuli) (Neugebauer & Li, 2003). Further, lesions of the (basolateral part of the) amygdala *stop* typical chronic pain *progression* compared to control rats with sham surgery (Li, Wang, Chen, Zhang, & Wan, 2013), a finding that suggests that amygdala structure may be actively contributing to chronic pain development.

If these neuroplastic changes are replicable and linked to chronic stress or extinction deficits, then there may be a crucial time window to combat these effects as an animal study that compared stress-induced neuroplastic changes in the amygdala and hippocampus found that whilst the resulting hypertrophy

in the hippocampus reversed quickly after cessation of the aversive situation, such hypertrophy persisted in the amygdala even three weeks later; as the animal study was not continued beyond this time frame it is unclear whether neuroplasticity reverses at a much later time point (Vyas, Pillai, & Chattarji, 2004) and it is unclear how big the effect would be in a human with chronic pain.

This emphasizes the urgency of further investigations into chronic pain-related amygdala plasticity but also into any factors that contribute to such alterations. One factor that is linked to chronic stress but has also been shown to contribute to extinction deficits on its own is 'uncertainty' as elaborated on in the following section.

1.4.3 Mechanism #3: Uncertainty

Chronic pain usually is linked to plenty of uncertainty: A lot of chronic pain patients experience great relief when, after a long odyssey, they are finally confronted with a plausible explanation as to the cause of their pain. On the one hand this may be because this knowledge allows relief over the worry that their pain might mean cancer or disease deterioration, which allows them to cope with the pain better, on the other hand, many patients feel being taken serious at last (Lillrank, 2003). Beyond this, there is usually not a 100% reliable pattern between certain movements and pain, patients do not usually know onset, intensity or duration of spontaneous pain, when or whether the next treatment will achieve sufficient pain relief, and if their condition will stabilize or deteriorate further (and neither do lab animals), making uncertainty stressful. But also after the diagnosis is established, chronic pain is often characterized by uncertainty in the form of sudden pain attacks that can vary in length and intensity. This is usually reflected in chronic stress and thus the influence of uncertainty on pain progression is intuitive rather than proven but there are empirical studies in healthy volunteers showing that uncertainty can modulate acute pain.

One such study showed that uncertainty about upcoming stimulus intensity was reflected in increased amygdala activity comparable to stimuli of

moderate pain intensity (Bornhovd et al., 2002) and that uncertainty can enhance perceived aversion of experimental pain depending on its intensity (Brown, Seymour, Boyle, El-Deredy, & Jones, 2008). Similarly, Meulders and Vlaeyen found that delivering pain with unpredictable onset caused more distress than delivering the same stimuli predictably (Meulders & Vlaeyen, 2013). Indeed, this slight difference in the experimental design led to "non-differential fear generalization" (Meulders & Vlaeyen, 2013) such that even healthy participants showed impairments in learning to differentiate threat and safety cues in the experiment[1].

This stimulus generalization is also the basis for the 'imprecision' pain progression model (Moseley & Vlaeyen, 2015) which states that imprecise cue-pain associations make pain likely to be triggered by stimuli that resemble the initial cue. This hypothesis, similar to the meta-hypothesis on extinction deficits worsening chronic pain, is not fully proven but plausible based on what we know from animal and human studies on conditioning as explained in the following.

Most conditioning studies have applied stimuli in a consistent fashion for the associative learning part and have also used such a reliability in the extinction phase (i.e., the conditioning stimulus is never followed by the unconditioned stimulus). Not all studies have however applied such a 100%-schedule and logically a less consistent conditioning pattern (called: partial reinforcement designs) implies some uncertainty in the acquisition phase. This in turn slows the extinction process or even impairs it completely, a phenomenon called the partial reinforcement extinction effect (PREE) in rats (Haselgrove, Aydin, & Pearce, 2004). Although likely translatable, the term is not used in human studies.

In a human conditioning study, Morriss et al., found a relationship between amygdala activity, self-reported intolerance of future threat-related uncertainty and performance in an aversive conditioning task to the extent

[1] For enhanced discussion, also refer to *Imprecision theory*, which states that uncertainty during repeated pain experience leads to "blurred encoding" of this experience and to "generalization", which makes it "more likely […] that pain will be triggered by more functionally distinct stimuli" (Moseley & Vlaeyen, 2015).

that the higher uncertainty intolerance, as indicated by the participants' self-report, was predictive of extinction deficits in the paradigm (Morriss, Christakou, & van Reekum, 2015).

Thus, uncertainty may contribute to extinction deficits and by this possibly to disease progression in chronic pain. Further, it seems that there is a direct link between uncertainty intolerance and amygdala activity, although this awaits replication, both in healthy volunteers and in a chronic pain cohort to understand its potential for pain management.

As much as uncertainty intolerance is linked with distress, it is also linked with fear as it is essentially fear of the unknown. Intuitively one expects overlap between trait anxiety and intolerance of uncertainty but the study shows that the two are not necessarily interchangeable as trait anxiety was not a good predictor (Morriss et al., 2015).

Therefore there is motivation to discuss the role of uncertainty on the amygdala in relation to chronic pain but it also integrates with what the amygdala is best known for, which is discussed as amygdala-pain progression mechanism nr. 4 in the next section.

1.4.4 Mechanism #4 – Fear, threat, salience and relevance processing

The amygdala is best associated with fear processing and fear is a powerful modulator of pain which can even lead to complete absence of pain (analgesia) or exaggerated pain (hyperalgesia) (Pare & Duvarci, 2012). Also threat (Isenberg et al., 1999; Ohman, 2005) and threat-safety discrimination (Ostroff, Cain, Bedont, Monfils, & LeDoux, 2010; Sangha, Chadick, & Janak, 2013) are regulated by the amygdala and are relevant in the present context as pain is inherently a threat cue to preserve physical integrity. Together these influences are often referred to as 'emotional modulation of pain'. Hashmi and colleagues observed that chronic pain "shifts brain representation from nociceptive to emotional circuits", including the left and right amygdala, in chronic back pain patients within a year after pain onset (Hashmi et al., 2013); three years later the same group reported that amygdala volume was the strongest predictor in a model that explained which individuals of this set

worsened and which improved on their pain levels a year later (Vachon-Presseau et al., 2016). This specific study will be discussed further in chapter 4 but this brief outline serves to support promise of emotional processing and the amygdala for pain progression.

Similarly relevant as related to threat-safety discrimination are vigilance, which the amygdala processes (Davis & Whalen, 2001; Davis, 1992; Whalen, 1998), and salience (or relevance or attention). Salience can have pro- but also anti-nociceptive effects, similar as fear (i.e. even fractures may not be noticed if individuals are deeply distracted by e.g., competitive sports games) and thus it does not surprise that discussions on human pain are often intrinsically linked with salience (see also discussion by Mouraux & Iannetti, 2009). It is less known that salience processing is claimed by some to be the amygdala's main function (Cunningham & Brosch, 2012) rather than fear processing.

Together with the other three proposed mechanisms this suggests that the amygdala could have a key role in discussions on pain, even if a lot of research is still to be done. As briefly described within each section, these mechanisms are complementary and provide links between mechanisms that the biopsychosocial model of pain was lacking: the amygdala is involved in 'emotional modulation of pain', psychological mechanisms (fear avoidance, threat and safety perception, conditioning), but also biological mechanisms (neurobiological bases of conditioning, chronic stress, arousal, amygdala hyperactivity); even social elements in pain are covered by the amygdala because oxytocin (the "social hormone") is important for signal processing in this structure (Debiec, 2005). This suggests that the amygdala is a promising structure to investigate with respect to pain chronification generally but also a structure that is not well understood (see box 3).

What is its role in contemporary pain research?

Box 3: What's the amygdala's main function? A micro-commentary

A thorough literature search will link the amygdala to many related neuropsychiatric disorders (e.g., depression, phobias, various anxiety disorders, obsessive-compulsive disorder- for review see (Zhang et al., 2018)) but also to different conditions such as autism, aggression, addiction, and studies on prediction error encoding in addition to relevance processing, and associative learning. The amygdala is best known to be linked to fear and emotional processing; it is less known that the amygdala is essential for associative learning although the latter as a more general function of the amygdala would allow more comprehensive explanations. For instance, abnormal safety-threat perception underlies various neuropsychiatric disorders and often originates from personal experience in which traumata are associated with certain behaviours, items, or places. If fear draws on associative learning (in order to evaluate a situation based on previous experience), as in this example, it is not contradictory that there are patients with bilaterally completely lesioned amygdalae (as result of a disorder) who are able to feel fear (Feinstein et al., 2013).

Another example where a focus on associative learning would allow further progress concerns habituation and replicability.

Facial expressions are the most reliable to elicit amygdala activity whilst most other experimental stimuli either yield rapid habituation, no discernible amygdala activity or replicability problems (summarized in (Boubela et al., 2015)). It is suggested here that the amygdala's activity to facial stimuli- rather than a general role in emotional processing- reflects that facial expressions are perhaps the most important cues in our environment to make behaviourally relevant decisions on safety versus threat, gain versus harm, and consequently for fight or flight decision. As facial expressions are dynamic and complex, they require a constant refinement of learned associations as well as a quick reassessment at every encounter of a person and if the amygdala's repository of learned associations is used to classify facial expressions into harm or safety

cues[2], it is sensible that there is a lower habituation rate of facial stimuli in neuroimaging studies in contrast to other objects that do not usually change their connotation (e.g., a cobra normally means danger).

1.5 The amygdala's role in human pain studies: limitations, confounding factors and limited awareness?

The volume of preclinical work (i.e., >200 published studies) and some of the pain progression models put forward suggests that the amygdala plays a major role in pain research which, on first sight, is corroborated by a meta-analysis on human pain studies (Simons, Moulton, et al., 2014).

However, the latter meta-analysis suffers from pre-selection bias as the amygdala was part of the search terms and yet it is based on merely 23 included human neuroimaging experimental pain studies and 17 studies on patients suffering from a heterogeneous mix of pain disorders. As pointed out by the authors, the insula with the same basic search terms yields 6 times as many papers on pubmed before filtering either search for exclusion criteria. There is also the fact that many of the included studies, are done with a poor spatial resolution for a small structure like the amygdala (1.5 Tesla scanners and PET studies with smoothing kernels of up to 17mm FWHM- N.B. amygdala volume is ca. 1cm^3 according to post-mortem studies (Brierley, Shaw, & David, 2002)). Judging by such data quality and by sheer numbers alone, the message may not have enough substance to motivate further human pain amygdala studies. In line with this, a systematic review of 266 cutaneous nociceptive fMRI experiments less than 5% reported amygdala activation (Tanasescu, Cottam, Condon, Tench, & Auer, 2016). This shows that the amygdala is not unheard of in the context of human pain but its reporting is sparse. It is tempting to conclude from this that the amygdala is not as important in pain as for instance the insula; this may be reflected by the fact that the amygdala is often not or only marginally mentioned in human pain whole brain meta-

[2] Importantly, the classification does not necessarily reflect that danger comes from a certain person but that there may be danger in the environment.

analyses (Peyron, Laurent, & Garcia-Larrea, 2000) and even if mentioned in the results it may be ignored or mentioned superficially in the discussion which most of the times is focused on the insula.

Yet, this conclusion may be premature as it ignores any possible influence of publication bias in combination with amygdala-specific technical and design issues which may limit amygdala results in human pain more than for instance findings regarding the insula.

Firstly, data quality as alluded in Simon and colleagues' report is an issue: The location of the amygdala makes it prone to signal drop out (due to magnetic field inhomogeneities) and thus is prone for type II errors. Its size and lack of clear borders pose further challenges and thus without the motivation to do region-of interest analyses findings in this structure are likely to become victim of necessary but stringent whole-brain multiple comparison corrections. On the whole, there may not be sufficient motivation for this as it is known that amygdala activity is prone to rapid habituation or to replicability problems. In this respect, motivation has been probably further diminished when it was reported that amygdala fMRI findings are even worse in their replicability when physiological noise correction is done (Boubela et al., 2015).

In addition, some of the few human studies mentioning the amygdala report its 'deactivation' during experimental pain or its anticipation (Rubio et al., 2015; Ziv, Tomer, Defrin, & Hendler, 2010) and thus probably added confusion.

But motivation and technical problems are not the only ones. In particular, Tracey and Mantyh mentioned the amygdala in their review about pain related brain regions as one of the regions whose activation depends "upon the particular set of circumstances for that individual" (Tracey & Mantyh, 2007), which hints at dependence on details of the experimental designs. This suggests that the fact that amygdala does not appear to be consistently involved in a majority of human pain studies may reflect a mechanistic pattern that relates to limitations of human pain studies as opposed to a diminished role of this structure in pain.

This receives support by the fact that authors of a meta-analysis on human fMRI studies claimed the amygdala to be exclusively active when confronted with aversive stimuli but not with painful ones (i.e. no overlap of brain areas) (Hayes & Northoff, 2012). Similarly, there is an inconsistent picture on neurosynth.org regarding the strength of the link between the term (both acute and chronic) 'pain' and 'amygdala' (z-score values) as this link is weaker than the link for "threat" (reverse inference, right amygdala: "pain" z-score max. 6.15 vs. "threat" z-score 9.86; left amygdala: "pain" z-score: 0 vs. "threat" z-score max. 12.4) despite the fact that "pain" is represented in this comparison with 420 studies while "threat" only yields 170, so this discrepancy is not due to power. This is visualized in figure 4.

Pain by default is meant to be aversive and threatening as an incentive to avoid behaviour that is likely to inflict damage on organisms. Such a discrepancy between neural circuits for pain and those for threat or aversion begs to question whether experimental pain stimuli were "painful enough" to be threatening to some degree, as pain normally is.

As far as neurosynth is concerned, this paradox may be simply based on authors not using the terms "threat" or "aversion" in their manuscripts- however, results from the meta-analysis cannot be explained this way and rather support the alternative explanation.

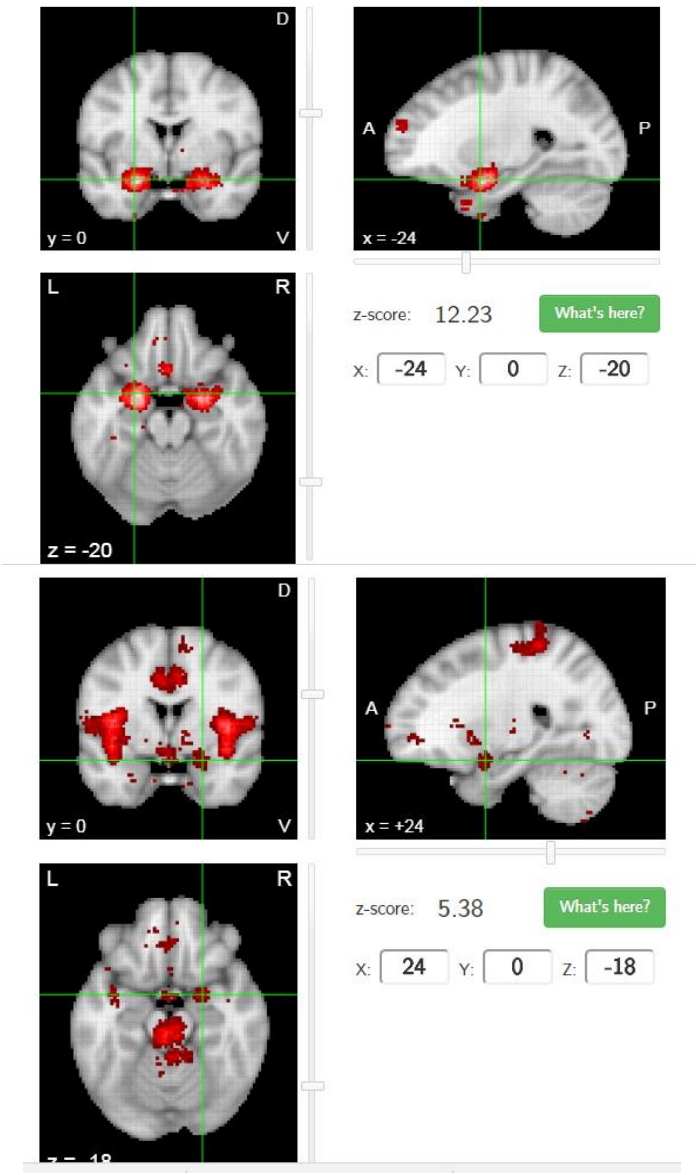


Figure 4: Automated meta-analysis of 170 studies on "threat" (top) and 420 studies on “pain” (bottom) -compiled by neurosynth.org

This figure shows that the association strength between the amygdala and the term pain or the term threat is very different: although the terms threat and amygdala have less than half of the number of studies compared to the terms pain and amygdala, the z-score is much higher which suggests that pain literature does not nearly as often mention the amygdala than literature on threat. Maximal z-scores displayed in this figure irrespective of laterality.

To some degree, safety perception also diminishes the level of aversion in aversion studies but depending on the stimuli used it may be argued that this effect is more detrimental in experimental pain studies which relates to the definition of pain and the stimuli used. As explained earlier in this chapter, pain is usually the next stage of aversion. Indeed, most human aversion studies use

electric shocks, which, as soon as they are applied above perception-threshold, are almost automatically aversive, and only at high levels become painful which are rarely used in these studies. Most human experimental pain studies, are however, not based on electric but on thermal, pressure, or pinprick pain stimuli which do not necessarily even elicit aversive experiences. Thus it would be wrong to conclude that amygdala findings across painful and aversive stimuli should be equally affected by limitations from study designs or ethics and should elicit the same, consistent amygdala activation.

Relatedly, discrepancies between animal and human pain studies are certainly helped by if not based on unequal ethical limitations: participants in experimental studies are "well informed and the element of threat is reduced to a minimum" (Ingvar, 1999) because they voluntarily choose to undergo painful procedures in a controlled context and know they can withdraw from an experiment at any time, know that the resulting pain will only be moderate in intensity, lasts only briefly and know that no resulting tissue damage is to be expected. This knowledge certainly reduces aversion but is not available to lab animals.

It is also clear that electric shocks are not subject to the same level of habituation that other experimental pain stimuli are prone. Conversely, the concept of habituation as a lack of consistency and a nuisance factor is rather prone to misconceptions: slower rates of habituation could reflect problems with safety learning, which in turn could reflect extinction deficits- making the rate of habituation a variable of considerable interest for pain research, especially when comparing habituation rates between pain patients and healthy volunteers. Indeed, anxiety disorder patients have been found to have abnormal responses to safety cues rather than abnormal responses to threat cues (Lissek et al., 2014) which makes clear that both safety learning (and thus "habituation") and aversive learning need to complement pain research. Conversely this means that habituation is not always a nuisance factor and a lack of amygdala activity is not necessarily a lack of consistency of this brain structure.

More generally, though, it is possible that some types of studies may elicit more reliable amygdala activations because they use paradigms that are more adequate to visualize the amygdala's function: as discussed in box 3, a lot of findings would be reconcilable if this meta-function is associative learning. In such a scenario, it would be natural that studies on aversive conditioning elicit the most consistent amygdala findings, alongside studies using facial or fear stimuli which would draw on aversive learning. The latter implies that timing should be considered as a factor which is usually not the case when responses are averaged across the entire paradigm. Kattoor and colleagues for example did not find amygdala activity in their human aversive conditioning study to either the pain or safety cue in the early stage of conditioning (Kattoor et al., 2013). The authors reported that halfway through the conditioning paradigm, the amygdala did respond to the cues but did not differentiate between them; this changed in due course and by the end of the acquisition phase the amplitude of the response was considerable but only for the pain cue (Kattoor et al., 2013). Had the authors averaged responses over the entire acquisition period, then this would probably have yielded type II errors which is probably reflective of many studies as few choose not to average across the whole paradigm.

1.6 Summary and outlook on next chapters

Whilst there is still no consensus on what drives pain progression, the amygdala is linked to four plausible and complementary mechanisms that could contribute to pain chronification. This discussion does not imply that the amygdala is the only relevant brain region for pain processing but it serves to outline its potential. It is pointed out that the amygdala's infrequent pain-related activity in human studies often leads to interpretations minimizing its importance and that most of such data is based on animal studies. Thus, the current discussion serves foremost to caution against evaluating this brain region's role in human pain studies before current limitations have not been addressed with carefully designed studies and analyses.

From the mechanisms outlined, chapters 4 and 5 elaborate on pain-related amygdala neuroplasticity, -activity and -connectivity before chapter 6 closes with an outlook how work on uncertainty management, as part of the third mechanism suggested here, could be implemented to benefit pain patients.

Chapter 2: Aims and objectives

The overarching aim of this thesis is to evaluate the role of the amygdala in human pain further, using MRI. Specific aims and objectives for the study chapters are stated below.

Amygdala structure in health and chronic knee OA pain (chapter 4)

Aim 1: To investigate whether the structure of the amygdala is altered in chronic pain.

Objective: Use of voxel-based morphometry to compare amygdala gray matter density in patients and healthy controls in a whole-brain analysis as well as a region-of-interest analysis.

Aim 2: To investigate whether any pain-related amygdala gray matter density alterations are enhanced in a female patient group and whether there are distinct pain-related amygdala structure in either sex.

Objective: The same analysis will be performed in separate subsamples of males and females.

Amygdala functional connectivity in health and chronic knee OA pain (chapter 5)

Aim1: To investigate whether seed-based amygdala connectivity maps are sensitive to the exact amygdala masks used for seed-based connectivity as this will help judge comparability of the main findings of this chapter against published literature.

Objective: Use seed-based connectivity analysis with different amygdala masks based on Harvard oxford and Juehlich atlas to compare within-group maps.

Aim2: To investigate whether amygdala networks differ in pain patients and healthy volunteers.

Objective: This will be addressed with a seed-based resting-state functional connectivity study comparing amygdala networks in chronic knee pain patients and healthy controls.

Aim3: To investigate whether there are distinct pain-related amygdala network alterations for females and for males.

Objective: This will be addressed with the same analysis as for aim 2 in separate sub-samples for males and females.

Chapter 3: Basic MRI physics

Magnetic resonance imaging (MRI) is the method of choice to investigate the human amygdala non-invasively in-vivo in humans as it produces brain images with relatively good spatial and temporal resolution. This chapter will describe essential principles of such data acquisition and pre-processing steps as it relates to data used in chapters 4 and 5. The following describes MRI in the context of brain imaging but the physics explanations are of course not limited to scans of the head.

3.1 MRI: the basics behind the signal:

The MR scanner comprises of a magnet that induces a strong static magnetic field (relative to the earth magnetic field; abbreviated B_0) that is as homogenous as possible over the head; shimming coils are meant to alleviate slight distortions in the static magnetic field due to the head of the person (individual shape) (Huettel, 2004).

This magnetic field is used to take advantage of an atomic property: that protons have dipoles and spin around their own axis ("precess"), which results in a small moving magnetic field that in turn comes with electrical current (Huettel, 2004). Importantly, only atoms with an odd number of protons (along with neutrons and electrons) have a net "spin" and thus magnetic resonance imaging can only use certain atoms, most prominently ^{1}H because these are part of water molecules that are abundant in organic tissue (Bitar et al., 2006). Under normal conditions, the spin axis points in random directions and therefore the tiny moving magnetic fields cancel each other out (Huettel, 2004) (see figure 1).

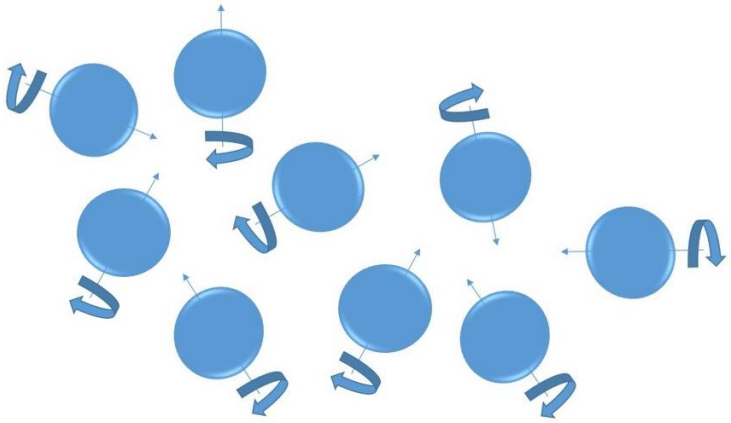


Figure 1: Proton dipoles and spins

Every proton spins but without an external magnetic field their spins point in all directions.

Spin axis direction can be visualized when a sufficiently strong magnetic field is applied- in which case the proton aligns with it such that its spin axis is either parallel or antiparallel relative to this magnetic field although the majority of protons aligns in a parallel way which is associated with a lower energy level (Huettel, 2004) (see also figure 2).

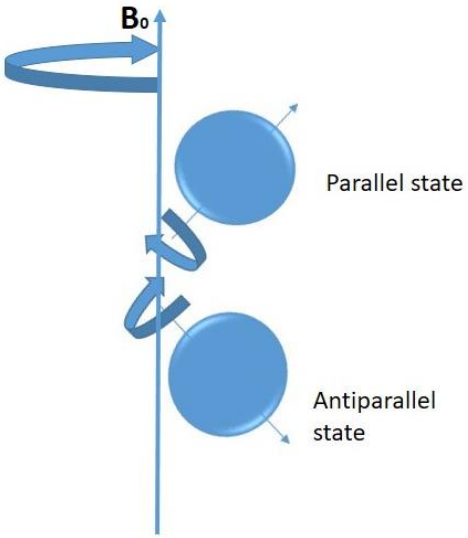


Figure 2: Proton alignment within a magnetic field

When a static magnetic field (B_0) is applied, protons align with this field in either a parallel or anti-parallel energy state.

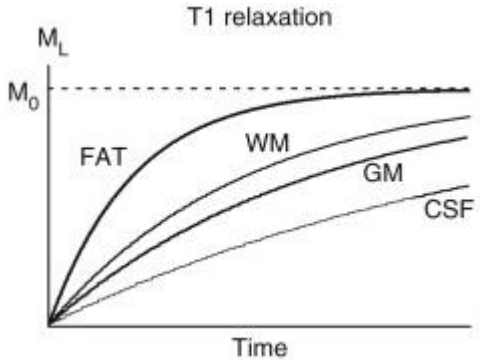
If a proton is hit by a radio frequency pulse of the right energy (which depends on a constant and the atom type as summarized in the Larmor equation) it will absorb this energy and excite to the antiparallel state. This way nearly all protons are in the antiparallel state and their spins synchronize ("precess in phase") (Huettel, 2004). This phenomenon is described by "resonance" and allows the micro-magnetic fields to sum up such that a receiver coil can detect a net magnetization because moving magnetic fields induce current flow. However, this effect is only short-lived because very quickly after excitation excited protons start to revert to the lower energy state and the spins start to get out of synchronization ("dephasing") (Huettel, 2004).

More specifically, this change in magnetization is often depicted as a vector that has a horizontal (transversal) and a vertical (longitudinal) component and thus one distinguishes between transversal relaxation for the steady loss of magnetization as protons revert to their lower energy state and between longitudinal relaxation that describes loss of magnetization as protons' spins get out of synchronization (Bitar et al., 2006). The built-up and loss of magnetization for either component is a continuous process and is described by T2 and T1-curves respectively which is important to mention as different tissues have different relaxation times and thus different T1 and T2 curves (Bitar et al., 2006). The transverse magnetization may be relatively higher than the longitudinal one or reverse which yields different contrasts on brain images and therefore one basic distinction is to name images as T1-weighted or T2-weighted (Huettel, 2004); this will be elaborated below but is also illustrated in figure 3.

More precisely, T2 curves depict theoretical dephasing speed whilst the actual decay is faster because of (remaining) unavoidable magnetic field or tissue inhomogeneities and is thus more accurately described as "T2*" (Huettel, 2004). Yet, a T1-T2* graph is simplified as the actual graph is a 3D spiral (because the magnetization vectors move to and from the receiver coil) (Goebel, 2007) but the simplified version allows to easily visualize optimal timing for receiving maximum signal.

Because signal is rapidly decaying, applying an inverse radiofrequency pulse allows to reverse the dephasing temporarily and by this maximise signal; this signal is called echo and the time between the radiofrequency pulse and the maximum signal, commonly abbreviated TE (Huettel, 2004). The time between one radiofrequency pulse and the next is called time-to-repeat, commonly abbreviated TR and usually denotes the time to acquire a set of slices that make up one volume that covers the whole brain (unless a smaller field-of-view was chosen) (Huettel, 2004).

A



C

Weighting	*TR*	*TE*
T1	*short*	*short*
T2	*long*	*long*
Proton Density	*long*	*short*

B

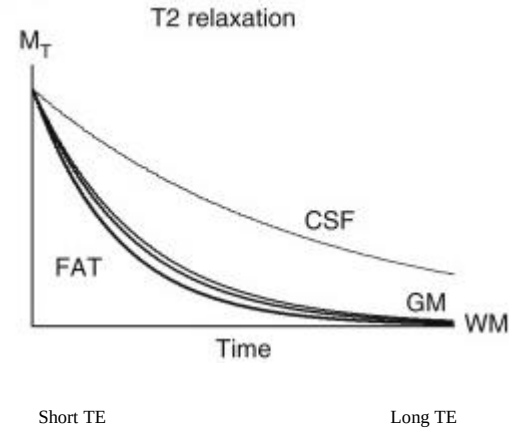


Figure 3: T1 and T2 weighting for different tissues and how they allow different tissue contrasts.

Figure A and B show T1 and T2 relaxation curves and thus illustrate longitudinal and transverse components of the net magnetization vectors after excitation through a radiofrequency pulse. As the figure illustrates, different tissue has different relaxation times. By selecting the moment in time when the T1 and T2 curves are most distinct between tissues, it is possible to obtain different contrast in the images later. The time point in turn depends on the scanning parameters TR and TE. Figure C: According to length of TE and TR, the images are called T1, T2, or Proton density weighted images.
Figures in A and B taken from (Sodickson & Sodickson, 2016)

3.2 Contrasts and sequences

The relative quantity of ^{1}H atoms is different between tissue components and thus is one of the principles that allows to distinguish different tissues on an image which explains why T1 and T2 curves for fat and water are different (Bitar et al., 2006). Additionally tissue with high water content does not allow protons to emit energy quickly and thus protons in tissue with high water content only slowly revert to the lower energy state; fat on the other hand absorbs energy better and thus the T1 relaxation time is much shorter (Bitar et al., 2006).

Hence a T1-T2* graph for each tissue type allows to find the optimal timing for RF sequences that are optimal for the tissue type in question. For this reason T1-weighted images (characterized by the use of a short TR) arguably have the best contrast for standard structural anatomical images (Bitar et al., 2006). Structural sequences that are better suited for suspected lesions or tumour detection are not relevant for the current work and thus not further discussed. One speaks of scanning sequences when more than one radiofrequency pulse is implemented; multiple pulses, at specific timings, enable to measure signal beyond dephasing because they reverse spin direction and thus allow spins again to briefly precess in synch (Huettel, 2004). Such pulses are called “echoes” and allow to prolong signal measurement (Huettel, 2004) but as much as one wishes to prolong this time, ultimately it is the impermanence that is one of the features that allow 3D image reconstruction from the signal measured- which will be explained below.

3.3 Functional magnetic resonance imaging (fMRI)

Functional MRI is based on a contrast that allows assumptions about the activity of certain areas. These assumptions rest on the principles that more active neurons need more oxygen and thus demand further blood supply and that oxygenated and deoxygenated haemoglobin have different magnetic properties (Goebel, 2007). FMRI is sensitive to a ratio between these two states of haemoglobin during a task relative to another task or baseline and

this ratio change serves as correlational evidence for increased neural activity; that is why fMRI is based on blood-oxygen-level dependent imaging (BOLD) (Goebel, 2007).

Conclusions from such correlations are not undebated, especially as the percent of BOLD signal change is often very small but there is strong support that hemodynamic responses at the very least reflect local neural processing (local field potentials) and neural input and thus fMRI remains an useful tool for some inferences about brain activity (Logothetis, 2008). Principles of fMRI are relevant to data for chapter 5 but the principles for localizing signal are the same as for structural images.

Box 1: What is BOLD signal change?

As explained above fMRI interpretation of brain activity requires that the measurement reflects a substraction: task A- task B or task A-baseline to obtain a map of brain regions that are relatively more active in a certain situation than in another. Mazaika describes a time series example where activity (plus noise) over time (x-axis) peaks at 200-500ms time, reflecting the correlate of increased neural activity shortly after onset of a task compared to a steady baseline before and after that time (100%)- consequently the task is thought to have induced a 2% signal change (because signal peak is at 102%).
(Mazaika, 2009).

3.4 Tracing signal to specific locations for image reconstruction

Any image reconstruction requires that signal at a specific time can be traced to a selected location before at another time signal is measured from another region (i.e., neighbouring tissue) hence the smaller the selected volume the more precise the reconstructed image; for this selectivity MRI scanners comprise of another two hardware components.

A gradient coil (slice selecting gradient) overlays another magnetic field on top of the static magnetic field during the radiofrequency pulse such that only a restricted "slice" of tissue is enabled to have the precession frequency required for excitation and by this the signal that is measured at a certain time is restricted to come from a certain slice of tissue (Goebel, 2007). Slice thickness is determined by the range of frequencies of the RF pulse and by the slope of the gradient field (Goebel, 2007).

Another frequency encoding gradient ensures further spatial localization by applying a gradient after the radio-frequency pulse to ensure that excited protons spin with different precession frequencies along the gradient (frequency encoding gradient) (Goebel, 2007). This ensures that the signal emitted by these protons differs systematically (from left to right) such that it is possible to make out the origin of the signal from a specific column along the selected slice (Goebel, 2007). Thus lastly, it is necessary to trace the signal origin within a column in order to reconstruct an image of the scanned tissue. This is done with a phase encoding gradient that is turned on along the horizontal axis (x-axis) for a short time after the radiofrequency pulse (Goebel, 2007). Applying the same principle as with the frequency encoding gradient this makes protons precess with different frequencies within the columns and thus allow precise localization of the signal within what is called a "voxel"- the 3D equivalent to the unit "pixel" in a 2D photo. Mathematically the received signal is mapped into a matrix (k-space) before its localization and image reconstruction is performed through Fourier transformation such that voxels are mapped on the resulting image by different shades of gray (Goebel, 2007).

Parallel imaging has been claimed to be the most significant advance for imaging around the late 1990s to early 2000s because it allows considerably faster speed in image acquisition (Larkman & Nunes, 2007); this was more recently optimized further as multiband imaging which applies the principles of slice excitation and image reconstruction (described above) on several interleaved slices in parallel (Feinberg & Setsompop, 2013). Similarly, more recent advances were made by sparse imaging which complements scanning time reduction efforts by interpolating images from undersampled data (Yang, Kretzler, Sudarski, Gulani, & Seiberlich, 2016). As these techniques are not applied in work pertinent to this thesis, they are not described further.

3.5 Artefacts

What is susceptibility?

The static magnetic field does not magnetize every material in the same, and thus different material has different susceptibility that denotes the extent of its magnetizability (McRobbie, Moore, Graves, & Prince, 2003). This concept allows contrasts but can also give rise to undesired artefacts where tissues with vastly different magnetizability interface because it creates different magnetic fields at the interface and in turn microscopic different gradients and accelerated dephasing, giving rise to signal drop out (McRobbie et al., 2003). This is most prominent at air-tissue or bone- tissue interfaces for instances (McRobbie et al., 2003). *Also metal in the body which stays secure in their position during the scan such as dentures, can yield such signal drop out.*

Artefacts can occur from various sources that perturb the image acquisition: aside from hardware problems or software settings (which are getting rarer with modern scanners), wrap around artefacts can occur when the field of view does not cover tissues adequately, and undersampling can produce ringing artefact (Huettel, 2004). In most cases, it is however rather subject movement causing a serious problem as it results in shifting signal from one slice to another during image collection, resulting in blurred images (Bitar et al., 2006). Related to this, physiological motion summarizes smaller movements due to respiration or heartbeat and can be corrected if not excessive (Huettel, 2004) (this will be discussed in more details in chapter 5). Also susceptibility artefacts can be serious in producing signal drop out and are very prominent at the

amygdala: As mentioned in chapter 1, the anatomical location of the amygdala in particular poses problems to MRI because of susceptibility-induced signal drop at tissue-air interfaces which contributes to magnetic field inhomogeneity and thus to less measurable signal and image distortion. This is in particular a problem for scanning amygdalae.

Chapter 4: Amygdala morphometry

4.1 Introduction

…. "It's not about you, could have happened to anyone." "But it does not happen to anyone. So many break a leg, recover after a few weeks and move on with their lives as before… but I'm one of those who are stuck with that pain although you say it is all healed… why does it have to be me? "

As much as this piece of a conversation between a patient and the treating doctor is fictive, it probably captures essences of realistic conversations in chronic pain treatment even if these elements are not necessarily spoken out loud: the desire to be able to predict pain progression better in order to advise patients and doctors alike and find explanations why individuals are affected. But how to find something to blame for severe perceived pain when there is little or no underlying detectable physiological defect? The classic example to illustrate this argument is osteoarthritis where correspondence between perceived pain and the underlying joint decay is known to be poor (Dieppe & Lohmander, 2005), with pain ratings ranging from zero to extreme despite comparable joint decay, or where 27-44% of successful total knee or hip joint replacements as last-resort measures have not diminished pain completely 3-4 years after the intervention, with 6-15% reporting "severe-extreme persistent pain" (percentages for hip and knee replacement respectively, n=632 knee and n=662 hip replacement patients)(Wylde et al., 2011). If supposedly all affected tissue is removed and the remaining tissue appears to have recovered well but the patient still expresses pain it is tempting to think 'it is in their patient's head'. Many pain patients feel let down and hurt by such open or covered scepticism (Newton, Southall, Raphael, Ashford, & LeMarchand, 2013) and whilst simulating is a possibility it does not seem probable that such a patient would agree to undergo surgery. What drives pain progression?

4.1.1 In search of vulnerability factors for chronic pain

Factors reviewed recently in relation to vulnerability to chronic pain include age, sex, personality and mental health, perceived stress, previous pain exposure and pain level at onset, as well as genetics and epigenetics, with the brief notion that preterm births predispose to “altered brain development and stress responses”, which in turn bears increased risk for chronic pain development (Denk & McMahon, 2017). Notably, this review skips over about two decades of human chronic pain brain imaging research that claimed to have found anomalies in brain structure and asked whether these are the “cause or the consequence” of chronic pain as May did half-way through that time (May, 2008).

The most popular approaches to make structural comparisons between patients and matched controls based on T1-weighted images for specific brain areas are in terms of volume, followed with some distance by gray matter density investigations and sometimes shape comparisons. Volumetry is popular because of its intuitive interpretations as well as the ease of automatic segmentation methods.

Gray matter density on the other hand, relatively easy to apply, seems also an intuitive even though indirect measure of structural alterations: this measure of signal intensity in T1-weighted images reflects the probability of a voxel being gray matter and consequently is thought to be a measure of density when averaged across a structure but not undebated (see below and discussion). Cortical thickness is a measure which is less debated but not applicable to subcortical structures, requires more computational work and is overall less frequently used. Shape measures are relatively new and comparatively scarcely used, perhaps for two reasons: on the one hand, preprocessing and normalization of images to enable further analyses might obscure smaller shape differences, on the other hand, inter-individual differences in brain areas can be reflective of many factors (e.g., genetic, nursing, environment, education, personal history etc.) and thus it is difficult to speculate where pathology might start, especially if normal shape of a

structure or subregions are not well-defined (i.e., amorphous). As a consequence voxel-based morphometry, which compares areas of interest voxel-by-voxel, dominates literature on pain-related neuroplasticity and is largely based on volumetry.

4.1.2 Human in-vivo studies on pain-related neuroplasticity

May compared studies on several chronic pain disorders and concluded that, whilst each aetiology had its own pattern, there was overlap in four brain regions that are commonly reported in studies on pain and thus have become part of the “pain matrix”, a network of brain regions supposedly in charge of pain processing (this will be discussed further in chapter 5). A year later the author’s team replied to their own question (‘cause or consequence’) more confidently with “consequence” (Rodriguez-Raecke, Niemeier, Ihle, Ruether, & May, 2009) but this did not stop research to pursue this question further. This is evidenced by the amount of meta-analyses and reviews emerging in the past decade, describing an inconsistent and in parts contradictory picture, even if results are not pooled over aetiologies (see Table 1).

Table 1: Summary of meta-analyses findings on pain-related neuroplastic alterations in brains of patients (relative to controls) investigated through MRI

Authors	Aetiology	sample size	Decreased gray matter	Increased gray matter
(Smallwood et al., 2013)	Various	500 chronic pain patients (23 studies)	putamen, posterior inferior frontal gyrus, anterior cingulate/proximity, medial frontal gyri, bilat. insula, superior temporal gyrus, left thalamus, medial frontal gyrus, cingulate, superior frontal gyrus, right anterior middle frontal gyrus	hippocampus and parahippocampal gyrus
(Cauda et al., 2014)	Various	700 patients	bilateral medial frontal gyri, bilateral superior frontal gyri, right pre- and post-central gyri, bilateral anterior insula, right cingulate cortex, basal ganglia, thalamus, PAG	bilateral post-central gyrus, left inferior parietal lobule, right pre-central gyrus, right post-central gyrus,]dorsal prefrontal areas, caudate, thalamus, cerebellum, pons
(Pan et al., 2015)	Neuro-pathic pain	n=240 + 263 controls	bilateral anterior insula, thalamus, right superior frontal gyrus, left postcentral gyrus	right medial frontal gyrus, right posterior insula
(Dai et al., 2015)	Migraine	n=222 + 230 controls	Post. Insula/operculum, prefrontal, ant. cingulate	
(Shi, Yuan, Dai, Ma, & Sheng, 2016)	Fibro-myalgia	n=180 +126 controls	bilateral anterior cingulate/paracingulate cortex/medial prefrontal cortex, bilateral posterior cingulate/paracingulate cortex, left parahippocampal gyrus/fusiform cortex, right parahippocampal gyrus/hippocampus	cerebellum
(Lin, Lee, & Weng, 2016)	Fibro-myalgia	n=180 +123 controls	left medial prefrontal cortex, right dorsal posterior cingulate cortex	

This demonstrates that despite of the amount of research carried out on this topic there does not seem to be a confident answer to the question whether there are pain-related neuroplasticity or structural alterations that predispose chronic pain development, even if some reviews might wish to give this impression.

One particular study should however be discussed in the present context because it investigated whether there are "brain signatures" that predict who will be suffering and who recovered from their injuries by comparing structural brain images from patients at onset of pain and images acquired a year later (Vachon-Presseau et al., 2016) and findings of this report point to right amygdala volume explaining an astonishing 90% of variance of the prediction (Vachon-Presseau et al., 2016). Yet, within the same study the authors rerun the same model with another software (which they claimed to yield more accurate segmentations) and pooled hemispheres which made the explained variance drop to 60% (Vachon-Presseau et al., 2016). The authors justified this choice because the latter model was based on amygdala volumes produced with different segmentations; although likely a practical reason, it is unclear why these segmentations were not done separately for each hemisphere when animal pain models do distinguish between left and right amygdalae (Ji & Neugebauer, 2009). This gives the impression that such findings, even though promising, seem to be dependent on technical details (i.e., 90% to 60% is a steep drop), especially when such findings have not been replicated, and begs the question whether findings on amygdala volume alterations are plausible in the first place.

Considering the fact that most meta-analyses and reviews on pain-related structural brain alterations did not mention the amygdala, the conclusion is negative, especially when results depend on technical details. The latter may however also be regarded as the biggest caveat associated with meta-analyses and reviews as they rarely address sources for type II errors; considering the small size of the amygdala, any pain-related neuroplastic changes would also be expected to be small-scaled and thus factors like large smoothing kernel

sizes, small field strength, whole-brain analysis approaches with stringent multiple comparison corrections are particularly likely to yield type II errors. It would therefore not be good scientific practice to equate a lack of amygdala findings in these meta-analyses with a lack of pain-related neuroplastic changes in this brain area.

4.1.3 Human in-vivo studies on pain-related neuroplasticity in the amygdala

The few reports that do report the amygdala, mention amygdala volume alterations in irritable bowel syndrome (Labus et al., 2014), in hemi-facial spasm (Bao et al., 2015), in fibromyalgia (Burgmer et al., 2009; Lutz et al., 2008), in migraine (Neeb et al., 2017), in the development of chronic back pain (Vachon-Presseau et al., 2016) and similarly increased gray matter in the amygdala after prolonged morphine consumption in treating the latter (Lin, Chu, et al., 2016), increased gray matter after successful treatment in complex regional pain syndrome (Erpelding et al., 2016), and increased gray matter after pain-relieving hip replacement for Osteoarthritis (Rodriguez-Raecke et al., 2009) (see table2) and thus this seems in line with the general trend in human pain literature reporting gray matter reductions.

Table 2: Voxel-based-Morphometry human studies that report pain-related amygdala gray matter de- or increases

Gray matter increase in amygdala (volume unless indicated otherwise)		
Report	**Aetiology**	**Finding**
(Labus et al., 2014)	Irritable Bowel Syndrome	Bilateral but only right amygdala when controlling for Early Trauma Inventory global score
(Bao et al., 2015)	Hemifacial spasm	Bilateral amygdala
(Lutz et al., 2008)	Fibromyalgia	Bilateral but possibly pooled in analysis
(Neeb et al., 2017)	Chronic migraine	Right amygdala
(Vachon-Presseau et al., 2016)	Chronic back pain	Right amygdala

(Rodriguez-Raecke et al., 2009)	Osteoarthritis pain, Treatment with surgery	Right amygdala gray matter density
(Mao et al., 2013)	Chronic back pain	Left amygdala
Gray matter decrease in amygdala		
(Burgmer et al., 2009)	fibromyalgia	Amygdala
(Mao & Yang, 2015)	Chronic back pain	Bilateral amygdala but more reduction on left
(Barad, Ueno, Younger, Chatterjee, & Mackey, 2014).	Complex regional pain syndrome	Left amygdala
(Valfre, Rainero, Bergui, & Pinessi, 2008)	chronic as opposed to episodically suffering migraine patients	Left amygdala
(Lin, Chu, et al., 2016)	Chronic back pain, Treatment with morphine	Bilateral amygdala, Right amygdala when controlling for placebo
(Erpelding et al., 2016),	complex regional pain syndrome, treatment	Increase in right amygdala after treatment

Yet, the opposite has also been reported: greater amygdala volume in chronic back pain relative to controls (Mao et al., 2013), in chronic as opposed to episodically suffering migraine patients (Valfre et al., 2008), and a positive correlation between amygdala volume and pain intensity in complex regional pain syndrome (Barad et al., 2014).

Contradictory findings can reflect several reasons: for one, gray matter alterations as detectable in T1-images do not have a definitive interpretation. They could represent decay or increase of cells, spines, synapses, axons or glia, simply changes in cell size; additionally gray matter increase could simply reflect temporary neuroinflammation (Davis & Moayedi, 2013). Secondly, on the basis of a T1 image alone any such alterations cannot be classified into maladaptive or adaptive plasticity: what appears to be hypotrophy for instance could reflect excitotoxic damage or recession of circuits acting to reduce pain

or condensation of pain maintaining circuits. Equally, hypertrophy or volume increase can reflect maladaptive or adaptive developments. The multitude of interpretations may have been a factor why this topic was not mentioned in the recent review on vulnerability factors for pain chronification (Denk & McMahon, 2017) and why the amygdala has not been investigated further.

Overall there are relatively few reports in human pain literature that found neuroplasticity in the amygdala, and thus the focus could be widened to include pain-relevant literature that may help to evaluate whether amygdala structure alterations in pain progression are likely.

Literature on PTSD for instance is relevant for pain for the similarities and hypothesized mechanistic overlap with chronic pain outlined in chapter 1.

The amygdala plays a pivotal role in PTSD literature with studies showing that anomalies in amygdala structure (amongst other brain regions) may be regarded as a "predisposition" to develop PTSD if individuals are subjected to adverse experiences (e.g., reviewed in (Admon, Milad, & Hendler, 2013) and (Ahmed-Leitao, Spies, van den Heuvel, & Seedat, 2016) and recent study in (Cacciaglia et al., 2017). Similarly, there are reports that PTSD is linked with smaller amygdala volume (Morey et al., 2012; Rogers et al., 2009) but also such that have not found any link between disease and amygdala structure (O'Doherty, Chitty, Saddiqui, Bennett, & Lagopoulos, 2015; Tench, 2018; Woon & Hedges, 2009)).

Naturally, this is not immune to the same criticism that such a finding may receive in pain literature and thus it is not surprising to find that there are discrepant meta-analyses on this topic, also because PTSD is not a homogenous disorder (Lanius et al., 2010). Yet, if functioning of the amygdala itself (and not only its structure) is repeatedly tied to PTSD (see review in (Diamond & Zoladz, 2016)) and if PTSD and chronic pain share extinction deficits as a common mechanism for disease progression (which does not imply exclusivity), findings that point to the amygdala as predictor may receive more attention in pain despite discrepant findings: As outlined in chapter 1,

there are claims that structural alterations in the amygdala likely "cause" rather than arise from impaired extinction (Farrell et al., 2013; Maroun et al., 2013).

Even if this statement is toned down as this link is promising but novel and insufficiently explored, stress-induced neuroplasticity in the amygdala is a less novel finding (Wilson, Grillo, Fadel, & Reagan, 2015). It seems unlikely that stress-induced neuroplasticity would not have an impact on disease progression seeing as extinction deficits are fostered by chronic stress as summarized in chapter 1.

Importantly, stress-induced alterations in the amygdala as opposed to other brain structures do not necessarily reverse shortly after cessation of the experiment (Vyas et al., 2004) even though they are based on relatively short-lived episodes (e.g., water maze). If short-lived experiments are capable of inducing detectable long-term stress-related neuroplasticity, then this strongly suggests that the impact of chronic pain on brain structure may be greater. This makes stress- and also pain-related structural anomalies in the amygdala conceivable and worthwhile of further investigation despite lack of such findings in meta-analyses.

4.1.2 Sources for discrepancies: Amygdala gray matter volume and density interactions

While human work often summarizes morphometry in terms of volume loss or increase, animal literature more often uses the terms hypertrophy and hypotrophy or atrophy.

Hypertrophy may be in the form of new neurons, glia, dendrites (length or count of), or new spines while hypotrophy relates to decreases of these structures; atrophy on the other hand refers to more substantial cell loss.

Similar to human work, discrepancies may be found in chronic stress literature where stress-induced hypertrophy in the amygdala is the most frequent scenario but the presence of atrophy and no-change findings cannot be ignored, either (Wilson et al., 2015).

Admittedly, this parallel is not immediately obvious because gray matter decrease and hypertrophy do not seem to have anything in common; this is because some studies that do not perform within-subject contrasts mislead with the term “decreased gray matter” or “gray matter loss” as opposed to wording it “smaller volume compared to healthy controls” - which may still seem equally incompatible with the term “hypertrophy” from histology. Acute stress and aversive learning studies in animals have shown decreased number and lengths of some dendritic branches, dendritic retraction but also increased spine density and more dendritic intersections in the amygdala (Heinrichs et al., 2013; Maroun et al., 2013). Thus it is possible that amygdala gray matter condenses, resulting in a smaller amygdala volume in patients compared to controls but this needs to be studied further. Clearly there is limited data to make direct inferences about the comparability of findings based on T1-images and histology in humans; within the scope of this chapter this argument merely serves to demonstrate that volume and gray matter density are complementary measures and that some findings may seem contradictory due to inaccurate labelling; the term volume loss or volume increase should be reserved for longitudinal study designs.

4.1.3 Methodological aspects of amygdala segmentation and parcellation

Discrepant findings could be due to further methodological aspects as demonstrated by Vacchon-Presseau and colleagues who used two different segmentation approaches performed in different software and found a remarkable drop in explained variance (Vachon-Presseau et al., 2016). The amygdala may indeed be one of the most challenging structures to segment given its size, some rather diffuse borders and anatomical proximity to its neighbour hippocampus. Thus, natural variance in software output would have a greater impact on this structure than on others, moreover if the amygdala consists of different cell groups that have different functions (Swanson & Petrovich, 1998). Its main subnuclei, the lateral and basolateral parts (see figure 1) form a unit with prefrontal and temporal areas while the central

nucleus is the main output nucleus (Veinante, Yalcin, & Barrot, 2013) and is rather implicated in the autonomic nervous system (Swanson & Petrovich, 1998) yet the superficial nucleus is almost exclusively reported in relation to olfactory social cues (Bzdok et al., 2013) and will not be further discussed.

Further subparcellations of these nuclei should be mentioned in the present context as the laterocapsular part of the central amygdaloid nucleus is referred to as the "nociceptive amygdala" in animal pain studies (Veinante et al., 2013). The minute "intercalated cell mass" within the amygdala on the other hand is a highly concentrated group of inhibitory interneurons which are essential mediators of extinction processes (Likhtik et al., 2008) and receive input from the medial ventral prefrontal cortex (Neugebauer, 2015). Failure of the intercalated cell mass to relay prefrontal control has been suggested to result in amygdala hyperactivity and in turn contribute to pain chronification (Neugebauer, 2015). This suggests that precision with regards to a small and heterogeneous structure such as the amygdala is crucial but may be very challenging: a recent MRI-based atlas on a large sample from the human connectome project suggests that subparcellation into 9 distinct amygdala nuclei is possible with high resolution anatomical images but it also excludes several nuclei such as the intercalated cell masses as these were too small or had ambiguous borders (Tyszka & Pauli, 2016).
Such subparcellation is not attempted with the two popularly used probabilistic atlases, one of which is based on post-mortem histology (n=10 subjects) and segments the amygdala into three nuclei: the laterobasal, centromedial, and superficial ((Amunts et al., 2005), as part of the Juehlich probablisitic atlas), the other one only segments the amygdala as one unit (Harvard-Oxford subcortical probabilistic atlas).

4.1.4 Amygdala structure and sex differences

There are debates whether total amygdala volume in females is smaller or equal to males ((Stuart J Ritchie, 2017) but see (Marwha, Halari, & Eliot, 2017))

and thus sex might obscure group differences. This potential discrepancy in a healthy volunteer population needs to be emphasized because sex differences are a recurring point of discussion in literature on pain, probably as frequently ignored as discussed but no discussion on chronic pain or on stress can omit sex if only for the fact that females are more prone to stress disorders (Tolin & Foa, 2006) or to extinction deficits (Baran, Armstrong, Niren, Hanna, & Conrad, 2009) than males (N.B. most animal extinction literature is based on male rats). Indeed, sex hormones can "exacerbate stress-mechanisms" (Farrell et al., 2013) and the amygdala is a hotspot for estrogen receptors (Farrell et al., 2013) which may explain part of increased vulnerability for females.

This can mean that increased risk for extinction deficits may be linked with increased sensitivity and a finer-tuned system to threat rather than safety cues. Indeed a longitudinal study on normally cycling women showed that left amygdala volume significantly varied within subjects depending on their cycle phase (which is marked by varying estrogen and progesterone levels) and that phase-related amygdala volume differences further correlated with negative affect scores after a stress-inducing paradigm (Ossewaarde et al., 2013). This provides tentative evidence that estrogen levels and amygdala structure are linked and that this relationship reflects on stress or threat reactivity which fits observations that women and their amygdalae seem to be more threat-sensitive when estrogen and progesterone levels peak (briefly reviewed in (Lischke et al., 2012)). This means that sex is an important factor for amygdala structure and possibly linked to sub-phenotypes that complicate analyses beyond what can be corrected by sex as nuisance factor. It has to be taken into account that estrogen levels in females fluctuate as part of a regular cycle and are subjected to ageing-related decrease until after menopause when levels stay low. In this light, it may not be a coincidence that this age group has the highest prevalence of chronic pain but even before menopause extremely low estrogen levels can occur, especially when hormonal contraceptives were used (see (Merz, 2018) for in-depth discussion).

4.1.5 The current work

This motivates the current work to investigate amygdala structure alterations in relation to chronic pain. We further aim to critically evaluate popular methods to investigate amygdala structure, as well as to investigate sex, as the most commonly implicated factor for discrepant findings.

4.1.6 Research questions and hypotheses

- Is there a relationship between amygdala gray matter density and chronic pain?

Animal literature suggests that the most frequent direction in chronic stress is hypertrophy in the amygdala which would be hypothesized to be reflected in increased gray matter density relative to controls.

- Are pain-related alterations to amygdala density mediated by sex?

It is known that females are more prone to develop chronic pain, anxiety disorders, or extinction deficits and thus any pain-related amygdala structure alterations would be expected to be enhanced in females.

- Do pain-related alterations to amygdala density differentiate pain patients who do better and patients who worsen on pain symptoms?

It line with hypothesis nr1 it is expected that 'non-improvers' show enhanced pain-related amygdala structure alterations compared to patients who are improving over the course of one year after pain onset.
This will be explored with a longitudinal data set.

These research questions will be explored with whole-brain GMD-VBM approach (Part A) as well as a region-of-interest GMD analysis (Part B).

4.2. Methods Part A

Data sets

For the current chapter, three analyses were run: the first analysis was done on T1 images from four different studies from the identical scanner (total n=64, for descriptive see table 3), the second data set was from another study from the identical scanner (n=103, for descriptive see table 4), the third analysis is based on a data set (n=41, see table 5 for descriptive) kindly made available through the open pain project (http://www.openpain.org). There is no overlap in data sets between these analyses.

Contributions to data acquisition are specified at the beginning of this chapter, the following describes the characteristics of each data set. Where available, depression scores are listed for each data set (Beck's Depression Index: BDI-II). Recent discussions challenge that the BDI questionnaire is adequate in diagnosing depression in chronic pain as the latter is often accompanied by somatic symptoms (e.g., insomnia) that do not necessarily reflect depression and thus a two factor structure was suggested (Knaster, Estlander, Karlsson, Kaprio, & Kalso, 2016). This however makes comparisons to healthy controls difficult if the latter were to be assessed with the original one factor scoring of the BDI questionnaire. Without the intention to accurately clinically diagnose depression, the one factor BDI score may be used to simply give indications about the participant's wellbeing; for this reason the original BDI score is reported here for both patients and controls.

4.2.1 Data set for study 1

The data set abbreviated as study 1 is an amalgamation of different local studies approved by the local Ethics committee; anonymised pooled data analysis, is part of these approvals. All participants provided informed consent. The IMPOA study (Ethics reference: REC 10/H0408/115) provided the majority of data sets to the following analyses. Patient data sets were part of IMPOA and IPRO studies (Ethics NRES reference: 14/EM/0189) while healthy controls data sets belonged to IMPOA, PAMIR (REC ref: 4/EM/0061 & IRAS: 124223),

BRaNDY (Ethics reference: 14/EM/0064) and VESPA studies (Ethics reference: 10/H0408/37).

Healthy controls from PAMIR, BRaNDY, VESPA were not subjected to the same thorough chronic pain screening in IMPOA but any potential chronic pain occurrence in the healthy controls data set is thought to be minimal. A detailed description of sample sizes of each study, sex and age distribution, as well as pain characteristics where available is listed in tables 3-5.

The sample size reflects data without excessive motion artefacts or incidental findings; exclusion on the bases of the latter two factors was decided without awareness of the specifications of the data set.

Table 3: Basic description of data sets in study 1

Osteoarthritis chronic knee pain patients (n=51 females, n=47 males)					
	age	pain severity (0-10) (n=96)	Pain Duration (yrs) (n=96)	BDI-II (n=95)	STAI T (n=86)
median	65	3	8	6.5	32
minimum	44	0	1	0	20
maximum	89	10	50	22	63

Study	N= males	N= females	Age in years
IMPOA	11	22	median 63, range: 43-84
PAMIR	4	12	median 70, range: 46-83
VESPA	1	10	median 51, range: 46-67
BRaNDY	1	3	median 52, range: 50-55

Healthy controls: Individuals from IMPOA study filled out the ICOAP questionnaire as part of the study, other studies did not include this measure. If there was no indication of chronic pain during screening procedure for these studies, the individual is considered to be healthy although a small risk of chronic pain is acknowledged here.

Scanning parameters

T1 images were acquired at a 3T scanner (GE, Discovery MR750) with a 32-channel head coil (FOV=256, voxel size 1 x1 x1, slice thickness 1mm, flip angle=12). TE and TR varied slightly between the different studies from 3.18ms to 3.3ms and 8.2 to 8.5ms, VESPA and BRaNDY data sets also varied slightly with respect to image dimensions (156x256x256 and 256x256x156, respectively).

4.2.2 Data set for study 2

The data set for study 2 is based on a more recent local pain patient and control study under the abbreviation INCOPE (Ethics reference: REC 10/H0408/115). Due to the fact that this data set lacked a sufficiently large sample of healthy controls this data set was enlarged by healthy controls from study 1 to achieve age and sex matched patient and control groups of nearly equal size. Characteristics of this data set are set out in table 4.

Table 4: Basic description of the sample for study 2 (INCOPE data)

Patients (n=22 females, n=26 males)						
	age	pain severity (0-100)	ICOAP	Pain Duration (yrs)	BDI-II	STAI T
median	64.50	30.00	48.86	6.50	9.00	35
minimum	48.00	0.00	0.00	0.00	0.00	0
maximum	79.00	90.00	97.73	48.00	40.00	66

No missing data.

Healthy (n=30 females, n=25 males)		
	age	BDI-II
median	66	3
minimum	48	0
maximum	79	19

All healthy controls from study 2 filled out the ICOAP questionnaire and are confirmed to be pain free.

Scanning parameters

Images were acquired at the identical scanner as data sets for study 1 (GE, MR750) and the identical head-coil. Parameters were TR=8.132ms, TE=3.164, voxel size= 1x1x1, slice thickness= 1mm, flip angle=12, FOV=256.

4.2.3 Data set for study 3 (subacute data set)

The third data set consisted of a subsample from the subacute data set that is made available at http://www.openpain.org, and which comprised baseline scans (visit 1) at pain onset as well as scans at one year follow-up visits (visit 4) along with psychometric data that allows to determine whether patients' health improved.

Selection of data sets:

Subjects were considered for this analysis if they had usable T1-weighted images for both visit 1 and visit 4. Improvers and non-improvers were selected according to the "pain disability index total score" (PDI), which can range from 0-70 and represents impact of pain on functioning in several life aspects (e.g., family, social, occupational), as follows: patients were included as improvers in the analysis if their PDI score at visit4 was below 20 and if they showed a notable reduction (difference score >5) in the PDI score from visit 1 to visit 4. One included subject only met the first criterion but because scores at both visits were low and the relative reduction from visit 1 to visit 4 was considerable (visit 1: 6, visit 4: 2), and because the PainDETECT score indicated that the person indeed suffered from pain (visit 1: 7, visit 4: 6), this subject was considered an improver also. Subjects were classified as non-improvers if their PDI score at visit 4 was >20 to indicate that pain notably impacts on their life and if their PDI score between visit 1 and visit 4 did not change, decreased by less than 5 points, or increased. The PDI score 20/100 as cut off is semi-arbitrary.

These criteria were chosen because a minimum 20% reduction in pain intensity scores, as applied by Vacchon-Presseau et al., did not seem to be an exhaustive criterion for claims on pain recovery for this analysis for the following reasons: a) pain intensity reduction is considered to be a secondary goal for primary care after improved coping b) the percentage criterion would include subjects whose scores on visit 1 and visit 4 differed minimally (e.g., from 4 to

3, or from 2 to 1.5 [3]) yet experience with human subjects shows that such minimal differences may simply reflect self-assessment inaccuracy c) in this data set PainDETECT was more prone to missing data compared to PDI questionnaire. This data set is described further in table 5.

Table 5: Basic description of data for study 3

Improvers (n=6 females, n=12 males)

	Age (yrs)	BDI-II, visit 1	BDI-II, visit4	avg pain intensity*, visit 1	avg pain intensity*, visit 4
median	46	8	3	60	30
minimum	27	0	0	30	0
maximum	66	18	17	90	80

*average pain intensity in the last 4 weeks (as part of PainDETECT questionnaire), multiplied by 10 to enable direct comparisons with other samples analysed in this thesis, such that the max. score is 100.

Non-improvers (n=12 females, n=11 males)

	Age (yrs)	BDI-II, visit 1	BDI-II, visit4	avg pain intensity*, visit 1	avg pain intensity*, visit 4
median	44	7	6	70	60
minimum	27	0	0	20	15
maximum	57	22	30	80	90

Scanning parameters

Anatomical images were collected with a 3T Siemens Trio scanner (TR=2.5ms, TE=3.36ms, voxel size 1x1x1, flip angle=9, FOV=256).

4.2.4 Analysis

The latest methodological advancement for vbm recommends segmentation before normalization to improve the latter and subsequent segmentation of the normalized GM images for analysis; this additional segmentation step distinguishes the "optimised vbm" from "standard vbm" procedures (see

[3] Vacchon-Presseau et al. did not specify which pain intensity scale was used as their criterion. Their data includes: PainDETECT total score, PainDETECT pain time course, PainDETECT average pain in the last 4 weeks, PainDETECT pain intensity at this moment, Neuropathy Pain Scale total score. The exemplary values are taken from their data on "PainDETECT average pain in the last 4 weeks".

figure 2 in (Mechelli, Price, Friston, & Ashburner, 2005)). This has been implemented in the current analyses; further in-house optimization is therefore referred to in this work as the in house optimized pipeline. As several analyses with combinations of software have been used for methodological comparisons on "detectability" of amygdala alterations, figure 2 gives a comprehensive overview of preprocessing and analyses steps for each.

Briefly, three different approaches have been tested on study 1 data set:

a) the latest standard pipeline in FSL (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSLVBM/UserGuide) that bases statistical group comparisons on random permutations; to comply with literature we chose to base this analysis on commonly reported number 5000 permutations and threshold-free cluster enhancement (advised in (Radua, Canales-Rodriguez, Pornarol-Clotet, & Salvador, 2014)) to determine significant clusters at corrected $p<0.05$.

b) the latest standard pipeline in SPM that utilizes DARTEL for template creation, normalization and the second segmentation, and GLM for group comparison at corrected $p<0.05$ (Ashburner, 2010: https://www.fil.ion.ucl.ac.uk/~john/misc/VBMclass10.pdf)

c) the in-house optimized pipeline where software associated with the most accurate performance was chosen for each preprocessing step: Images were N4 bias-field corrected (ANTs), affinely registered to ICBM 152 template with 12 degrees of freedom (FSL FLIRT), skull-stripped (Freesurfer's watershed algorithm) and reoriented to anterior cingulate (origin) for tissue classification, segmentation through SPM12's New Segment, and template creation and normalization in SPM DARTEL (SPM recommended for these steps in (Klein et al., 2009), (Kazemi & Noorizadeh, 2014)). The statistical analysis was done with permutation testing in FSL because this is less vulnerable to a false error rate due to imbalanced group sample sizes compared to a two-samples t-test. 15,000 permutations were chosen for this step because of evidence that

>10,000 but <20,000 permutations are necessary to minimize the false error rate (Dickie et al., 2015).

Data from study 2 was available after study 1 and was therefore used to test replicability of findings in a similar patient cohort. Additionally, analyses were also performed on the data set for study 3 to identify structural alterations in chronic pain improvers and non-improvers a year after onset of pain.

In all analyses age and sex were included as covariates of no interest. The standard smoothing kernels of FWHM= 8mm was chosen in SPM DARTEL which corresponds approximately to smoothing with sigma= 3mm in FSL in the current analyses. Approaches b-c used the "preserve concentration" option instead of modulation as recommended for gray matter density analyses (Radua et al., 2014); such an option was not available in FSL.

4.3.1 Results Part A

Analyses performed with approaches a-b did not yield any significant clusters even when significance thresholds were lowered to p <0.1 for exploratory analyses.

The optimized-optimized approach (outlined as option c in methods) presented a small cluster in the right frontal pole that was 'denser' in healthy controls relative to patients at exploratory corrected p <0.1 when analysing study 1 data; interestingly, the same contrast with the same approach yielded a cluster in the right hippocampus extending into the putamen and several smaller cortical clusters, in particular in the paracentral lobule, at a significance threshold of corrected p <0.05 in study 2.

Sub-analyses on females with the same approach and contrast yielded a small cluster in the left superior temporal gyrus that was significantly 'denser' in female controls than female patients in study 1 data set but did not replicate in study 2 data set. Male subgroup analyses did not yield any cluster even at

exploratory p-values (<0.1). This is illustrated in figure 4 with corresponding information in table 6.

The reverse contrast (Patients > Controls) did not yield any clusters in any approach with either study 1 or study 2 data set not even at exploratory corrected p-values.

The study 3 data set yielded a trend for a small cluster in the left putamen when contrasting patients who improved over the course of one year with those who did not (table 6).

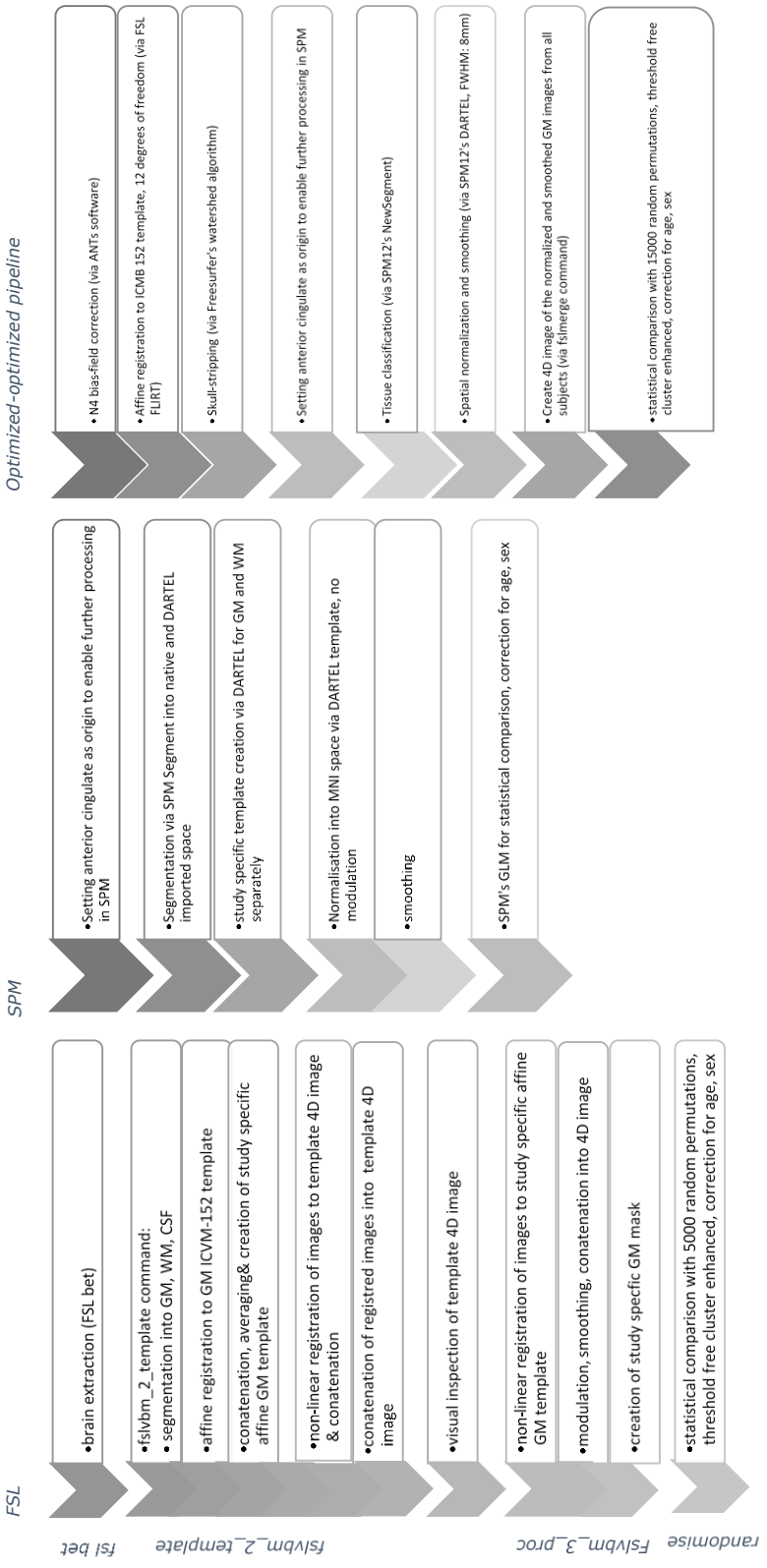


Figure 1: Overview over whole-brain vbm analyses in this chapter

This figure demonstrates the different software approaches that were used: the "classic" FSL VBM pipeline (left part), the most recent SPM-DARTEL VBM pipeline (middle), and the in-house optimized pipeline that uses a combination of these as well as other software to maximize accuracy of analyses (right).

Table 6: Results of whole-brain optimized-optimized vbm analysis for Healthy controls > Pain patients at 15,000 permutations.

	label	*Voxel count*	*X*	*Y*	*Z*	*p*
Study 1	R frontal pole	243	30	117	48	0.069
Females Study 1	L STG	215	103	65	57	0.038
Study 2	Several areas, of which:	8384*				
	R Hippocampus	453*	41	67	39	<0.05
	R Putamen	419*	41	79	46	<0.05
	cerebellum	318*	38	43	17	<0.05
	Cortical clusters	5715*				<0.05
Study 3: Improvers > non-improvers at visit 4	L Putamen	287	110	131	74	0.079

* The voxel count number supplied in this table is based on ITK-SNAP software: load thresholded results ($p < 0.05$) map as segmentation and click 'Volume & statistics'. ITK-SNAP was used to supply voxel counts for each separate cluster by manually labelling the cluster and extract the corresponding voxel count. Other voxel counts in this table were supplied by FSL and checked in ITK-SNAP.

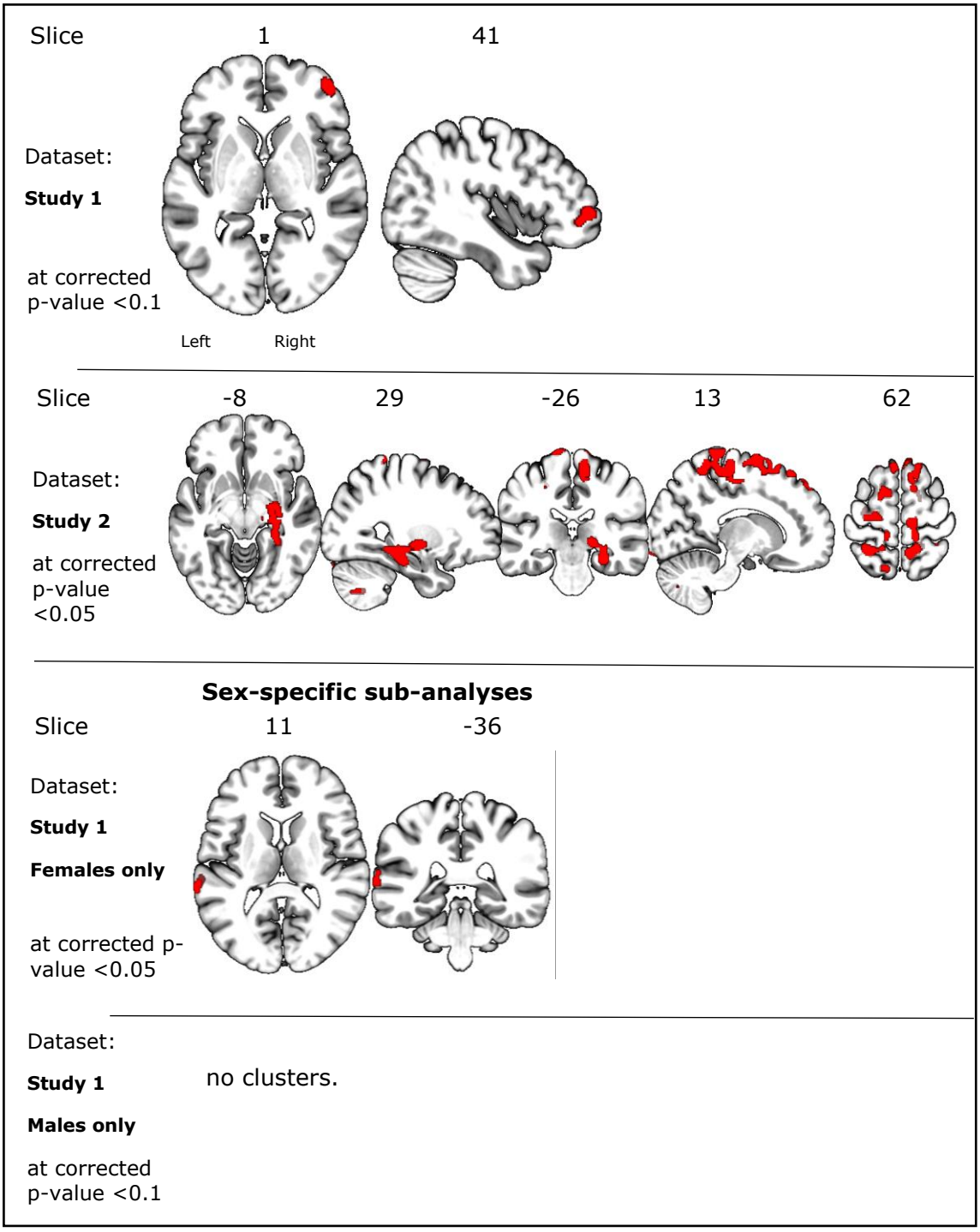


Figure 2: Results of whole brain vbm analysis with the optimized-optimized approach for contrast healthy controls > pain patients at 15,000 permutations.

This figure shows that the same contrast gives different and non-overlapping brain areas in the two main data sets and another different cluster when restricting the analysis to females from study 1 data set. No clusters were found for males nor for the reverse contrast in either data set.

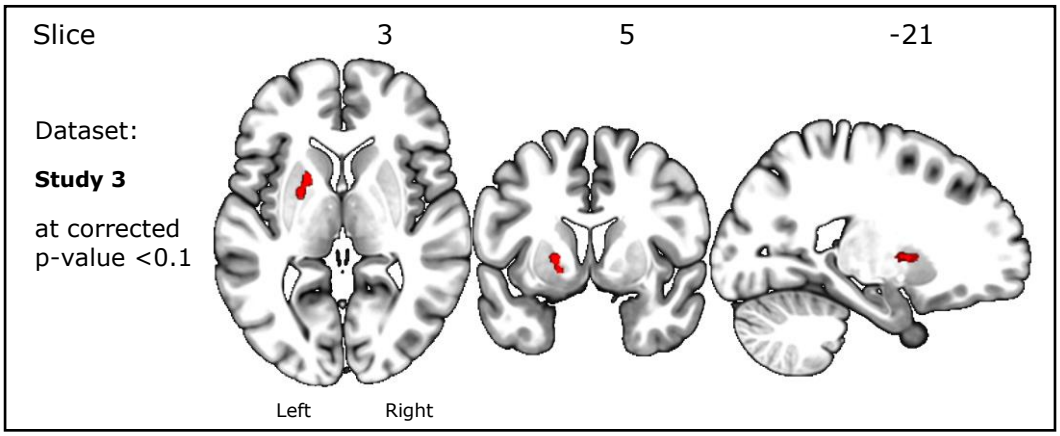


Figure 3: Results of whole brain vbm analysis with the optimized-optimized approach for improvers over non-improvers from the subacute pain patient data set at 15,000 permutations.

This figure shows a trend for a cluster in the left putamen to be 'denser' in improvers relative to those patients who did not experience pain relief over the course of one year.

4.4.1 Discussion part A: Gray matter density based whole-brain voxel-based morphometry

The aim of this section is to identify pain-related structural alterations in the amygdalae in humans, however with gray matter density as the parameter of interest. The popularity of whole brain analyses within human pain research makes such an approach an adequate starting point because it allows comparisons to literature and its possible type II errors. As already outlined in the introduction, relatively few reports link the amygdala and pain-related neuroplasticity and accordingly it was hypothesized that the whole-brain analysis in the current work would not reveal the amygdala either.

Indeed the amygdala was not found to have altered gray matter densities with this approach, neither in the comparison of pain patients and healthy controls based on a large data set, nor the smaller pain patient and controls comparison, nor in sex-specific subgroup comparisons, nor in comparisons of pain patients who improved or who did not improve a year after pain onset.

Overall it can be concluded that the analyses do not replicate findings in similar published work and do not replicate across two similar samples that were acquired with identical hardware. Sample size could be a reason for discrepancies because it needs to be acknowledged that these data sets' sample sizes differed nearly by a factor of 2 for pain patients yet according to Scarpazza et al., especially unbalanced sample sizes substantially contribute to type I errors (Scarpazza, Tognin, Frisciata, Sartori, & Mechelli, 2015). Both data sets' sample sizes are well above commonly reported sample sizes for morphometry (which have been as low as n=14) which suggests that a small sample size itself is not an exhaustive explanation for replicability issues but power issues cannot be simply dismissed (Button et al., 2013).

Indeed, voxel-based morphometry itself has received a lot of criticism and defense. One argument states that voxel-based morphometry is reliable unless comparing groups with unbalanced sample sizes which enhances false positive rates (Scarpazza et al., 2015). Scarpazza et al. argue that such an enhanced false error rate would be accompanied with significant voxels "randomly distributed across the brain" (Scarpazza et al., 2015). Overall this is argument is not

applicable to the current analyses as all but one comparisons were based on equal sample sizes and did not show any significant clusters; however, the sample size was unequal in the largest data set analysed herein and thus the one relatively small cluster that was found with the optimized-optimized vbm approach could be spurious.

VBM has also been criticised for spatial imprecision that comes with any normalization because it is impossible to perfectly register every brain structure at the same time (Ridgway et al., 2008); this argument can be used for both type I and II errors and thus unavoidably is also applicable to the current analyses. Whilst there has been discussion on whether vbm has sufficient sensitivity to detect mesoscopic volume differences (but see (Ashburner & Friston, 2001)), morphometric analyses in human pain have not been accompanied by informed guesses about the expected scale of morphometric alterations in the various brain structures. These effects may be microscopic in human pain and thus beyond vbm's detectability. This may be enhanced for gray matter density alterations which may not be as pronounced as volumetric differences.

In this light, the significant clusters from the current chapter are not further discussed here moreover as the focus for this chapter rests on the amygdala; the interested reader may however be referred to the next chapter which discusses the putamen, hippocampus, cerebellum, and frontal regions as part of amygdala networks.

For the amygdala, the arguments above can in part be addressed with more sensitivity through region-of-interest (ROI)-based group comparisons which avoids issues with stringent multiple comparison correction or voxel-based morphometry and smoothing. This shall be analysed and discussed in the next section.

4.2.2 Methods for part B: ROI based analysis of gray matter density

ROI approaches require a mask that limits the analysis to the structure in question. Commonly, such a mask is created either by applying a sphere with a certain diameter on coordinates reported to be the structure of interest or by using a probabilistic atlas that is based on histological work and thus labels each voxel according to how likely it is part of a certain structure across subjects (e.g., 50% probability means it is part of structure X in 5 out of 10 individuals).

Creating suitable masks for gray matter density analyses for region of interest analyses (ROI) GMD extraction

In order to perform a region of interest analysis (ROI), a mask of the respective brain region is needed. The following section of the methods refers to the brief exploration of which amygdala mask is most appropriate. Masks will be created using the two most commonly used probabilistic brain atlases. Probabilistic atlases are popular to create masks, not least due to the intuitive meaning and ease of use as only a threshold (0-100) has to be chosen which represents the probability of a voxel within the mask belonging to the brain region in question. On the downside the thresholds are semi-arbitrary because reports do not motivate the selected cut-off other than by reference to a classic report which does not provide a justification (seminal work by (Roy et al., 2009) applied a 50% threshold).

Such a justification could be that several thresholds are applied and the resulting masks visually compared on the basis of anatomical borders and reasonable volume; this will be elaborated below. Also specified below are details on the two popular probabilistic atlases that have been used and compared in this section.

a) Juehlich atlas.

This atlas is based on post-mortem brains from 10 healthy subjects, which have been dissected, stained and labelled thoroughly. With respect to the amygdala, this atlas offers labels for laterobasal and mediocentral amygdaloid nuclei in both hemispheres and happens to be the practical implementation of one of the classic manual amygdala segmentation protocol (specifically: (Amunts et al., 2005), cited 558 times by October 2018).

The paper by (Roy et al., 2009) has been frequently taken as a reference on amygdala segmentation using this atlas and consequently a 50% threshold is commonly used; for replicability and comparability to this report, 50% threshold was chosen. For a more conservative evaluation 80% threshold was used to create the respective mask on the MNI152 template in fslview. Representative slices are shown in figure 4.

b) Harvard-Oxford atlas

In contrast to the Juehlich atlas, further differentiation into amygdaloid subnuclei is not provided in the Harvard-Oxford atlas. The critical evaluation compared masks produced with the same thresholds as above. Representative slices are shown in figure 4.

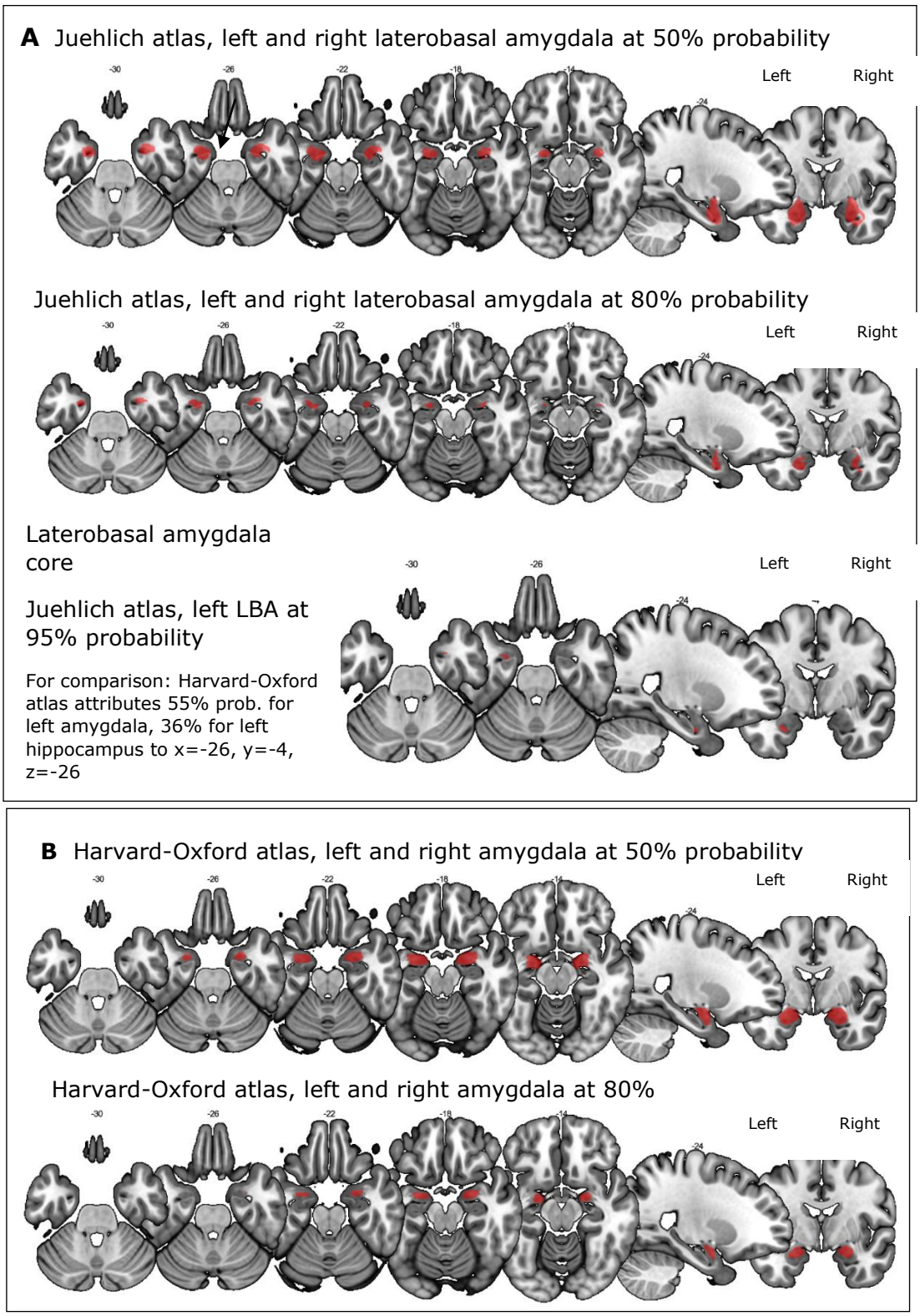


Figure4: Comparison of amygdala masks from two probabilistic atlases
4A shows laterobasal amygdala masks based on Juehlich atlas at 50 and 80% probability while 4B shows amygdala masks at these probabilities created through the Harvard-Oxford subcortical atlas.

Further analyses used the mask that passed visual quality control best. Unsmoothed preprocessed data as described in chapter 3 was used to extract average amygdala GMD for each hemisphere of each subject using fslmaths command. These values were compared in either independent samples t-tests to distinguish patient and healthy groups or a paired t-tests to test for a progression effect between visit 1 and visit 4 of the subacute pain sample using SPSS.

4.3.2 Results part B: ROI based analysis of gray matter density

The independent samples t-tests between gray matter density extracted from the amygdalae of healthy controls and pain patients from the large sample (table 7), as well as independent samples t-tests between these groups in the small INCOPE sample (table 8), and the paired samples t-tests between improvers and non-improvers from the subacute pain study (table 9) were not significant (all $t < 1$). Consequently there was no need to apply any multiple comparison correction. Inspection of group means reveals that all groups have a similar probability of > 95% on average per voxel of either amygdala to belong to gray matter, with minute standard deviations and standard errors of the mean and thus the lack of significance is not surprising. With this result, no further analyses were performed.

Table 7: Group comparisons of amygdala GMD in study 1 data

Amygdala	Group	N=	Mean	Std. Deviation	Std. Error Mean
Right	Patients	74	0.957	0.0537	0.00625
	controls	64	0.958	0.0447	0.00560
Left	Patients	74	0.959	0.0492	0.00573
	controls	64	0.962	0.0369	0.00461

Table 8: Group comparisons of amygdala GMD in study 2 data

Amygdala	Group	N=	Mean	Std. Deviation	Std. Error Mean
Left	Patients	29	0.959	0.0315	0.00876
	controls	13	0.961	0.0623	0.01158
Right	Patients	29	0.968	0.0234	0.00649
	controls	13	0.958	0.0465	0.00865

Table 9: Group comparisons of amygdala GMD in study 3 data set

Amygdala	Group	Mean	N=	Std. Deviation	Std. Error Mean
Left	Improvers at visit 1	0.985	18	0.0154	0.0036
	Improvers at visit 4	0.983	18	0.0170	0.0040
Right	Improvers at visit 1	0.980	18	0.0163	0.0038
	Improvers at visit 4	0.980	18	0.0146	0.0034
Left	Non-improv. at visit 1	0.981	23	0.0199	0.0041
	Non-improv. at visit 4	0.979	23	0.0225	0.0046
Right	Non-improv. at visit 1	0.977	23	0.0184	0.0038
	Non-improv. at visit 4	0.976	23	0.0187	0.0039

4.4.2 Discussion part B: ROI based analysis of gray matter density

This section focused on extracting mean amygdala gray matter densities and investigate if there are group differences for healthy controls and pain patients. Secondly it will be investigated whether there is a group difference between pain patients at the onset and a year after they contracted pain, divided into a group that improved with regards to how pain affects them and those that did not improve.

For this, amygdala masks were necessary that would delineate the volume from which the mean gray matter density is calculated from. We found that the popular Juehlich atlas does not allow fine amygdala delineation as the common but arbitrary threshold of 50% probability mask includes white matter and hippocampus. Considering the fact that this atlas was based on only 10 post-mortem brains (Amunts et al., 2005) but normalization was performed with the MNI template that is based on data from 152 subjects, discrepancies are not unexpected. Yet even a conservative 80% threshold did not delineate the amygdala accurately.

Conversely, the Harvard-Oxford atlas was deemed to give a good but slightly conservative amygdala delineation at 80% threshold and hence this mask was chosen for group comparisons. The mask based on this atlas gives an amygdala volume of ca. 0.7 cm^3 that is close to reported histology-based amygdala volumes from human post-mortem studies (maximum 1 cm^3 (Brierley et al., 2002)). With this mask no significant group difference or between-session difference (subacute data set) was found which was not surprising when all groups had a mean > 95% probability of gray matter with either left or right amygdala mask and a minute standard deviation.

We expected very small group differences because a) this analysis had been restricted to very conservative and small amygdala masks and b) while this mask has been chosen to reliably compare the same brain region across subjects, it is conceivable that not all voxels within this mask would be affected by pain-related changes in spine density as parts of the amygdala have

subspecialisations (i.e., search volume would be even smaller) c) no atrophies but rather minute changes in spine density in the amygdala were expected akin to animal stress models, which may also be restricted to small subparts of the amygdala. Indeed, some studies on amygdala plasticity in rats have applied further anatomical precision and reported that the same stress model led to decreased spine density in medial but the opposite in the basolateral amygdala (in wild-type sample) (Bennur et al., 2007). The mask applied in the current analysis is likely to reflect more basolateral amygdala parts but a clear cut cannot be made and hence any opposing effects that have been found in chronic stress, if also present in the current sample, could partially cancel each other out. Yet, the size of the effect being below detectability for in-vivo MRI measures is not an exhaustive explanation for the current lack of group differences.

Whilst it is logical to assume that a region with average higher probability for voxels to belong to gray matter is 'denser' than a region where such an average is considerably lower, this parameter is only a very indirect measure of neuronal density as acknowledged in the original description of this parameter for VBM (Ashburner & Friston, 2000). Correspondingly, there has been at least one study showing that gray matter density of samples from human temporal lobes as per T1-images does not correspond to histological measures of spine density in the same sample (Eriksson et al., 2009). On the other hand there is data that suggest that VBM-GMD may be a reasonable proxy for actual spine density as assessed with histological techniques in rats that were subjected to a fear conditioning paradigm (Keifer et al., 2015).

Yet, the lack of any variance and the fact that probability was close to 100% in most subjects in the current analysis suggests that the parameter is a reflection of segmentation quality rather than any neurobiological underpinning, seeing as segmentation quality was checked thoroughly and the mask was selected carefully to be conservative and representative of the amygdala mask. Of note is further that the current exploration was based on unsmoothed data and without the need to correct for gray matter volume because identical masks

were used for every subject. This suggests that published group differences on gray matter density should be carefully examined, especially where they concern the amygdala and no explicit atrophy is expected.

The limitation of using this mask is the obvious lack to investigate amygdala sub-nuclei but considering that gray matter density did not seem to be linked to neurobiological underpinnings, this would very likely not have made a difference with the present parameter.

4.4.3 General Discussion

The motivation for morphometric investigations of the amygdala is based on the suggestion that extinction deficits can be "caused" by structural abnormalities in this region and thus the aim of this study was to examine any pain-related alterations in the amygdala while critically reflecting on the applied methods and any explanatory variables given the myriad of conflicting results in the literature. It was investigated whether gray matter density of the amygdala differs between pain patients and controls, both through the popular whole-brain VBM approach as well as a region of interest analysis. The latter revealed that the parameter is unlikely to have a neurobiological meaning in the current context which makes consideration of further limitations (e.g., between-subjects design, medication use) and factors that drive these results redundant.

Overall, the motivation to pursue research questions of this chapter is unaltered but it is questionable what a reliable parameter for amygdala morphology may be in-vivo if no atrophies are expected (i.e., shape analysis is not suitable for amorphous structures like the amygdala, cortical thickness is not applicable for subcortical structures). Hence, there may not be an alternative to studying amygdala structure alterations in animal pain models.

As the motivation for this study rests on findings of stress-induced hyper- and hypotrophy in animals, the chapter concludes with a short discussion on translatable factors that may drive these.

A study has shown for instance that when Sprague-Dawley rats were subjected to a situation that arguably involves considerable stress for the animal (i.e., placed on an elevated and brightly lit platform in the middle of water) no difference was to be seen in the structure of the amygdala on 7T MRI brain scans, however amygdala volumes in F344 rats subjected to the same condition increased by 45% (Bourgin et al., 2015) as the latter rats did not "habituate" to the stress protocol. What can be simply summarized by 'breed' in animal studies would encompass genetic factors and sex, personality traits, cultural influence, upbringing (in particular reactions to threat), gender, personal experience and past stress exposure (i.e., frequency, magnitude). Beyond these internal factors, however, the context and details of stress exposure can also explain unexpected findings.

Vyas and colleagues provided an example of dependence on details when they observed hypertrophy in amygdalae of rats subjected to chronic immobilization stress but not rats that were subjected to chronic but unpredictable stress (Vyas, Mitra, Rao, & Chattarji, 2002). For chronic pain this sounds both interesting and counterintuitive as unpredictability of stressful situations usually makes these worse: unknown onsets or durations or intensity of stressful stimuli make these considerably more stressful for some individuals. However Vyas et al used a very diverse "stress protocol" for the unpredictable stress condition (e.g., '3-4 minutes forced swim' whereas on other days it would involve changes to the usual light-dark cycle or food deprivation) and clearly some forms of stress may not be comparable to being immobilized regularly. Hence the overall stress level in the "diverse" condition might have been lower than in the predictable stress condition and hence have had less impact on amygdala structure. On the other hand, with no pattern in the stress protocol at all, no repeated or previous exposure of the same stimuli, this condition might not have been targeting the amygdala at all as associative learning is usually based on patterns.

Translating this discussion to a human sample, it is possible that the quality and level of stress is different between chronic pain samples may be different and that the origin of the study may play a substantial role for findings: Patients

from the UK where for instance national health system, pension funds, possibly employment contracts that give more security, and the prospect of some financial support in case of unemployment, might experience the disorder less stressful compared to the low back pain in a country where for many people on average or low income poor health can quickly become a financial and existential disaster in addition to the stress caused by the disorder itself. Clearly different stress levels would be expected to yield differential impact on amygdala structure. This makes clear that analyses on human pain will most likely never be free from confounds but extensive sample descriptions can mitigate these to some extent by placing results into the respective context.

Chapter 5: Functional connectivity of the amygdala in health and chronic pain

5.1 Introduction

The previous chapter focused on structural alterations within the amygdala but brain regions do not work in isolation. Which brain areas interact closest with the amygdalae?

5.1.1 Anatomical connections of the amygdala

At first sight a myriad of brain regions are candidates for an amygdala network because this structure has abundant anatomical connections and whilst there is overlap in the specified brain regions it is interesting to note that each textbook and published report on this topic describes a slightly different amygdala brain network in a healthy population: Felten and Shetty's for instance specify in their textbook the centromedial amygdala to receive information from the olfactory bulb, septal nuclei, hypothalamus, thalamus, bed nucleus of stria terminalis and brain stem areas (parabrachial nucleus, periaqueductal gray matter, ventral tegmental area, raphe nucleus, locus coeruleus) as well as indirect signals from the central amygdala nucleus and basolateral amygdala which in turn receives input from prefrontal areas, cingulate, insula, parts of the hippocampus, as well as sensory focused brain areas such as the thalamus, sensory association cortex amongst others (see figure on page 419 in Felten, & Shetty). The list of brain areas that the amygdala complex relays information to is not any shorter and overlaps in parts with the mentioned efferent connections such that the amygdala complex possibly modifies signals before relaying them back

Such a picture urges caution about the common remark in the literature that the amygdala's main output comes from the centromedial amygdaloid nucleus although it is said to be of special focus for pain processing with its laterocapsular part sometimes referred to as "the nociceptive amygdala" (Veinante et al., 2013). Veinante et al. additionally describe connections

between the amygdala and the basal nucleus of Meynert or the substantia nigra (Veinante et al., 2013) as well as other regions already described by Felten and Shetty.

This is not an exhaustive overview and may need expanding in view of a co-activation meta-analysis from 2013 based on fMRI studies (270 experiments) on the amygdala that additionally specifies the precuneus, superior temporal gyrus, middle frontal gyrus, putamen, thalamus, and cerebellum as shown in figure 1 (Bzdok et al., 2013).

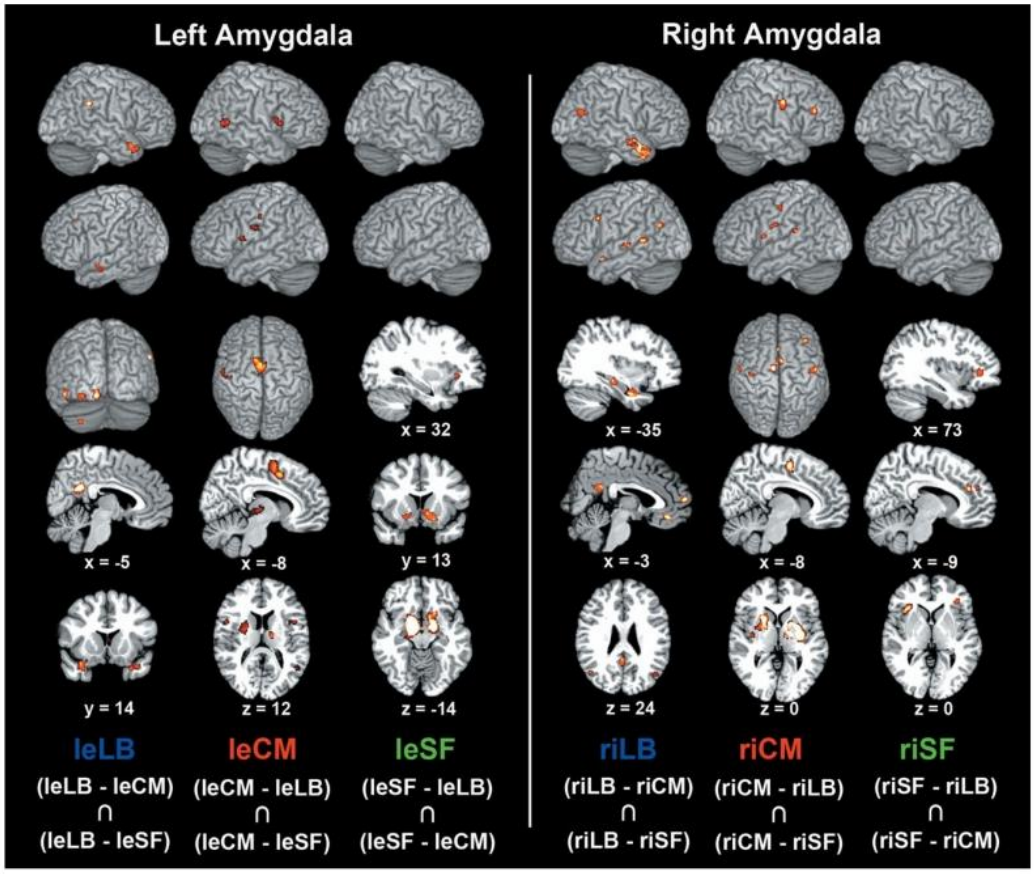


Figure 1: Meta-analytic co-activation of amygdala: figure taken from findings on 270 fMRI experiments from (Bzdok, Laird, Zilles, Fox, & Eickhoff, 2013).

The figure shows that the main amygdala subnuclei are frequently active alongside the putamina, anterior insula, precuneus, and prefrontal areas, paracingulate and thalamus across various fMRI experiments. *Abbreviations: le: left, ri: right, LB: laterobasal, CM: centromedial, SF: superficial amygdaloid subnuclei.*

Extensive connections to and from the hippocampus should not be ignored (for more details see appendix) but are not further discussed here because in-vivo

functional imaging does not allow to study amygdala-hippocampus interactions separately from each other.

5.1.2 Extinction, anxiety, and stress networks

The most obvious start in such a discussion is linked to a network that regulates fear and anxiety, emotions which many believe to be the amygdala's main function and which undoubtedly influence pain perception. Stein and colleagues investigated this question with an fMRI paradigm involving fearful stimuli (Stein et al., 2007). Using effective functional connectivity they identified causal influence by the amygdala on activity in the parahippocampus and subgenual cingulate as well as indirect influence on the supragenual cingulate (via subgenual cingulate) and posterior cingulate (via supragenual cingulate), and indirect influences on the insula via the subgenual cingulate; in turn, posterior cingulate, supragenual cingulate, the insula, but also the orbitofrontal cortex regulate activity in the amygdala (see figure 2 in (Stein et al., 2007)). BA 46, which is part of the dorsolateral prefrontal cortex, is also an indirect member of this amygdala-network because it is regulated by the orbitofrontal cortex (Stein et al., 2007). Similarly, it seems to be a common finding to link hyperactivity between the left amygdala and the orbitofrontal cortex with subclinical anxiety levels (Blackmon et al., 2011).

The orbitofrontal cortex is part of the vmPFC which in turn is commonly linked to neuropsychiatric diseases such as depression or PTSD (e.g., review by (Hiser & Koenigs, 2018)). It is likely that this brain region is not only involved in chronic pain comorbidities (such as depression, anxiety, indeed see (Mehta et al., 2018)) but also contributes pain chronification mechanisms, which has been suggested based on animal studies:

Which of these brain areas has a relationship with the amygdala that is of relevance to chronic pain and its progression? The following intends to put some of these connections into the context of networks that are relevant for pain processing.

Neugebauer's model on pain progression points to a lack of inhibition on amygdala activity by the vmPFC as fundamentally contributing to pain progression (Neugebauer, 2015). Human studies also point to the vmPFC for regulating amygdala activity (Motzkin, Philippi, Wolf, Baskaya, & Koenigs, 2015) although the evidence is not as strong as from animal work.

This also fits an amygdala driven pain progression mechanism focused on extinction deficits because the vmPFC, along with the hippocampus and the amygdala are key to the extinction network (Marek, Sun, & Sah, 2018). Deficient safety learning is related to concepts on threat and safety processing and perception of uncertainty which implicate most of the brain structures mentioned above as amygdala network (Grupe & Nitschke, 2013). Indeed it has been suggested that the vmPFC is generally involved in uncertainty "coping", safety and threat evaluations, but also suppression of "emotional responses as a reaction to threatening situations" (Wever, Smeets, & Sternheim, 2015).

Of note, parcellations between vmPFC, mPFC and dmPFC may differ slightly between studies and may also be referred to by other labels; figure 2 in box 1 illustrates this.

Box 1: Prefrontal labels

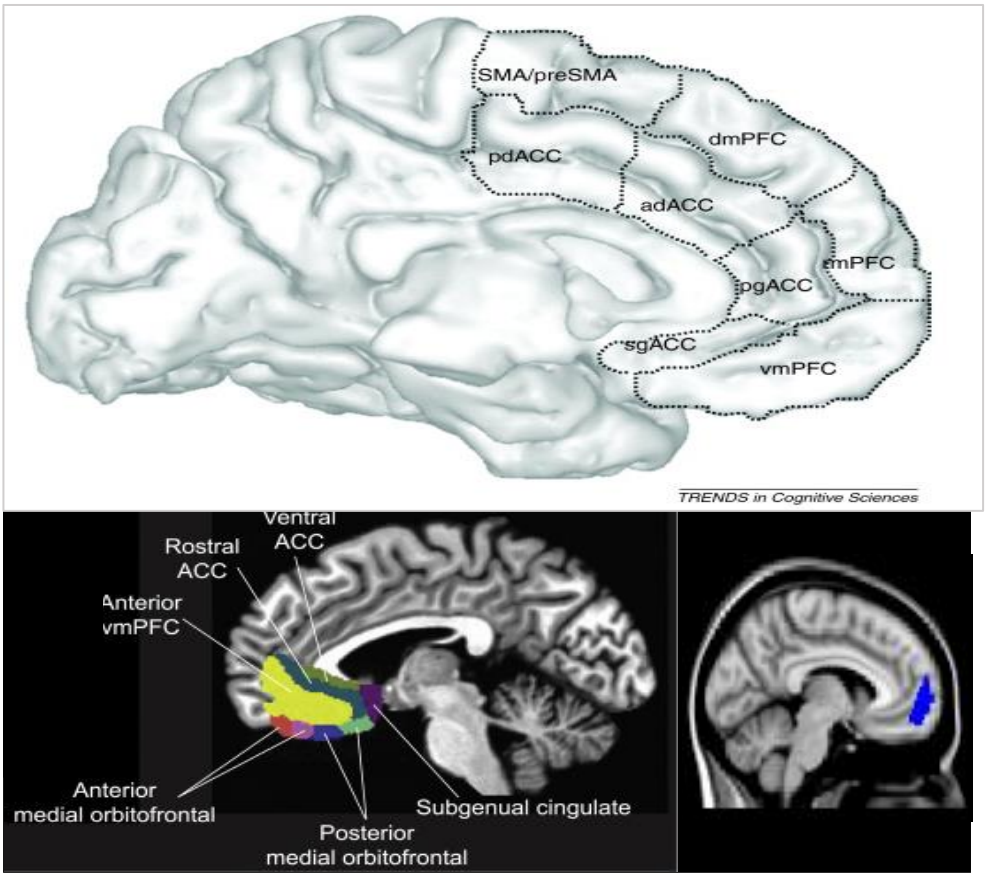


Figure 2: Parcellation of prefrontal areas

Figure on the top: adapted from (Etkin, Egner, & Kalisch, 2011), bottom left adapted from (Jalbrzikowski et al., 2017), bottom right: mask kindly provided by Arman Tajibaev, underlying brain is MN152 template.

The orbitofrontal cortex as well as the subgenual cingulate, rostral and ventral anterior cingulate are parts of the ventromedial prefrontal cortex (vmPFC). Care should be taken to distinguish the vmPFC as defined in (Jalbrzikowski et al., 2017) from the mPFC region defined in Etkin et al. (Etkin et al., 2011). Similarly, what sometimes is referred to as the dorsal anterior cingulate can be equivalent to the vmPFC in rats (e.g., in [3]). This brief note serves to caution that anatomical labels between studies may differ slightly, especially regarding boundaries of the vmPFC which is even sometimes labelled in results as anterior cingulate (e.g., see (Roy et al., 2009), elaborated in discussion of this work).

The vmPFC and mPFC also overlap largely with Broadman area 10 which is shown in the bottom figure. Conversely clusters in the dmPFC can appear labelled in studies as cingulate (e.g., see (Roy et al., 2009), elaborated in discussion of this work). The frontal pole is another label that comprises a large part of the dorsal prefrontal cortex.

Another relevant prefrontal area in pain is the dorsomedial prefrontal cortex (dmPFC), which Grupe and Nitschke link in their review to exaggerated predictions about threat outcomes and their probability (Grupe & Nitschke, 2013). This suggests amygdala upregulation (in contrast to downregulation by other parts of the vmPFC). Indeed, amygdala activity seems to correlate positively with that of the dorsomedial prefrontal cortex (dmPFC) during anxious anticipation, which was even suggested as part of a mechanism to 'maintain anxiety' (Vytal, Overstreet, Charney, Robinson, & Grillon, 2014), especially as but this brain area did not become active during safe trials. Another group confirmed dmPFC-amygdala coupling which however had a U-shaped relationship with anxiety levels (Kim, Gee, Loucks, Davis, & Whalen, 2011). Given that anxiety and pain perception are known to interact, both findings suggest to study the dmPFC's role in pain progression.

Furthermore, the dmPFC has been linked to empathy and social pain (Masten, Morelli, & Eisenberger, 2011) as well as is part of the default mode network (DMN) and has been frequently linked to chronic pain through DMN alterations (Baliki, Geha, Apkarian, & Chialvo, 2008; Baliki, Mansour, Baria, & Apkarian, 2014; Cottam, Iwabuchi, Drabek, Reckziegel, & Auer, 2018). Beyond the DMN, the dmPFC receives relatively little attention in pain research which may in parts be obscured by nomenclature differences as it can also be referred to the more frequently reported BA9 (Tanasescu et al., 2016).

Similarly, in the context of uncertainty coupling between the dorsolateral prefrontal cortex (dlPFC) and the amygdala should be mentioned; the former is claimed to "facilitate[ ] threat detection" (Wever et al., 2015) thus, unsurprisingly, the dlPFC is similarly hyperactive as the amygdala in highly uncertainty intolerant individuals (Wever et al., 2015). Related to this, literature on acute and chronic stress suggests that dlPFC-amygdala coupling is important in stress recovery (Quaedflieg et al., 2015). Specifically, Quaedflieg and colleagues measured baseline-, stress-task induced as well as recovery-induced alterations in amygdala functional connectivity and found cortisol reactivity to interact with amygdala –left dlPFC coupling such that the authors

conclude reduced coupling may index increased stress vulnerability in some individuals; also amygdala-mPFC coupling showed cortisol differentiation. In fact, PTSD patients have been shown to have negative coupling between the amygdala and the dlPFC which was not apparent in trauma exposed controls and healthy volunteers (Diener et al., 2016). Linked to this Nicholson and colleagues found that the dlPFC is prominent in amygdala downregulation via neurofeedback in PTSD patients (Nicholson et al., 2018). As PTSD is linked to stress, it is important to mention that the amygdala stands out by its content of corticotrophin-releasing factor (CRF) (Gray & Bingaman, 1996) and that CRF mediated neuroplastic changes in the amygdala have been suggested to considerably contribute to pathologic anxiety and PTSD development (Shekhar, Truitt, Rainnie, & Sajdyk, 2005). Furthermore, Rouwette and colleagues directly suggest CRF within the amygdala to be a key to switch "pain on and off" because it can have pronociceptive effects at low concentrations and analgesic effects if administered in high concentration (Rouwette et al., 2012). Rouwette and colleagues further describe a model of pain chronification in which the normal endocrine cascade to pain involving an initial release of CRF which is then substituted by other neurotransmitters, fails and is characterised by a continued release of CRF (Rouwette et al., 2012). Judging by the importance that CRF within the amygdala receives in this model based on animal work, it also highlights how important the dlPFC –amygdala link could be in pain chronification when this connection has been shown to be important in human stress studies.

Independently of such PTSD and stress studies, the dlPFC has received interest in human pain research as evidenced in a recent review that concludes that the dlPFC is important for "the suppression of pain and maintenance of pain inhibition" (Seminowicz & Moayedi, 2017) yet the amygdala is only mentioned in the latter review in a figure as part of the reward/fear network in which the authors also implicate the dlPFC (Seminowicz & Moayedi, 2017).

What is known about the amygdala in relation to a brain network directly serving pain processing?

5.1.3 Amygdala and the pain network

Animal experiments have demonstrated that parts of the amygdala receive information from the thalamus and the sensory cortex, the laterocapsular amygdala which is also referred to as the "nociceptive amygdala" even receives "purely nociceptive information via the spino-parabrachio-amygdaloid tract" (Neugebauer, 2015) which provides strong evidence for involvement of the amygdala in pain processing. The aforementioned reciprocal connections with the insula, ACC, hypothalamus, and PAG further strengthen such involvement because all these structures, especially the insula, are frequently reported in human pain literature (Tanasescu et al., 2016) (see also overlap with the more recent "dynamic pain connectome" (Kucyi & Davis, 2015)).

Indeed sometimes the amygdala is referred to as natural element of the "pain matrix" without further elaboration- mostly as part of the medial pain system, a subnetwork of the pain matrix (see box 1 for more explanation). Others attribute very little or no importance to it in this context, apart from perhaps mentioning it briefly as part of the "limbic system" or briefly referring to its role in "emotional modulation of pain", which mirrors the amygdala's underreporting in human pain studies (Tanasescu et al., 2016).

All of this is in sharp contrast to statements that the "amygdala connectivity may serve as an indicator of psychological treatment response in pain patients" (Simons, Pielech, et al., 2014).

5.1.4 Amygdala functional connectivity studies in human pain

The latter was concluded by Simons and colleagues who studied amygdala-FC in paediatric complex regional pain syndrome and found that patients had enhanced functional connectivity between the left amygdala and several other brain regions, notably the prefrontal cortex, bilateral middle cingulate, left putamen, and left nucleus accumbens yet decreased connectivity with the left precuneus or parts of the occipital lobe in a small sample of patients and age and sex matched controls (n=12+12) (Simons, Pielech, et al., 2014). The right amygdala on the other hand had increased connectivity in patients in Simons'

report in the right precuneus or supramarginal gyrus, the calcarine sulcus but no decreased connectivity. Chen et al. investigated FC of the amygdala in chronic (n=16) and episodic migraine pain patients (n=18) as well as healthy controls (n=18) (Chen et al., 2017) and also assessed volume but only FC displayed differences between groups and served as predictor of sleep disturbance. They found that episodic migraine relative to healthy controls produced enhanced functional connectivity between the left amygdala and the left middle cingulate and the left precuneus, which fits the previously cited study. Chronic migraine patients, however, showed enhanced connectivity between the right amygdala and parts of the right occipital lobe. In a FC study on fibromyalgia, the amygdala was one of the regions whose connectivity "might be fundamental in understanding inhibitory pain modulation" as the authors claimed when citing previous work, but did not discuss these structures individually and they used a small sample of patients and healthy controls (n=9+11) (Cifre et al., 2012).

Whilst in a few more reports efforts have been made to discuss FC amygdala findings in human pain in a paragraph of the discussion (Chong et al., 2017; Qi et al., 2016; Yu et al., 2017), the conclusion in other reports is not as strong: Schwedt et al. also investigated migraine (n=20+20 patients and controls) and included the amygdalae as ROIs in their functional connectivity analyses; amongst other regions the amygdalae showed positive functional connectivity with at least two regions of the pain matrix but from the reported figures it cannot be determined which regions these specifically are (Schwedt et al., 2013). The authors focused on insula connectivity, and only briefly discuss the link between the amygdala and the insula and elaborate on the amygdala in one general paragraph on its pro- and anti-nociceptive activity by reference to animal studies by Neugebauer's group.

Another migraine FC study (n=38+20 patients and controls) mentioned their amygdala findings in relation to the insula in one sentence (Schwedt et al., 2014)- similar to a recent meta-analysis on such analyses in this patient group (Maleki & Gollub, 2016) and similar to another FC pain study which only mentioned the amygdala in one sentence of the discussion although this region

was amongst the regions of interest masks and was found to have stronger connections, functionally and structurally, with the anterior insula in the context of pain in a healthy population (Wiech, Jbabdi, Lin, Andersson, & Tracey, 2014). Interestingly, another functional connectivity pain study was entitled with 'amygdala' as 'the missing link' and found enhanced connectivity between amygdalae and insulae (as well as the thalamus amongst other regions) in migraine patients relative to controls but discussed the amygdala only in one sentence before focusing on the insula for the discussion (Hadjikhani et al., 2013).

5.1.5 Summary and motivation for current study

Overall this suggests that interest in pain-related functional connectivity of the amygdala is promising but based on small sample sizes and has not received much attention despite its potential to understand chronic pain better.

This motivates the current study to specifically investigate resting state amygdala functional connectivity in chronic knee OA pain patients relative to controls as this pain aetiology has not been reported in amygdala functional connectivity studies before.

Additionally reliability of any network alterations and methodology shall be tested by using different amygdala masks and two different OA and healthy controls samples which have been scanned at the same site with identical hardware.

5.1.6 Research questions and hypotheses:

A. Are findings from seed-based connectivity analyses using different amygdala masks comparable?

This question is exploratory and investigated because most literature uses either of two popular probabilistic atlases to create seed masks of the amygdalae.

B. Is the amygdala connectome altered in chronic knee OA pain?

It is hypothesized that connectivity between the amygdalae and vmPFC would be weaker in chronic pain because a lack of inhibition from the vmPFC (through amygdala inhibitory interneurons) is claimed to be a main contributor to an amygdala-focused pain progression model in animals (Neugebauer, 2015).
It is further hypothesized that connectivity between the amygdalae and dmPFC, and dlPFC is enhanced because these structures have been discussed to facilitate threat processing or mediate stress responses.
The insulae are also particularly relevant because they are the most frequently reported structures in human pain (Tanasescu et al., 2016). It is hypothesized that chronic pain would strengthen positive time series correlations between the amygdalae-insulae.

C. Are any pain-related alterations to the amygdala connectome sex-specific?

There is tentative evidence for sex differences in amygdala networks in a healthy population showing greater connectivity for the right amygdala for men and greater connectivity for the left amygdala in women, particularly regarding the mPFC (Kilpatrick, Zald, Pardo, & Cahill, 2006).
Overall, however, pain-related sex-differences in amygdala FC have not been investigated yet and therefore this remains a largely exploratory question; it is suggested here that the higher prevalence of females suffering from chronic pain may be linked to this.

5.2 Methods

The current analyses used data from two big single-site studies at the University of Nottingham, abbreviated here under the acronyms INCOPE and IMPOA, approved by the local ethics committee and performed in accordance with the declaration of Helsinki. All participants gave full informed consent; the data analysis for this chapter meets data protection and approved use as outlined in the study protocol. Participants were recruited through GP surgeries, posters, or through consent in previous studies permitting to contact them again for further studies. Only data relevant for the current analyses is reported here.

Teams recruiting and collecting data for these studies are described in detail in the acknowledgement and contributions chapter.

5.2.1 Participants and sample sizes

Participants were excluded from these studies if they were not able to give informed consent, be under 18 years of age, were unsafe or unfit for MRI, were pregnant, or had any significant neurological, psychiatric or other significant health problem with the exception of chronic knee OA pain for the patient group. Patients were included in the study if they had unilateral knee OA only with pain. The criterion for chronic knee pain was met if participants reported to feel pain most of the times and on most days for at least 3 subsequent months. Patients were not admitted to the study if prescribed opioids.

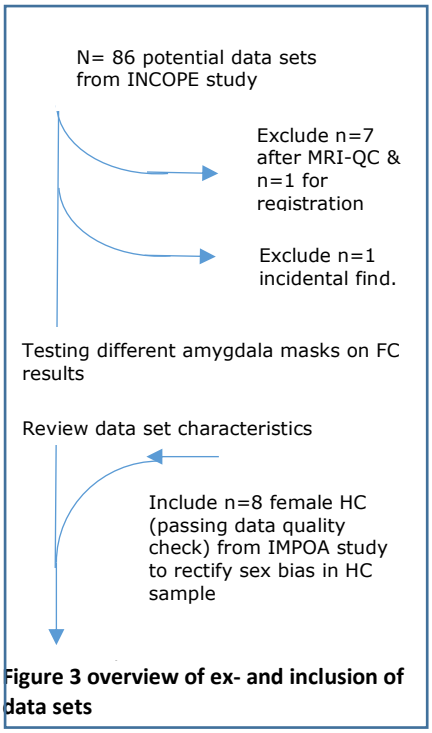


Figure 3 overview of ex- and inclusion of data sets

At time of this analysis 86 data sets with resting state from this study were available.

Criteria for exclusion of data sets are described in the section on quality control. N=7 were excluded after quality control, N= 1 excluded because of registration failure. Quality control is described further in the next section. An overview over characteristics of data sets included in the analysis (e.g., sample sizes of subjects, age, sex distribution, pain parameters) is given in table 1 in the results section.

Given that the female healthy controls were considerably underrepresented, n=8 female healthy controls from IMPOA study were included. In earlier analyses that are not included in this chapter IMPOA study data was found to overall suffer from poor data quality (i.e., only 8 from 17 female HC were of acceptable quality); the n=8 data sets from this study passed quality controlled in the same way as for the INCOPE study. The selection was blinded to characteristics other than sex, grouping (healthy controls only) and image quality. The choice to focus on female controls only was based on the fact that

female controls are underrepresented in the INCOPE data set and because time constraints did not allow to screen the whole IMPOA sample for more usable data.

5.2.2 Data used for the present analyses

Scans

Participants were scanned at the identical 3T scanner (MR 750 Discovery, GE Healthcare) and the same 32-channel head coil at the Queen's Medical Centre, Nottingham. Scans included a 1mm isotropic T1-weighted image with gradient echo sequence (TE 3.3ms, TR 8.5s, TI 450ms, slice gap 1mm, flip angle 12 degrees, FOV 256 with 256x256 matrix) and an echo planar sequence (TE 32ms, TR 2s, voxel: 3 x 3 x 3.5, 35 axial slices acquired in interleaved fashion with flip angle 77 degrees, FOV 240 and matrix 64X64) during resting state. Length of resting-state scans was 5 min 30s. Participants were instructed to relax during the scan and focus on a fixation cross.

Eight additional healthy volunteer subjects were scanned with the identical hardware but slightly different scanning parameters (flip angle: 80 degrees, TE 30ms, sagittal slice acquisition) and were asked to keep their eyes closed. The 8 additional healthy control data sets were acquired before a DV24 software upgrade on the scanner. In consultation with the current radiographer (who joined the team after the upgrade), it is not thought that this upgrade noticeably altered images but this possibility cannot be excluded unless comparing scans from the same person shortly before and shortly after the upgrade that were acquired with the same scanning parameters. To the best of our knowledge such a data set was not acquired.

Demographics and questionnaire data

Self-reported data used for the present analyses included age, sex, as well as questionnaires on trait anxiety (Spielberger, 1983), and perceived stress (Cohen, 1994), which were filled out by participants at their homes. Additionally, average pain intensity (0-10 scale) and self-reported duration of pain (in years), as well as their level of schooling and attended higher education on a 8-level scale (similar to (Egerton & Mullan, 2008) where 1 is no formal education and 8 reflects accomplished postgraduate studies) were self-assessed via short questions. Other questionnaires are not reported here as not relevant to the current study.

5.2.2 Image quality control and preprocessing

These steps involved quality control through MRI-QC software (see recommendation in (Esteban et al., 2017)) and, as a consequence, exclusion of images with framewise displacement >3mm or average framewise displacement >1mm; similarly incomplete resting-state data sets were excluded. All quality assurance checks were performed blinded to details of the subject's data set (e.g., belonging to patients or controls).

FSL software (www.fmrib.ox.ac.uk/fsl) was used for brain extraction of the T1-weighted images using FSL's BET (Brain Extraction Tool (Smith, 2002), additional setting: -f 0.3). Resting-state images were preprocessed via FSL's FEAT using McFlirt (Jenkinson, Bannister, Brady, & Smith, 2002) for motion correction, corrected for slice timing (interleaved acquisitions), brain extracted, spatially smoothed (Gaussian kernel's FWHM: 5mm), intensity normalized (one multiplicative factor on entire 4D data set), high-pass filtered (Gaussian-weighted least-squares straight line fitting, sigma=30s), and registered to the brain-extracted T1 image using FSL's FLIRT after the latter has been registered nonlinearly to standard space using FSL's FNIRT (Andersson, 2007a, 2007b).

Thereafter, ICA-AROMA was used to denoise the functional data as recommended in (Pruim, Mennes, Buitelaar, & Beckmann, 2015), using masks

in subjects' space that were produced by brain-extracting one functional volume. FSL's FAST was used to segment T1-weighted images into cerebrospinal fluid (CSF) and white matter (WM) which were then thresholded (volume x probability) to avoid partial volume effects using an in-house matlab script (kindly provided by Alireza Mohammadinezhad Kisomi to Sarina and William). The eroded WM and CSF images were registered to subject space via FSL's FLIRT and used to extract CSF and WM timeseries for each data set (using fslmeants) from the denoised functional images in order to include these as regressors of no interest in the first level analyses.

All 8 data sets were confirmed to have adequately registered amygdala masks to denoised resting state data that were not overlapping or bordering signal drop out. The latter is an issue largely applicable to IMPOA data and time constraints did not permit to screen the whole sample for more includable data sets from males and patients. As coverage of amygdala areas in INCOPE study was remarkably better, this extra quality control (overlaying individually registered amygdala masks on each resting state data) was performed only on a random selection of data sets (n=20 out of 77).

5.2.3 Amygdala seed-based connectivity analysis

Masks

Seed-based FC analyses requires extraction of time series from the seed via a mask and then correlation of this time series with time series of every other GM voxel and subsequent multiple comparison correction. This makes clear that the accuracy of the analyses depends on choosing adequate amygdalae masks; given the intention to critically evaluate the available methodology, this chapter set out to compare results produced with several amygdala masks for the left and right hemispheric seeds separately: a) laterobasal amygdala masks at 50% probability threshold based on the Juehlich atlas and as implemented in (Roy et al., 2009), additionally centromedial amygdala masks from this atlas at 60% probability threshold b) conservative Harvard-Oxford amygdala masks at 80% probability thresholds c) masks comprising of a 6mm sphere centred on

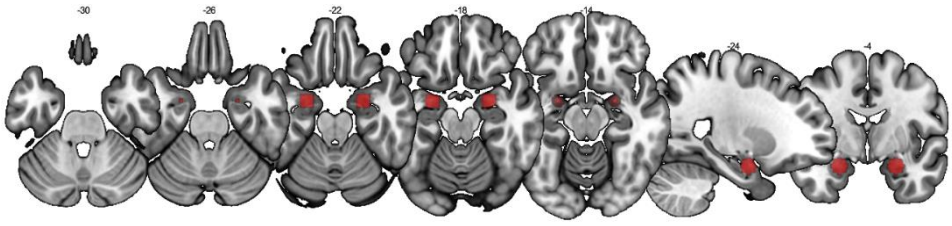
the coordinate with the highest amygdala probability according to the Harvard Oxford atlas (94% and 98%).

The latter was chosen for comparison because analyses regarding other seed regions in our group have been performed with 6mm spheres.

Masks are identical to those used in chapter 4, the additional mask for c is shown in box 1.

The methodological evaluation of the effect of different amygdala seeds was performed on a sample that comprised all subjects until data was reviewed (i.e., all but the additional n=8 female HC, see figure 4). All masks were registered to the individual space of the denoised data sets.

Box 1: Amygdala masks based on 6mm spheres



Left Right

This shows amygdala masks based on a 6mm sphere around x=-24 (or 24 for right), y=-4 and z=-20; the figure overlaid left and right masks. The centre coordinates were chosen based on the Harvard Oxford subcortical atlas: the centre voxel for the left mask received 98% probability for the left amygdala, while the corresponding voxel on the right hemisphere yielded 94% probability for the right amygdala.

First level analysis

First level analysis of the time-series was performed on the denoised data separately on left and right amygdala time series using FILM in FSL's FEAT for autocorrelation correction (prewhitening) (Woolrich, Ripley, Brady, & Smith, 2001), specifying positive and negative contrasts whilst correcting for CSF and WM as covariates of no interest. All other settings were left at default.

Second level analysis

Second level analysis was also done with FSL's FEAT specifying four contrasts: a mean amygdala network for the patient group, a mean amygdala network for the healthy control group, strengthened network in patients relative to controls, and strengthened network in healthy relative to controls. Mean relative motion was entered as a covariate of no interest. A gray matter mask was used to limit multiple comparison correction to gray matter voxels only.

In 2017 the standard z threshold for group statistics in FSL was increased to 3.1 both at for corrected cluster significance thresholds of p=0.05 (see release notes from 25.04.2017 in minor revision history https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/WhatsNew) in an attempt to reduce type I errors. Hence this recent standard is applied to the current work but because most published reports are based on the former threshold the analyses have been performed separately with both 3.1 and 2.3 z- thresholds to allow visual comparisons of results to published reports. Separate contrasts were specified for negative and positive correlations.

Second level analyses did not control for covariates of no interest other than CSF and WM; sex and age were reasonably well balanced between groups. Education and mean depression levels are systematically higher in patients but these variables have not been controlled for. Depression is a frequent comorbidity and several items on the depression score can also reflect impact of pain regardless of depression (e.g., insomnia). The difference in education levels reflects recruitment bias because healthy volunteers are more likely to be willing to participate in studies for "the greater good" if they have higher education whilst patients are more likely to consider their individual efforts to improve treatment options for themselves but also for future patients. Whilst the aim of each study is to compare samples that are as well matched as possible to minimize confounds there is no report that education levels directly affect resting state networks; more specifically there are no grounds to speculate that education would affect amygdala networks given that this brain area's activity is based on automatic, unconscious processes and hence education was not controlled for.

Overview of all seed-based whole brain FC analyses in this chapter

After evaluation of the effect of different amygdala masks, the seed mask that was considered adequate was used to carry out further analyses.

Multiple comparisons correction for the number of different seed-based functional connectivity analyses were not performed because within each analysis stringent multiple comparison correction was applied; furthermore small significant clusters that are not hypothesized and only appear in one sub-analysis will be interpreted with the necessary caution regarding the risk for type I error.

5.3 Results

5.3.1 General findings

Tables 1 and 2 describes both samples for final analyses with respect to their sample sizes, sexes, average pain severity, pain duration, and depression score (BDI-II) as well as their scores for perceived stress, trait anxiety where such data was available.

It shows that median depression scores are slightly higher than in healthy controls but either median score is too low to speak of any clinically relevant depression level (scores 0-13 reflect minimal depression, 14-19 mild depression, 20-28 moderate depression, 29-63 severe depression (Beck, Steer, & Brown, 1996)). However, while the maximum depression score in the healthy control sample is suggestive of mild depression, the maximum score in the patient sample is indicative of severe depression according to scoring classification provided by the BDI questionnaire. This classification has to be taken with scrutiny though as depression is often linked with chronic pain and conversely often misdiagnosed in chronic pain because sleeping problems and the lessened ability to enjoy some activities are side-effects of chronic pain and impaired mobility; for these reasons depression scores are not an exclusion criterion in this study nor a nuisance factor (because that would reduce variability inherent to pain) but serve here to describe the data set.

The data set is further described by the following: an age range of 65-81 years which is nearly equal for both samples, a pain duration for the patient sample ranging from 9 months to 48 years with a median of 7 years, and an ICOAP total score ranging from 4.5- 98 with a median of 46.5 (the maximum possible score is 100, minimum is 0), which indicates a moderate level of pain impact overall for the sample.

The patient sample is further characterised by low perceived stress levels (median 13 which is low according to Cohen, 1994) although the maximum score reflects high stress levels (maximum 36, scores 27-40 are considered to reflect high stress levels according to Cohen, 1994). Similarly, trait anxiety levels are low in the patient sample although there were exceptions (here: median score 35, maximum 66, maximal possible range: 20-80).

Reported medication use for painkillers within 24h prior to the appointment, any reported regular use of opioids and antidepressants are listed in table 3. Other medication taken regularly (e.g., for diabetes) is not listed here. Some antidepressants may also be prescribed off-label for pain relief (in particular Amitryptiline). Ten patients did not report to have taken any medication neither within 24h nor any regular use of pain killers or antidepressants.

Table 1: Description of samples for current analyses

Patients (n=25 females, n=23 males)				
	age	ICOAP total score	Pain Duration (yrs)	BDI-II
median	65	46.5	7	9
minimum	34	4.5*	0.75**	0
maximum	80	98	48	40
Healthy (n=17 females, n=20 males)				
	age			BDI-II
median	70			5
minimum	44			0
maximum	81			19

*2 patient data sets had missing data for this questionnaire. They were not excluded because a) one reported an average pain severity of 75 on a scale of 100. b) the other data set missed pain-related questionnaires but had a self-reported pain duration of 30 years

**one data set missed this information. It was nevertheless included because the ICOAP score for intermittent pain was 17 (but 0 for constant pain).

Table 2: Psychometric description of samples for current analyses

Patients	IntUnc.	Stress	Anxiety
median	52.5	13	35
minimum	27	2	0
maximum	112	36	66
n	42	48	47

Table 3: Painkiller and antidepressants use reported by patients

Painkillers 24h prior to scan	**Regular opioids**	**Other regular pain killers**	**Antidepressants***
Ibuprofen 400 mg		Ibuprofen	
Tramadol / paracetamol 50mg / 500mg	Marol (Tramadol HCl) Tramadol	Aspirin Fenbid gel (Ibuprofen)	
Co-dydramol 2x 10 mg&500 mg	Co-dydramol	Diclofenac gel	
Paracetamol 1000 mg		Paracetamol	
Tramadol 100 mg	Tramadol	Paracetamol Rizatriptan	Amitriptyline Citalopram
Paracetamol/ Codeine 2 x 500mg/ 30mg			
Paracetamol 1 x 500 mg		Aspirin Paracetamol	Mirtazapine
Tramadol/ Nefopam/ Lyrica (Pregabalin)/ Ibuprofen 2x 50 mg / 2x 30 mg / 2x 300 mg / 2x 400 mg	Tramadol	Ibuprofen Nefopam Paracetamol	
Aspirin 75 mg	Zapain (Codeine Phosphate/Paracetamol)	Aspirin, gabapentin	Citalopram
Naproxin/ Co-codamol/ Nefopam 500 mg/ 30mg&500mg/ 60 mg	Co-codamol	Fenbid Forte gel Naproxen Nefopam	Amitriptyline
Paracetamol/ Volterol 2 x 500mg/n.a.			
Amitryptiline 20mg			Amitriptyline
Gabapentin/Tramadol/ Dihydrocodeine/ Ibuprofen/ Paracetamol 500mg/ 1 tablet (dosage n.a.)/ 20mg/ 400mg/ 2 tablets (dosage n.a.)	Tramadol (?), Dihydrocodeine (20mg)	Ibuprofen (400mg), Paracetamol	
Paracetamol 1500mg		Paracetamol	

Duloxetine 30mg			Duloxetine (30mg), Amitriptyline (50mg)
Paracetamol n.a.		Paracetamol, Capsaicin, Lidocaine, Aspirin (75 mg)	
Ibuprofen 2 tablets (dosage n.a.)			
Naproxen 500 mg	Codeine phosphate (15mg)	Naproxen(500mg), Paracetamol(500mg)	
Co-codamol/ Naproxen 2 x 300mg or 500 mg/ 50 mg	Co-codamol (codeine)	Aspirin, Naproxen	
Zapain 1 x 30mg&500 mg	Zapain (Codeine) (30mg)	Paracetamol(500mg), Fenbid (Ibuprofen)	
Paracetamol/ Neurofen 2 x 500 mg/2 x 200 mg		Paracetamol, Ibuprofen	
Paracetamol 2x 500mg		Aspirin 75 mg	Amitriptyline (30mg)
Paracetamol 2 tablets (dosage n.a.)		Paracetamol, Aspirin,	
Dihydrocodeine/ Pregabalin 2 x 60 mg/1 x 60 mg	Dihydrocodeine (240mg/pd)	Pregabalin	Amitriptyline 75mg
Paracetamol 2 tablets (dosage n.a.)			
		Naproxen	Amitriptyline
Paracetamol 2 x 500 mg			
		Aspirin Fenbid gel	
			Citalopram
	Cocodamol Codeine		
		Ibuprofen	
		Paracetamol, Ibuprofen	
		Cortison injections 1-2/year, Gabapentin	
		Naproxen (500mg)	

Medication usage for healthy volunteers: One participant reported to have taken Ibuprofen within 24h, one reported paracetamol. 1 person reported to regularly take Voltarol gel, Paracetamol and Amitryptiline. Four healthy volunteers reported to take Aspirin regularly, one regularly uses Ibuprofen cream. Two more healthy controls regularly only take Amitryptiline.

Are findings from seed-based connectivity analyses using different amygdala masks comparable?

As figure 4 and 5 show, positive connectivity of the left and the right amygdalae in healthy volunteers are similar both across hemispheres as well as across different seed masks with exception of left centromedial amygdala networks based on Juehlich histological atlas. There were hardly any brain regions that correlated negatively with activity of either amygdala in healthy volunteers as figure 6 shows.

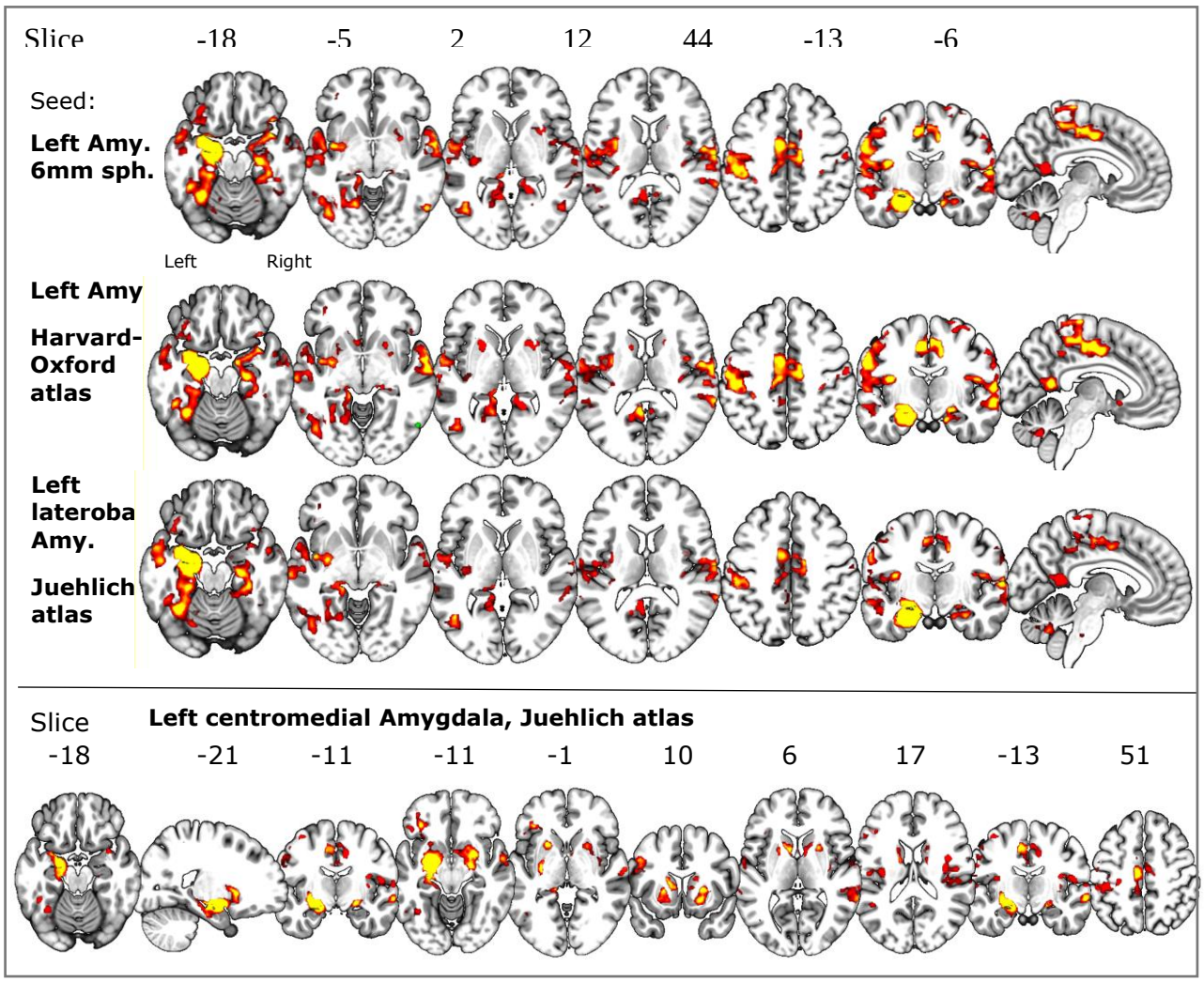


Figure 4: Left amygdala centred functional connectivity network (pos. correl.) for healthy controls from INCOPE data set
Figure 4 shows that the amygdala centred network is mostly comparable across findings applying different amygdala masks (left) column, apart from the bilateral putamen, and a small cluster in the right insula at a lower threshold which does not appear when using the left laterobasal amygdala as seed region.
Yellow: z>3.1, red: z>2.3

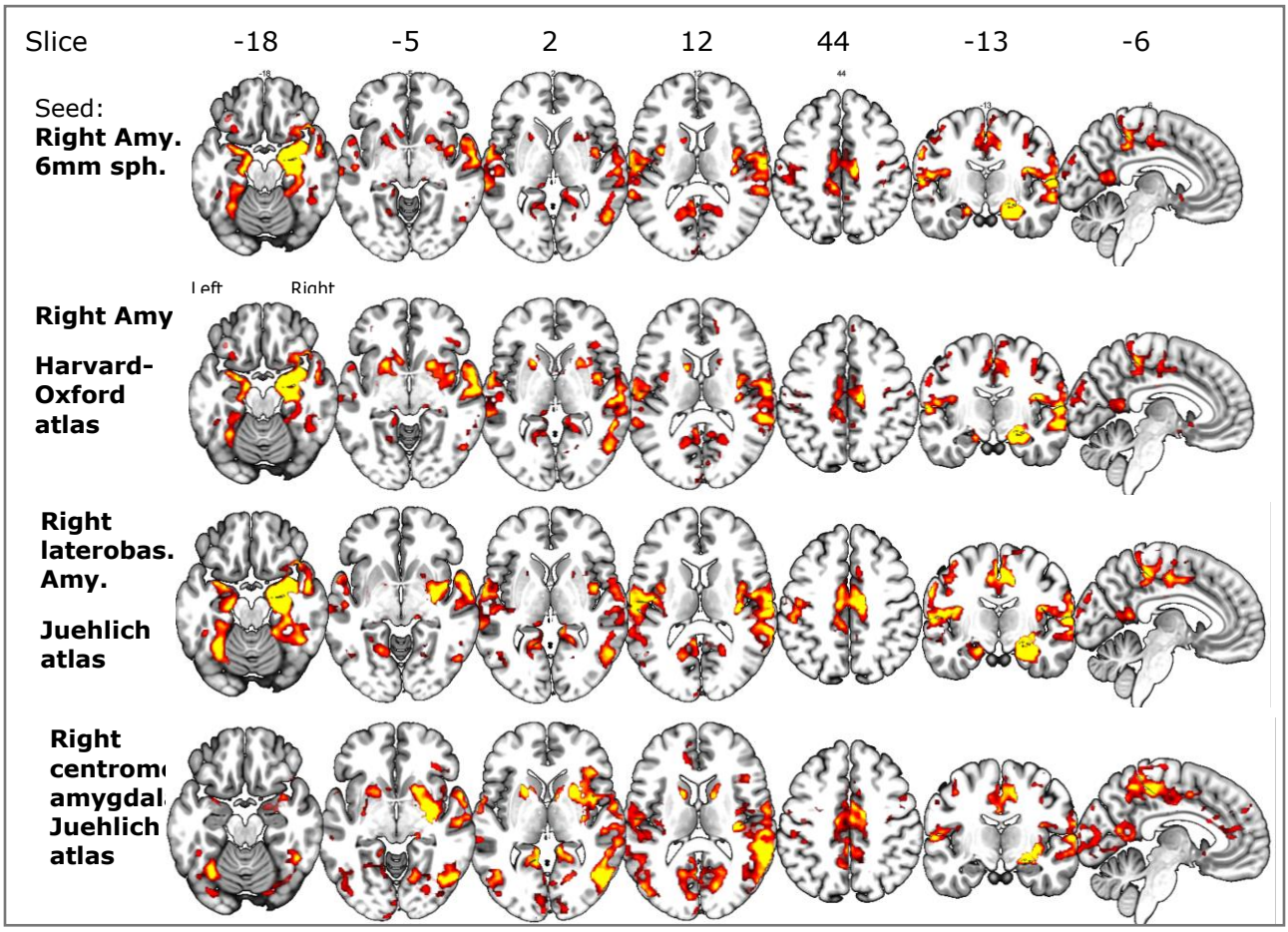


Figure 5: Right amygdala centred functional connectivity network (pos. correl.) for healthy controls from INCOPE data

Figure 5 shows that the use of different amygdala masks (left column) made negligible differences to the amygdala centred network.

Yellow: z>3.1, red: z>2.3

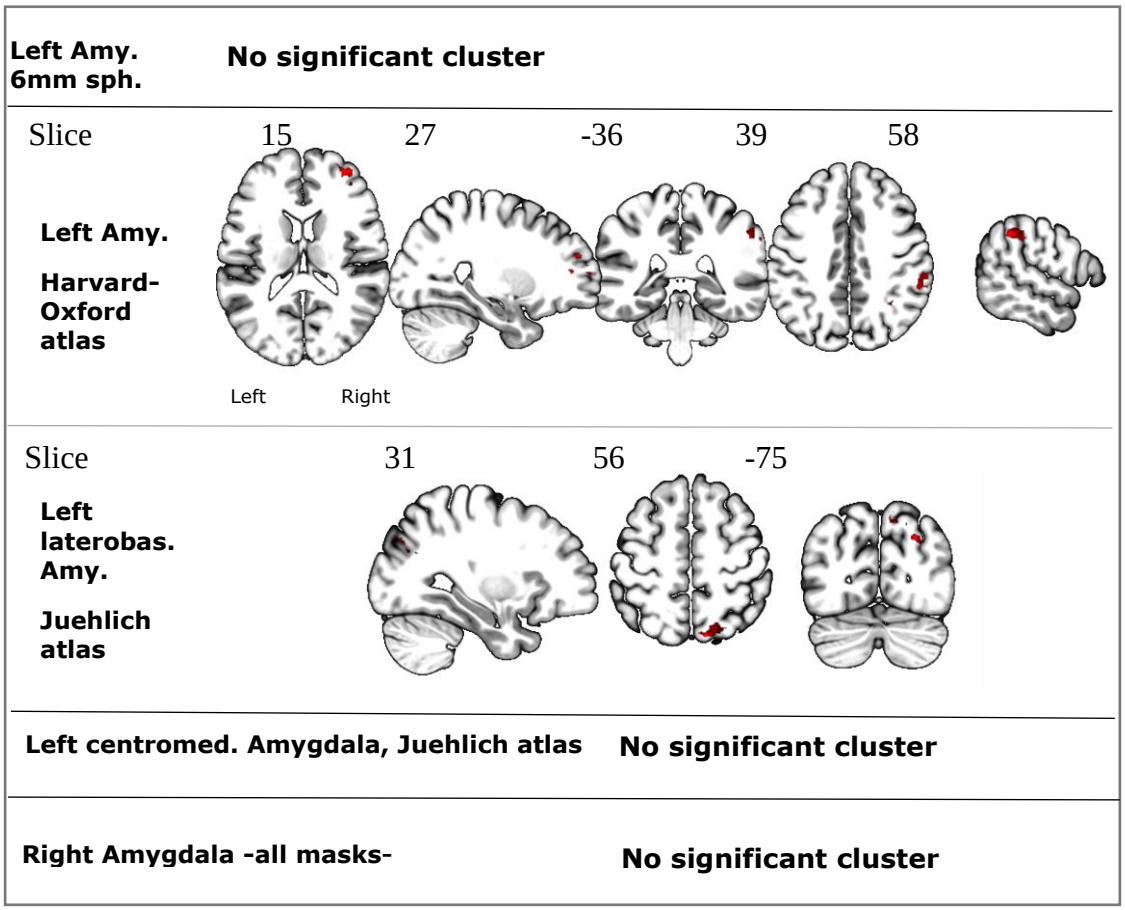


Figure 6: Amygdala centred functional connectivity network (neg. correl.) for healthy controls
Red indicates sig. clusters at 3.1<z>2.3; no clusters survived the new default z threshold.
There is no consistency between results produced with different left amygdala masks.
Abbreviations: sph.= sphere, Harv.-Oxf.= Harvard-Oxford, laterobas. = laterobasal, neg.=negative, correl.=correlation

5.3.2 Hypothesized results

Is the amygdala connectome altered in chronic knee OA pain?

Across both groups brain areas which positively correlated with amygdalae activity comprised prefrontal areas, insulae, putamina, but also fusiform gyrus, pre- and post-central gyrus (figures 7-9) as well as hippocampi which were however not considered a separate finding in the current analysis because of the proximity (enhanced by spatial smoothing) to the seed region. Negative coupling to the amygdalae was observed in the left amygdala network regarding the precuneus even extending into the cuneus.
A complete overview of clusters found for in this analysis as well as those discussed in further sections is given in tables 4 and 5.

Pain related alterations to amygdala networks
Despite of similarities between patients' and controls' amygdalae networks there were also visible group differences. Compared to healthy controls, amygdala networks in patients were reduced and distinct (figures 7-9). Unexpectedly, we found that the left amygdala was significantly less positively coupled with the left postcentral gyrus in pain patients; this was also visible in the right amygdala networks although only significant at the reduced z-threshold (figure 10). However there was significantly less positive coupling between the dmPFC and the right amygdala in patients than in controls (figure 10); this connection was hypothesized but the direction of this coupling was the opposite.

For comparability with literature findings, figure 10 shows that the dmPFC cluster found in the current work borders the dmPFC cluster that was reported in relation to "aversive amplication" (Robinson et al., 2012) and thus the cluster that the current hypothesis was based on.

Table 4: Overview of significant clusters for amygdala networks at z> 3.1 from seed-to-voxel FC analyses

Contrast	Anatomical Label	Cluster size (voxels)	p-value	Z- max	X (mm)	Y (mm)	Z (mm)
Positive coupling with RIGHT amygdala							
Pat	R Amy, hippocamp, Fusiform C, temporal pole	6295	<0.0001	13.1	20	-4	-18
	Cingulate G/ Precuneus	613	<0.0001	4.67	-4	-52	22
	L Pre/Postcentral G	256	<0.0001	4.25	-60	-8	36
	mPFC	224	0.000269	3.98	-2	56	-10
HC	R Amy, hippocamp, fusiform C, temporal pole	3586	<0.0001	10.8	20	-4	-18
	L Amy, hippocamp, putamen	903	<0.0001	6.18	-26	-2	-12
	R Supramarginal G	633	<0.0001	4.78	56	-42	12
	dmPFC	440	<0.0001	5.45	6	52	30
	dmPFC	260	<0.0001	4.76	-8	46	32
	L Precuneus	202	0.000564	4.32	-18	-58	8
	R Fusiform C	167	0.00193	4.25	44	-38	-20
	L post insula/planum polare	146	0.00422	5.71	-60	-8	4
	L Frontal orbital C	139	0.00552	4.68	-30	20	-18
	L Superior Temp G	89	0.0431	4.38	-62	-42	10
Pat>HC	**none**						
HC>Pat	**dmPFC**	**189**	**0.000883**	**4.35**	**8**	**56**	**24**
	R Superior Frontal G	**98**	**0.0292**	**4.32**	**10**	**-14**	**70**
Negative coupling with RIGHT amygdala							
Pat	none						
HC	none						
Positive coupling with LEFT amygdala							
Pat	L Amy extending to hippocampus and temporal pole/brainstem	5206	<0.0001	13.7	-20	-6	-16
	R Amy/hippocamp, temporal pole	2929	<0.0001	7.81	16	-6	-18
	L Precentral G	225	0.000308	4.52	-56	-14	28
	L Supramarg. G	213	0.000455	5.04	-60	-46	10
	Precuneus	206	0.000574	4.44	-6	-54	16
	Cerebellum	199	0.000726	4.71	48	-68	-30
	Frontal pole	194	0.00086	4.81	-10	50	42
	Brainstem	151	0.00394	3.71	-20	-62	-20
	brainstem	116	0.0151	3.91	22	-36	-36
HC	L Amy, hippocamp, putamen, insula	3478	<0.0001	11.2	-20	-6	-16
	R Amy, hippocamp, fusiform C	1498	<0.0001	5.5	18	-12	-22
	L Postcentr G	1456	<0.0001	5.27	-30	-26	50
	L Precentr G	487	<0.0001	4.9	-2	-28	72
	R Middle temporal G	383	<0.0001	5.2	66	-44	8
	R Superior Temporal G	375	<0.0001	5.43	60	-2	-12
	Postcentral G, Precentral G	366	<0.0001	4.81	16	-30	74
	L Fusiform C	127	0.0098	4.41	-34	-62	-8
	L Frontal orbital C	115	0.0158	3.91	-44	32	-12
	L Lateral Occip. C	105	0.0237	4	-46	-70	0
	R Precentral G	96	0.0345	4.09	32	-8	66
Pat>HC	**none**	**314**	**<0.0001**	**4.93**	**-36**	**-40**	**60**
HC>Pat	**Postcentral G**	**159**	**0.00294**	**4.48**	**-10**	**-38**	**56**
	Postcentral G	**131**	**0.00839**	**3.79**	**8**	**-38**	**64**
	R Superior Frontal G	**112**	**0.0178**	**4.31**	**16**	**0**	**60**

Table continues on next page.

Contrast	Anatomical Label	Cluster size (voxels)	p-value	Z- max	X (mm)	Y (mm)	Z (mm)
Negative coupling with LEFT amygdala							
Pat	R Lateral Occipital C	1166	<0.0001	4.95	18	-64	60
	L Precuneus/Superior parietal lobule	616	<0.0001	4.63	-38	-54	50
	R Precentral G	112	0.0178	4.21	46	0	42
	R middle frontal G	101	0.028	4.4	44	32	28
HC	R Precuneus	180	0.00139	3.97	56	-40	42
	R Frontal pole	135	0.00719	4.41	36	54	18

Abbreviations: R: Right, L: Left, Amy: Amygdala, hippocamp: hippocampus, ant: anterior, post: posterior, C: cortex, G: gyrus, Cerebell: Cerebellum

Table 5: Overview of significant clusters for amygdala networks at z> 3.1 for sex-specific analyses

Contrast	Anatomical Label	Cluster size (voxels)	p-value	Z- max	X (mm)	Y (mm)	Z (mm)
FEMALES - Positive coupling with LEFT amygdala							
Pat	L Amy, hippocamp,	580	<0.0001	5.78	-18	-8	-20
HC	L Amy, hippocamp,	442	<0.0001	5.84	-18	-10	-18
	dmPFC	122	0.0143	4	-6	54	12
	L Middle Temporal G	103	0.0302	3.78	-54	0	-18
Pat>HC	**R ant insula**	**97**	**0.0386**	**4.38**	**44**	**14**	**-2**
HC> Pat	**None**						
FEMALES - Negative coupling with LEFT amygdala							
Pat	R Precuneus	220	0.00048	4.29	30	-62	52
HC	R Precuneus	240	0.000257	4.31	54	-36	44
	R Precuneus/Supramarg. G	152	0.00472	4.48	24	-48	56
FEMALES - Positive coupling with RIGHT amygdala							
Pat	Cerebellum	120	0.0139	3.73	-6	-68	-38
HC	L dmPFC	706	<0.0001	5.08	-4	52	34
	R dmPFC	675	<0.0001	4.93	6	54	24
	R Amy	494	<0.0001	5.72	20	-4	-18
	R Superior Temporal G	101	0.03	4.53	56	12	-12
Pat > HC	**None**						
HC > Pat	**L dmPFC**	**150**	**0.00447**	**4.33**	**6**	**52**	**32**
	R dmPFC	**123**	**0.0124**	**4.24**	**-4**	**52**	**34**
FEMALES - Negative coupling with RIGHT amygdala							
Pat	None						
HC	L Angular G	342	<0.0001	4.54	-46	-52	42
	L Supramarginal G	191	0.00105	4.22	56	-34	44
	L Frontal pole	148	0.00481	4.07	-40	40	28
MALES - Positive coupling with RIGHT amygdala							
Pat	R Amy, hippocamp, temporal pole	1788	<0.0001	10.3	24	0	-18
	L Amy, hippocamp, temporal pole	1237	<0.0001	5.83	-24	0	-20
	L Precentral G	164	0.000979	3.92	-50	-8	32
HC	R Amy, hippocamp,	1574	<0.0001	8.69	24	0	-18
	R Superior Temporal G	1234	<0.0001	4.82	58	2	-12
	Post Cingulate	1172	<0.0001	5.86	14	-20	42
	L post insula/planum polare	544	<0.0001	4.98	-60	-12	8
	L Putamen	521	<0.0001	5.24	-22	14	0
	R lateral occipital C	184	0.000442	4.12	52	-66	-10
	R Superior Temporal G	172	0.00071	4.4	-62	-42	10
	L Precuneus G/ parietal operculum	108	0.011	4.68	-52	-36	24
	Cerebellum	82	0.0385	4.2	-34	-52	-22

Pat>HC	**None**						

HC>Pat	**L Postcentral G**	**184**	**0.000442**	**4.98**	**-10**	**-38**	**52**
	R Postcentral G	**135**	**0.00329**	**4.32**	**8**	**-36**	**52**
MALES- Negative coupling with RIGHT amygdala							
Pat	None						
HC	None						
MALES - Positive coupling with LEFT amygdala							
Pat	L Amy, hippocamp,	2365	<0.0001	10.8	-20	-6	-16
	R Amy, hippocamp,	945	<0.0001	6.49	16	-12	-14
	L Precentral G	161	0.00128	4.79	-50	-8	34
	L Superior Temporal G	141	0.0029	3.87	-56	-6	-12
HC	Posterior Cingulate	1540	<0.0001	5.36	-10	-14	48
	L Precentral G	1326	<0.0001	5.1	-54	-4	32
	L Amy, hippocamp	1279	<0.0001	9.77	-20	-6	-16
	R post insula/superior temporal G	422	<0.0001	4.72	66	-12	8
	R Amy, hippocamp	356	<0.0001	4.68	22	-12	-12
	L insula	276	<0.0001	4.62	-32	0	10
	L Fusiform C	182	0.00056	4.69	-34	-56	-18
Pat>HC	**none**						
HC>Pat	**R Postcentral G**	**205**	**0.000235**	**4.68**	**10**	**-42**	**58**
	L Postcentral G	**188**	**0.000445**	**4.28**	**-10**	**-36**	**54**
	Post Cingulate	**118**	**0.00779**	**4.59**	**-8**	**-10**	**50**
MALES- Negative coupling with LEFT amygdala							
Pat	R Precuneus	136	0.00357	3.96	8	-76	44
	Ant Cingulate	85	0.0361	4.08	6	26	22
HC	None						

Abbreviations: R: Right, L: Left, Amy: Amygdala, hippocamp: hippocampus, ant: anterior, post: posterior, C: cortex, G: gyrus, Cerebell: Cerebellum

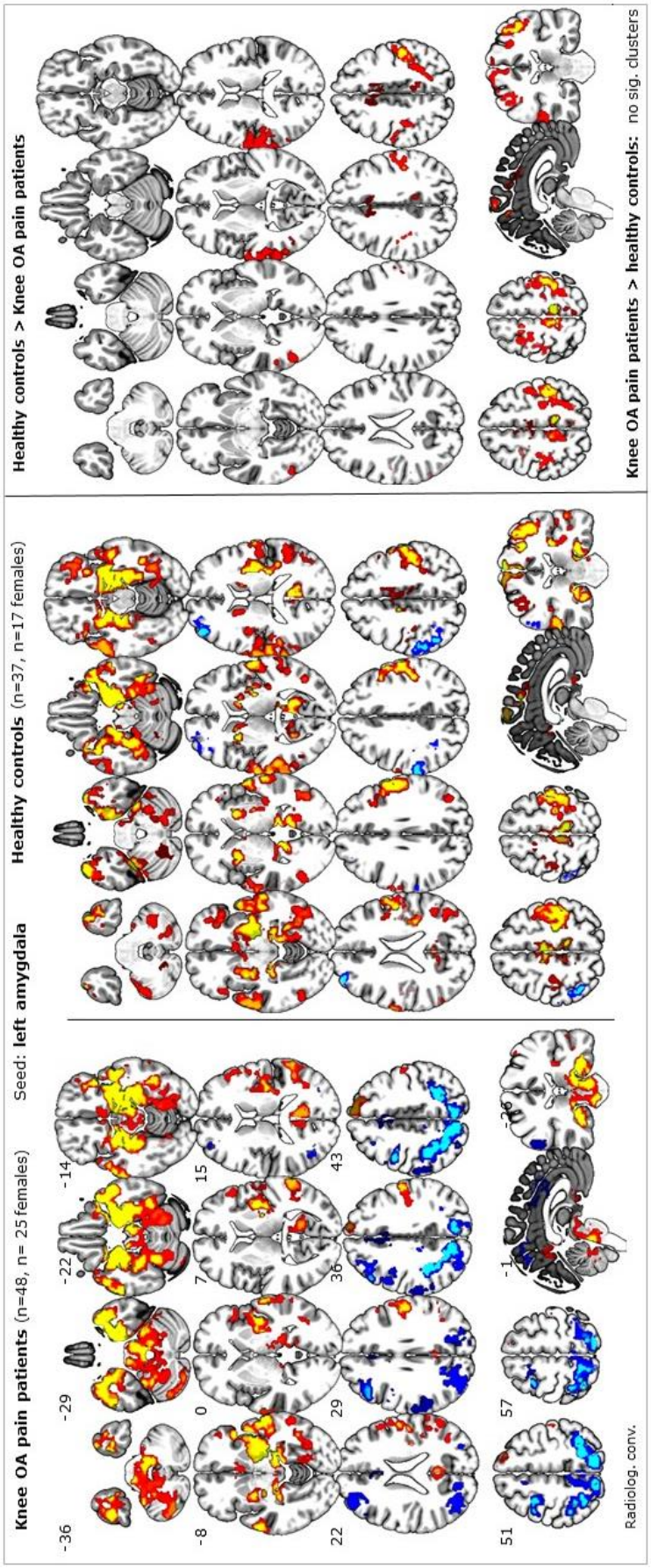


Figure 7: Left amygdala networks in pain patients and controls

This figure shows functional networks in patients (left) and in controls (middle) with the left amygdala as seed region. Clusters that positively coupled with the left amygdala are shown in yellow (for z > 3.1) and in red (for z >2.3) while clusters that negatively coupled with this seed are shown in cyan (for z >3.1) or blue (for z>2.3).

This right part of this figure shows group differences in functional networks of the left amygdala when comparing patients and controls. It shows several regions that are more positively coupled with the left amygdala in healthy controls than in pain patients or conversely less negatively coupled in pain patients than in controls (yellow: z> 3.1, red: z>2.3). There were no significant clusters for the reverse contrast.

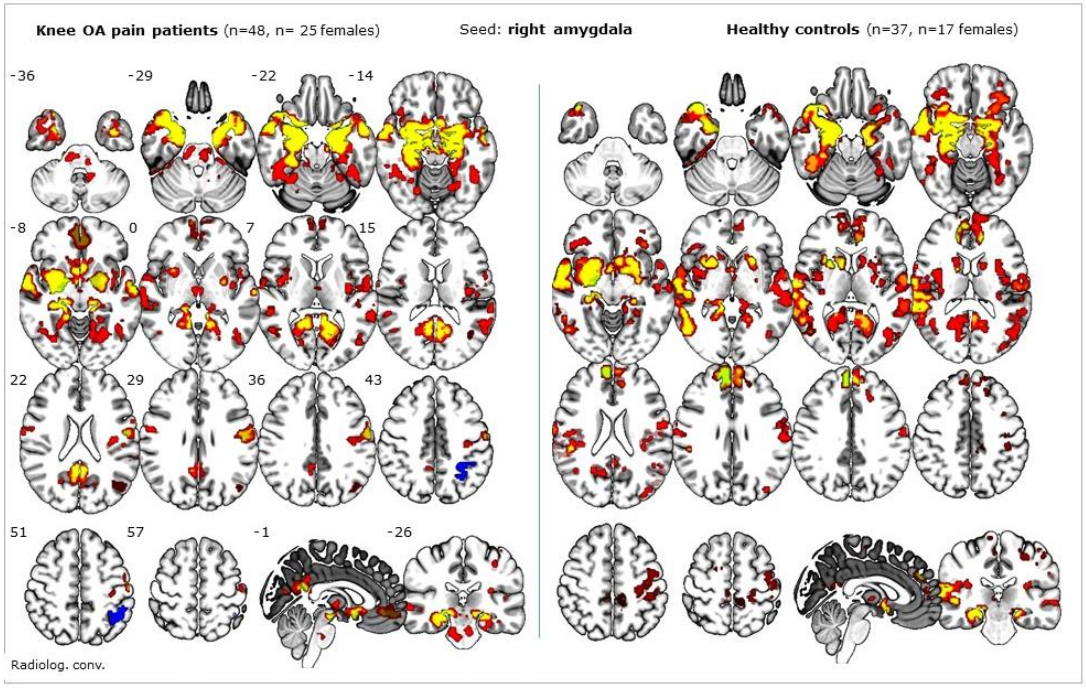


Figure 8: Right amygdala networks in pain patients and controls

This figure shows functional networks in patients (left) and in controls (right) with the right amygdala as seed region. Clusters that positively coupled with the right amygdala are shown in yellow (for z > 3.1) and in red (for z >2.3) while clusters that negatively coupled with this seed are shown in cyan (for z >3.1) or blue (for z>2.3).

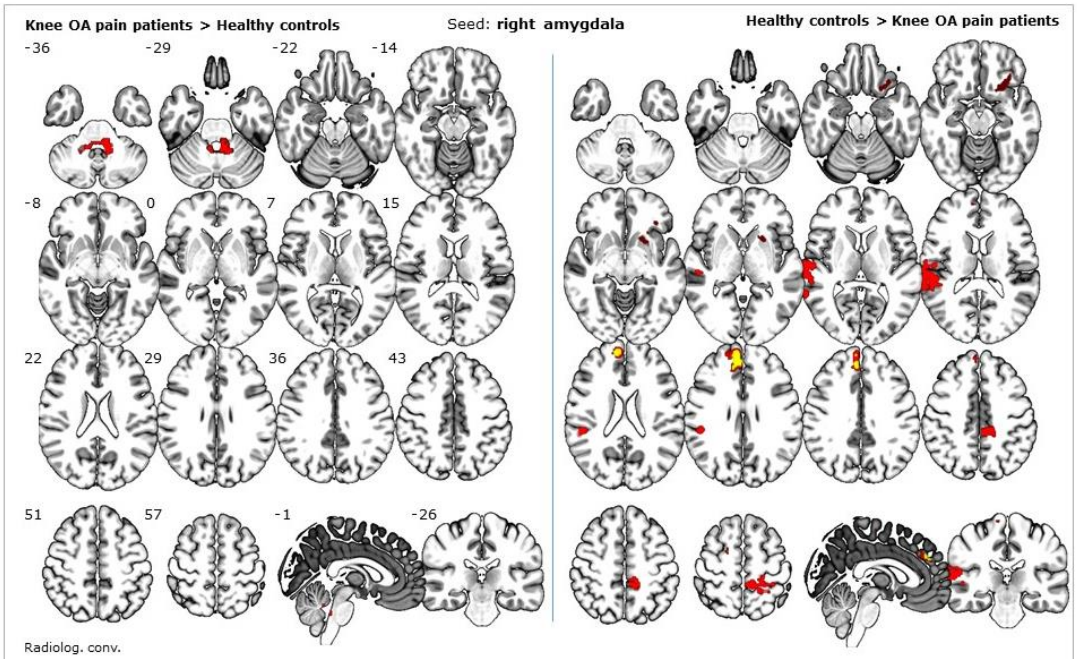


Figure 9: Right amygdala networks group differences

This figure shows group differences in functional networks of the right amygdala when comparing patients and controls. The left figure shows that a cluster in the brainstem is more positively coupled with the right amygdala in pain patients than in controls or conversely less negatively coupled in pain patients than in controls but only when examining networks at 3.1 < z >2.3. The right figure shows clusters that are more positively coupled with the right amygdala in healthy controls than in pain patients or conversely less negatively coupled in pain patients than in controls. Yellow: z> 3.1, red: z>2.3.

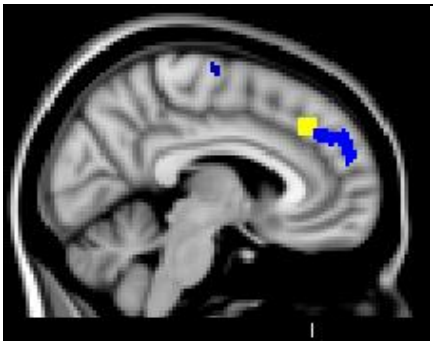


Figure 10: Comparison of dmPFC clusters from current and previous work

Blue: significant cluster in current work for right amygdala seed in HC > Patients, Yellow: sphere centred on coordinates from a study that links this cluster to "aversive amplification" by Robinson et al., 2012.
This figure shows that both clusters are immediate neighbours although not overlapping.

Are any pain-related alterations to the amygdala connectome sex-specific?

Subgroup analyses showed that the networks for female healthy controls and patients are similar to the main findings but also have distinct features. These analyses showed that positive coupling between left amygdalae and left insula was more pronounced in female patients than in controls or in males although no comparisons between sexes were undertaken (figures 11-15). This cluster was also seen in the main findings but was not significant there. Interestingly, comparisons between these networks from females suggest that the dmPFC cluster that appeared significant in the main group comparisons might have been biased by the female healthy control group because this cluster was not part of female patients' right amygdala networks; however, laterality effects were not formally tested.

The male subgroup analysis does not hint at any amygdala-insula group difference (figures 14-15) but on the other hand may have biased main findings regarding the postcentral gyrus in both amygdala networks because male healthy controls but not patients had significantly more positive coupling with this area in both amygdala networks; this was not seen in females but a statistical comparison between sexes was not done.

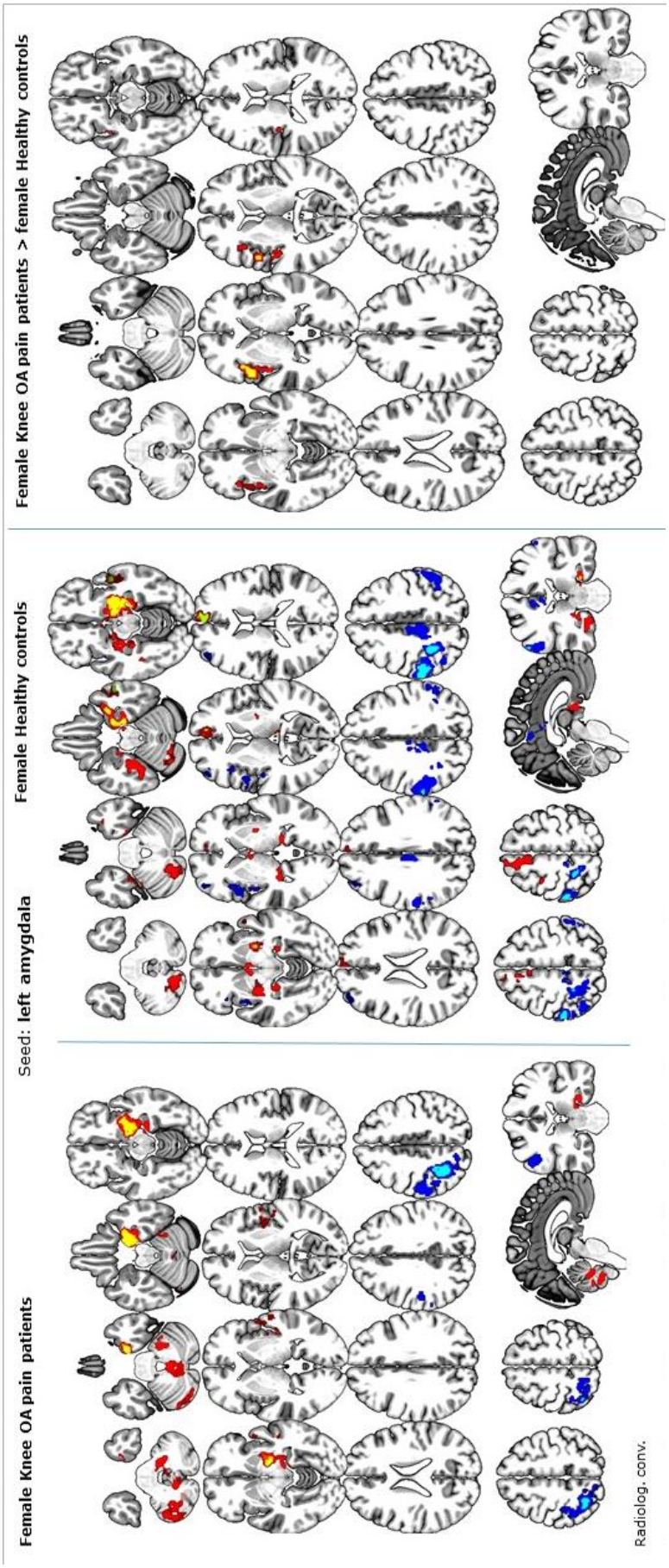


Figure 11: Left amygdala networks in female pain patients and controls

This figure shows functional networks in female patients (left) and in female controls (middle) with the left amygdala as seed region. Clusters that positively coupled with the left amygdala are shown in yellow (for z > 3.1) and in red (for z >2.3) while clusters that negatively coupled with this seed are shown in cyan (for z >3.1) or blue (for z>2.3).

The figure on the right shows group differences in functional networks of the left amygdala when comparing female patients and female controls. A cluster in the right insula is more positively coupled with the left amygdala in female pain patients than in controls or conversely less negatively coupled in pain patients than in controls. The reverse contrast did not yield any significant clusters. Yellow: z> 3.1, red: z>2.3.

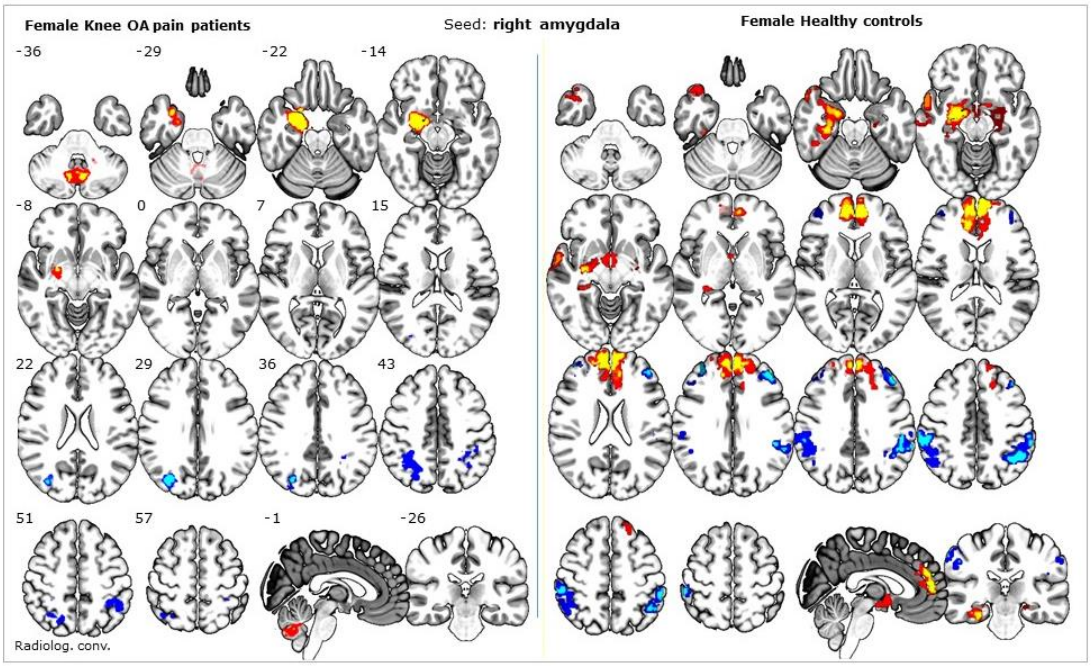


Figure 12: Right amygdala networks in female pain patients and controls

This figure shows functional networks in female patients (left) and in female controls (right) with the right amygdala as seed region. Clusters that positively coupled with the right amygdala are shown in yellow (for z > 3.1) and in red (for z >2.3) while clusters that negatively coupled with this seed are shown in cyan (for z >3.1) or blue (for z>2.3).

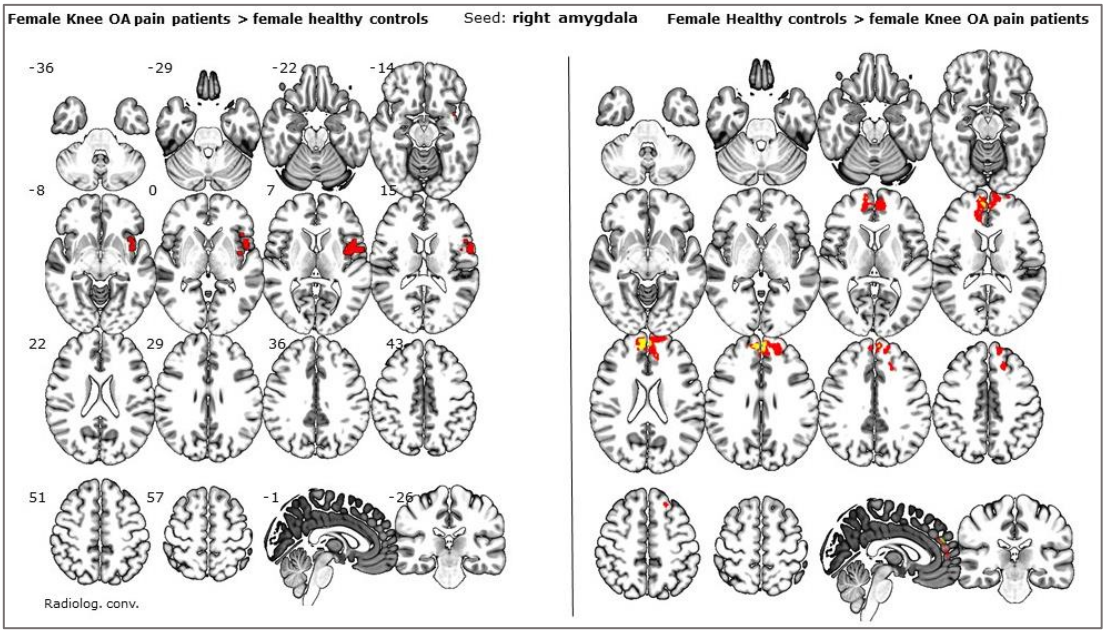


Figure 13: Right amygdala networks group differences for females

This figure shows group differences in functional networks of the right amygdala when comparing female patients and controls. The left figure shows that a cluster at a z-threshold of 2.3 in the right insula that more positively coupled with the right amygdala in pain patients than in controls or conversely less negatively coupled in pain patients than in controls. The right figure shows a cluster in the mPFC/dmPFC that is more positively coupled with the right amygdala in female healthy controls than in pain patients or conversely less negatively coupled in pain

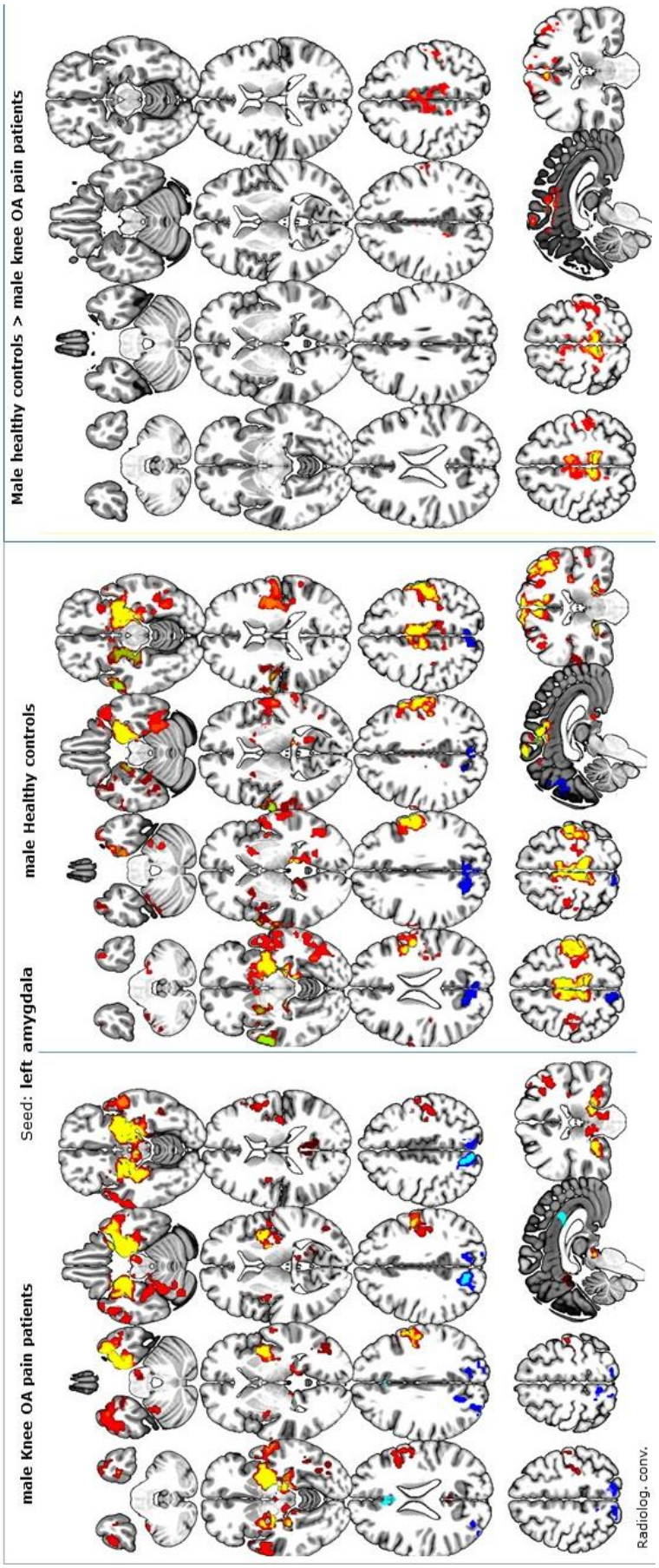


Figure 14: Left amygdala networks in male pain patients and controls

This figure shows functional networks in male patients (left) and in male controls (right) with the left amygdala as seed region. Clusters that positively coupled with the left amygdala are shown in yellow (for z > 3.1) and in red (for z >2.3) while clusters that negatively coupled with this seed are shown in cyan (for z >3.1) or blue (for z >2.3).

The figure on the right shows group differences in functional networks of the left amygdala when comparing male patients and male controls. The postcentral gyrus and posterior cingulate are more positively coupled with the left amygdala in male pain patients than in controls or conversely less negatively coupled in pain patients than in controls. The reverse contrast did not yield any significant clusters. Yellow: z> 3.1, red: z>2.3.

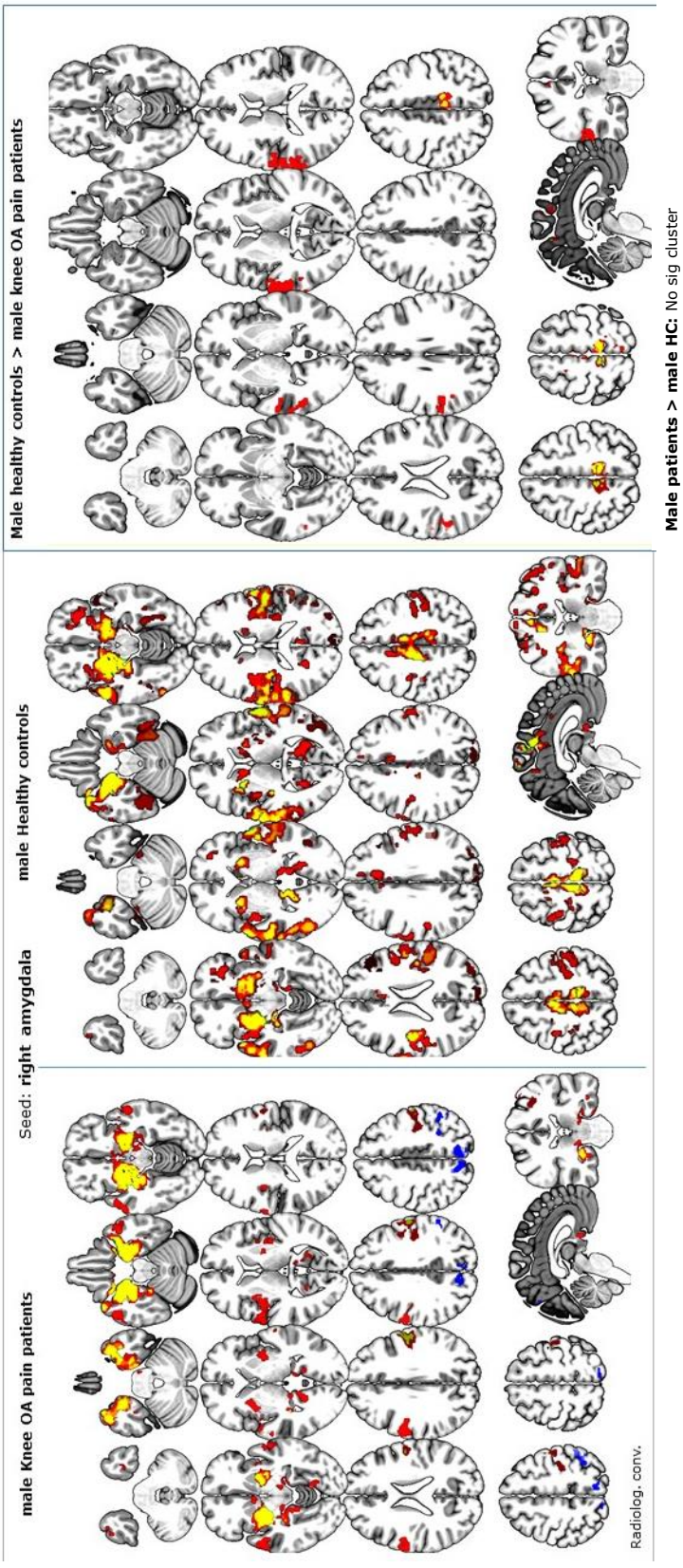


Figure 15: Right amygdala networks in male pain patients and controls

This figure shows functional networks in male patients (left) and in male controls (middle) with the right amygdala as seed region. Clusters that positively coupled with the right amygdala are shown in yellow (for z > 3.1) and in red (for z >2.3) while clusters that negatively coupled with this seed are shown in cyan (for z >3.1) or blue (for z>2.3). The figure on the right shows group differences in functional networks of the right amygdala when comparing male patients and male controls. Clusters postcentral gyrus and posterior cingulate are more positively coupled with the left amygdala in male pain patients than in controls or conversely less negatively coupled in pain patients than in controls. The reverse contrast did not yield any significant clusters. Yellow: z> 3.1, red: z>2.3.

5.4 Discussion

This study's primary aim was to investigate whether there are alterations in amygdala functional networks in chronic pain patients relative to controls by analysing resting-state seed based functional connectivity whilst critically evaluating the influence of different amygdala masks. The following will discuss the findings in relation to each research question.

5.4.1 Are findings from seed-based connectivity analyses using different amygdala masks comparable?

It became clear that, whilst the Harvard-Oxford atlas based and Juehlich atlas based amygdala masks visually differ in that the Harvard-Oxford atlas derived mask included white matter and substantial parts of the hippocampus, the networks based on these seed-regions were surprisingly comparable when applying a 5mm smoothing kernel with the exception of the network based on the centromedial amygdala subregion mask from Juehlich atlas. The latter mask contains large parts of the putamen, has a maximal probability for any voxel in this mask of ca 60%, and is visually not comparable to the other masks. Hence a lack of comparability between a network based on this specific amygdala mask relative to findings based on the other amygdala masks is expected. Due to concerns on the anatomical precision of this mask (see also chapter 4), it was decided not to pursue networks on amygdala subnuclei.

This suggests that functional connectivity results based on subdivisions of the amygdala as seeds may need to be interpreted carefully. Yet, comparison to literature that used Juehlich atlas' laterobasal amygdala masks as for instance in seminal work by Roy et al. 2009 may be possible without vast discrepancies (when using 5mm FWHM spatial smoothing kernels) even though the main group comparisons and sub-analyses in this chapter were performed with the more precise Harvard-Oxford amygdala masks.

It should be noted that the following focuses on clusters that were significant at $z > 3.1$ threshold because the former default ($z>2.3$) is no longer deemed sufficient by FSL developers to control type I errors but wherever a brain area,

which is by consensus in the field integral to pain processing (e.g., insula), appeared as significant cluster at the lower but not the most recent threshold this will be pointed out.

5.4.2 Is the amygdala connectome altered in chronic knee OA pain?

Overview of amygdala connectome

Generally, both amygdala networks in healthy controls and in patients were extensive and covered most regions that were reported in amygdala functional connectivity analyses before: similar to (Roy et al., 2009) we found positive coupling between either amygdala and the prefrontal areas, insulae, but also fusiform gyrus, and postcentral gyri in controls, while negative coupling was observed in the left amygdala network regarding mostly the precuneus similar to (Roy et al., 2009); a comprehensive list of all significant brain regions found in the current analyses is included in tables 4 and 5.

Whilst it is known that some results in brain maps may be spurious, especially if clusters are small, all significant results have been reported in the current work and without filtering according to networks in order not to preclude any future discussions or potentially introduce type II errors in coordinate based meta-analyses. It was also decided not to apply any cluster size threshold on the results because the amygdala itself as a small brain region illustrates that the size of a cluster may not necessarily be the best criterion to judge a cluster's relevance, especially when thresholds for cluster size are chosen semi-arbitrary; however, none of the clusters in the current results are below commonly applied arbitrary cluster size thresholds (i.e., <20 voxels). Furthermore, as the discussion on the amygdala- postcentral gyrus below exemplifies, it is possible that further empirical studies reveal additional mechanisms involving brain regions that have not received much attention so far and thus future work may focus on brain regions that are part of the current results but not discussed in the current work. The following discussion focuses on selected brain regions whose connectivity with the amygdala in the context

of chronic pain is considered particularly interesting based on currently available literature before elaborating on subanalyses and sex differences.

Contrary to Roy et al., we did not find any cluster in the thalamus for either amygdala network; also the cerebellum that had positive coupling between the left and right laterobasal amygdalae in Roy et al., was only found in the current analyses in the patient group. We also found positive coupling between amygdalae and the dmPFC which was very close to a cluster that displayed negative coupling in Roy et al.'s report and was inaccurately labelled anterior cingulate in the latter report (coordinates in Roy et al.: -2 30 36).
Roy et al. also report the vmPFC in relation to the amygdala which was intrinsically mostly excluded from the current analyses due to signal drop out but labelled these clusters as anterior cingulate or middle frontal gyrus (coordinates: 2 42 -16 and 0 34 -18), instead there were tentative clusters in the current work in subgroup analyses pointing at the mPFC which will be discussed in a separate section below. Connections to the hippocampi, reported in Roy et al. were also found in the current analyses but not considered a separate finding because of the proximity to the seed region. It needs to be stated, however, that Roy et al., used bigger and less precise amygdala masks as well as a slightly larger smoothing kernel and global demeaning and thus some discrepancies are expected.
Overlap in brain regions is also found in the report by Pagliacchio et al. on amygdala functional connectivity in a large paediatric-adolescent sample although the latter study, too, reported more brain areas that had negative coupling with the amygdalae (Pagliaccio et al., 2015).

The corresponding networks in healthy controls included parts of the paracingulate, postcentral gyri, putamina, but almost no negative coupling in the precuneus or any other region. The right amygdala network in patients showed less positive coupling with a very small cluster in the precuneus for negative coupling compared to the network with left amygdala seed in this group. On the other hand the right amygdala network had significantly more

positive coupling with the dmPFC in healthy controls than in patients; only at reduced thresholds there was another significant group difference for this contrast pointing at the right superior temporal gyrus but as reduced thresholds should not be used and this region was not hypothesized it is not further discussed here.

Amygdalae-postcentral gyri

The surprise finding of this report relates to the postcentral gyri which was significantly more positively coupled to the amygdalae in healthy controls than in patients and thus hypoconnected in the latter. Recently a direct anatomical connection between these brain areas was found in a diffusion tensor imaging study on emotional stimuli (Grezes, Valabregue, Gholipour, & Chevallier, 2014) which suggests the finding is plausible even if unexpected but the direction and laterality of coupling is at odds with previous reports: for instance a study found that PTSD patients had increased positive functional connectivity between the right amygdala and left postcentral gyrus during fearful compared to neutral facial stimuli (Stevens et al., 2013). However the same study also showed that PTSD patients had lower responsivity in the latter gyrus (Stevens et al., 2013) which suggests that its activity might help the amygdala to differentiate safety and threat cue and the valence might determine coupling direction. Related to this, an extinction study in healthy volunteers showed the right postcentral gyrus to be involved in the safety learning process when it was more active in the CS- (safe) relative to the CS+ (aversive) cues at the beginning of the extinction phase compared to late extinction phase (Morriss et al., 2015). This observation replicated in the same study for the left postcentral gyrus (amongst other regions in the same cluster) with a dependence on the individual's intolerance of uncertainty level (Morriss et al., 2015) which is most relevant to the current work because extinction deficits are hypothesized to contribute to pain chronification.

In contrast, a large combined genetic and functional connectivity study in a paediatric sample found less negative connections between the left amygdala and the left postcentral gyrus during resting state to be linked to increased risk

factors for depression, anxiety, PTSD, and HPA-axis malfunction as well as reduced emotion regulation abilities in adolescents (Pagliaccio et al., 2015). Together this suggests that left amygdala-left postcentral gyrus connectivity has potentially an important role in extinction mechanisms and in chronic pain development although coupling direction and potentially also laterality are details that require further investigation; the current data set showed significantly reduced coupling between the left amygdala and the left postcentral gyrus but at a lower significance threshold the right amygdala network also displayed a cluster in this area. It is plausible that coupling direction and laterality may determine the nature of the mechanism being maladaptive or compensatory but the paucity of reports on this gyrus makes conclusions challenging because the postcentral gyri, even when part of findings in some reports, received no attention with the exception of (Pagliaccio et al., 2015). Indeed, this gyrus also appeared in other chronic pain FC studies on migraine patients (Chen et al., 2017; Chong et al., 2017; Hadjikhani et al., 2013) and was activated during electric shocks alongside the amygdala and other brain areas (Gold, Morey, & McCarthy, 2015) but were not further discussed apart from briefly and inaccurately referencing three reports on experimental pain on several brain areas (i.e., only one cited report showed a small cluster in the postcentral gyrus but did not discuss it).

The mechanism through which the postcentral gyrus is linked to chronic pain may not be as straightforward as the genetic study suggests when linking it with vulnerability to anxiety disorders because comparisons in the current study between patients split into high and low scores on this measure have not revealed these gyri in relation to the amygdala. As mentioned before, such an absence may be a type II error because a complete reduction of amygdala networks is in disagreement with literature on anxiety disorders (Baur, Hanggi, Langer, & Jancke, 2013; Etkin, Prater, Schatzberg, Menon, & Greicius, 2009; Hahn et al., 2011) but remains to be investigated further. Independently, the present finding suggests that the link between amygdala-postcentral gyrus should be investigated further as it is potentially of clinical interest for chronic pain and in particular its progression

dmPFC –amygdala

The only other cluster that was significant at z> 3.1 for the group comparison lays in the dmPFC which was more positively coupled to the right amygdala in healthy controls than in patients. The directionality of this finding seems counterintuitive at first with claims that more positive right amygdala-dmPFC connections comprise an “aversive amplification” circuit (Robinson, Charney, Overstreet, Vytal, & Grillon, 2012) and may be the “switch” for clinical anxiety (Robinson et al., 2014b). Yet the initial reports from which these terms originate did not consider the possibility that in a healthy population enhanced connectivity between these two regions during threat cues can be a compensatory rather than maladaptive mechanism. The claim that this circuit creates ‘negative bias’ (Robinson et al., 2013) is not entirely comprehensible when subjects were merely asked during fMRI to classify facial stimuli as fearful or happy but showed indifferent reaction times. Nevertheless this interpretation has been used in four reports by the same first author (Robinson et al., 2012; Robinson et al., 2014a, 2014b; Robinson et al., 2013) and cited by similar studies.

A compensatory mechanism would also be in line with the current findings that this region was also more positively coupled with the right amygdala in highly uncertainty intolerant and highly stressed pain patients compared to mildly uncertainty intolerant and mildly stressed pain patients in the current study even though the lack of this cluster in the latter group was not significant at statistical group comparison. It should be stated, though, that the dmPFC cluster from the current work is very close but does not overlap with the mask created by coordinates from Robinson et al., 2012 and hence discrepancies could arise if there is functional sub-parcellation of this brain area.

This particular coupling has been observed to be permanently enhanced in anxiety disorders, which is reviewed by Robinson et al. (2014b) however this does not preclude the possibility that it may serve an compensatory mechanism that is not sufficiently efficient to tackle pathology but attempts to alleviate it until the circuit fatigues. Thus it would be necessary to investigate

this coupling further in anxiety disorders at different stages in order to judge whether it should be up- or downregulated for treatment optimization.

It is interesting to note that the same dmPFC cluster, although larger than in the current study, also linked in the aforementioned genetic-functional connectivity study (on vulnerability for depression and stress disorders) with the left amygdala seed (Pagliaccio et al., 2015) which suggests that the mechanism of this circuit may be further complicated by a laterality effect similar to the discussion about the postcentral gyrus. Independent of this important detail, these studies underline a focus on this circuit in aversive settings which certainly includes chronic pain. This is corroborated by a study that found positive correlations between amygdala-dmPFC FC and inflammatory stress responses (Muscatell et al., 2015).

Given that the amygdala-dmPFC circuit is serotonergic (Robinson et al., 2013) there is immediate clinical motivation to investigate this further as already one week of antidepressants can reduce amygdala-dmPFC coupling in healthy volunteers (McCabe et al., 2011). This motivation is further enhanced as the present cluster is in close proximity to what has been referred to as the "dorsal nexus" in some reports which was found to be a key link between the primary three resting state networks and depression (Sheline, Price, Yan, & Mintun, 2010). Overall, the present results suggest that the role of this cluster in chronic pain should be investigated further.

Amygdalae-Insulae

Our data sets showed that the insulae formed part of both the healthy as well as the pain patients' amygdala networks although each network was characterized by visually distinct parts of the insulae at reduced significance thresholds which was not accompanied by a statistically significant group difference. This could relate to a power issue for the presence of sub-phenotypes in the data set and hence should be followed up in further work.

5.4.2 Are any pain-related alterations to the amygdala connectome sex-specific?

Amygdala laterality and sex differences

Lastly, it should be highlighted that results were largely linked to the right rather than the left amygdala.

The discussion here is not oblivious to the possibility of phase encoding direction artefacts with regards to amygdala laterality as suggested in (Mathiak, Zvyagintsev, Ackermann, & Mathiak, 2012) but given that the current study's scanning protocol implemented orthogonal acquisitions laterality effects are not biased by this artefact and need to be voiced in relation to chronic pain. Indeed, there is literature to substantiate such a motivation: Animal studies have shown a "dominant role of the right amygdala" (Simons, Moulton, et al., 2014); specifically, it has been shown that only neurons in parts of the right but not the left amygdala "develop increased responsiveness" in an arthritis pain model (Ji & Neugebauer, 2009). Similarly a key protein in pain has been shown in another animal study to modulate inflammation-induced hypersensitivity only through the right amygdala (Carrasquillo & Gereau, 2008) and it has been suggested that there is a right hemisphere bias for the serotonin system (Arato et al., 1991; Fitzgerald, 2012) (for animal studies see also Andersen & Teicher, 1999; Bradbury, Costall, Domeney, & Naylor, 1985). In this context it should be highlighted that depression frequently co-occurs with chronic pain and nowadays also the use of antidepressants as off-label treatment of chronic pain per se rather than co-morbid depression is pursued both in primary care and further clinical trials (for review on efficacy see (Khouzam, 2016)).

Interesting is also that a resting-state on untreated depressed individuals found more amygdala activity compared to controls in the left hemisphere but only a trend in the same direction for the right amygdala (Peluso et al., 2009). Detailed discussion of depression fMRI studies are beyond the scope of the current chapter and hence this brief reference serves to raise awareness for

the possibility that amygdala laterality could play an important aspect in understanding disease progression.

Similarly, a previous amygdala-seed functional connectivity study in migraine patients linked the left but not right amygdala to pain progression (by comparing networks of chronic and episodic migraine sufferers and healthy controls)(Chen et al., 2017). This begs the question how amygdala laterality is linked with pain chronification because the only report discussing it in human pain is (Chen et al., 2017) which seems to conform to human depression literature but conflict with findings from with animal work.

Concerns about translatability could serve as generic explanations on discrepancies but they do not convince when considering further literature relevant to the topic. Such literature showed for instance that the right amygdala's activity correlated with extinction measures (Morriss et al., 2015) and represented anxious arousal in ex-warriors with PTSD (Pietrzak et al., 2015), or its volume to be linked with childhood trauma PTSD (Veer et al., 2015), and similarly, influence of fear conditioning was stronger on the right than the left amygdala (Baker & Kim, 2004). Similar to pain literature, though, there are also seemingly conflicting reports that suggest a focus on the left amygdala in PTSD (Starcevic et al., 2014), or in PTSD to unpredictable (vs. predictable) threat (Dretsch et al., 2016), left amygdala volume in childhood anxiety (but not right amygdala volume)(Qin et al., 2014), or in a study on spider phobia that showed patients could overcome their fear when memory reconsolidation was disrupted in the left amygdala (Bjorkstrand et al., 2016).

Additionally, there is bias for left amygdala activity in emotional processing in fMRI studies in general (Baas, Aleman, & Kahn, 2004) and in sympathetic arousal (Beissner, Meissner, Bar, & Napadow, 2013) yet an easy distinction, equalling left amygdala with negativity, does not hold because for instance electric stimulation of the left amygdala was shown to induce "pleasant and unpleasant" emotions (Lanteaume et al., 2007). Hence it does not surprise that there are few theories on amygdala lateralization; even the meta-analysis on amygdala in studies on emotional processing (Baas et al., 2004) does not discuss rationales for the overall leftward bias. Theories on amygdala

lateralization can be summarized with amygdala laterality to be linked with temporal dynamics of stimuli (Sergerie, Chochol, & Armony, 2008), conscious versus unconscious processing (Morris, Ohman, & Dolan, 1998), or language-related versus visual information (Markowitsch, 1998), however an explanation based on valence, or sex (Cahill, Uncapher, Kilpatrick, Alkire, & Turner, 2004; Schneider et al., 2011), or the combination of both (Killgore & Yurgelun-Todd, 2001), seems far more intuitive.

In this respect it is interesting to mention human conditioning studies that showed that memory regarding the same negative arousing pictures elicit stronger links with the right amygdala in men but the left amygdala in women with respect to negatively valenced stimuli (Cahill et al., 2004).

Interestingly, a laterality-sex interaction may also exist in the current data set because a cluster overlapping with the dmPFC from the main group comparisons of right amygdala networks was significant in subgroup comparison of female but not male patients against their healthy counterparts. In this respect it should be highlighted again that there is a considerably higher female prevalence for anxiety and stress disorders (Breslau, Davis, Andreski, Peterson, & Schultz, 1997), as it is known for chronic pain, although most people experience traumas and injuries in their lives that are sufficiently aversive to trigger PTSD and chronic pain yet only a fraction of exposed individuals develop this disorder (Frans et al., 2005) whereby exposure rates generally do not distinguish men and women. This suggests to link hypoconnectivity between the right amygdala and dmPFC to a lack of resilience which would support the suggestion of a compensatory mechanism that was discussed earlier in this manuscript; however, a logic-driven conclusion cannot substitute empirical data from future studies.

5.4.3 Conclusions and future outlook

Beyond enhancing our understanding of chronic pain processing and suggesting potential knowledge gaps in antidepressant treatment, the current work aids applications of Transcranial magnetic stimulation (TMS) which is being trialled for chronic pain treatment. Studies so far have applied TMS over

the motor cortex or the dlPFC- the current study suggests that the location with respect to prefrontal areas needs to be carefully chosen and interactions between different subparts of the prefrontal cortex should be studied as the effect of TMS is not restricted to the brain area directly underneath the coil; in particular the current work showed that the role of the dmPFC in pain progression may be important but is not understood given surprising findings in the current work and this brain area is likely to be affected as well if the dlPFC is stimulated through TMS.

5.4.4 Limitations

The discussion on resilience versus vulnerability should also critically involve the hippocampus to which an entire theory ("neurotoxicity hypothesis") was devoted in PTSD literature linking smaller hippocampi volume to increased risk (Gilbertson et al., 2002) and which, along with the amygdala and the vmPFC, is fundamentally involved in extinction mechanisms as outlined in the introduction chapter; however due to smoothing and anatomical proximity, as mentioned earlier, this region could not be separated from correlations with the seed region itself. Future amygdala FC studies are therefore advised to explore statistical approaches that do not depend on smoothing in order to investigate its relationship to the amygdala and their link to chronic pain. Such alternatives could not be pursued for both the lack of programming expertise and time. These endeavours would probably also benefit from higher spatial resolution through 7T. Equally, scan parameters should be adjusted for improved spatial resolution in the brainstem area. More importantly, though, future studies should make sure to avoid signal drop out in the vmPFC; even though data in the current study was not entirely compromised in this respect, a considerable proportion of this area was affected yet the vmPFC and especially the orbitofrontal cortex have so far been suggested in the literature to be the most important regulators of amygdala activity.

If hypotheses on the relationship between the amygdala and certain brain regions exist, future studies are also advised to complement resting-state FC analyses with task-related fMRI that is designed to disentangle different

interpretations, as for instance with the dmPFC. Similarly, interactions between the amygdala and vmPFC might be investigated best by the contrast between resting state and a threatening task. It needs to be highlighted that resting state for the current analysis was an advantageous choice because it allowed to study interactions without restrictions to a particular paradigm and allow to consider brain areas that were previously not hypothesized (i.e., postcentral gyrus) and that might otherwise have received marginal attention when shadowed by the amygdala-vmPFC connection for instance. The arguments pro resting state are however not oblivious to inherent problems with this task-free approach because their thoughts may vary widely from ruminating about ongoing worries to complete relaxation and it is rather common that participants get sleepy during such a scan, all of which produces considerable heterogeneity in brain activation.

Thirdly, as mentioned earlier, some obvious but insignificant group differences may have been due to a lack of power. This remains a limitation despite that the current sample size exceeds many published reports because it is not clear how many sub-phenotypes exist in OA; more than one phenotype is possible if chronic pain results from either weakened circuits for resilience or enhanced circuits that drive disease development or the (weighted) combination of both in addition to tissue pathology and other factors. This strengthens demands for maximal possible single-site acquired sample sizes for such analyses, ideally with equal numbers of healthy volunteers who are not only matched for age and sex but also trauma exposure, stoicism and other factors. Clearly, the number of factors make it a challenge to replicate studies but at least detailed sample descriptions allow to place findings in the respective context within aetiologies.

On the other hand, fMRI studies are also prone to type II errors. Of particular note to this point is the observation by Boubela et al. about amygdala fMRI studies (Boubela et al., 2015): in short, the amygdala's proximity to the basal vein of Rosenthal, which drains large parts of the medial temporal lobe, makes it likely that measured signal in the amygdala stems to some extent from

nearby regions like the insula, striatum, and especially the fusiform gyrus; an effect that is enhanced with larger smoothing kernels. As drastic as this limitation sounds, it is thought to apply to a minimal extent to the current data for three reasons: Firstly, the smoothing kernel in the current data set is smaller than those that Boubela and colleagues tested (Boubela et al.: 6-10mm; current data set: 5mm and cannot be further reduced for a pipeline that requires smoothing as the kernel needs to be bigger than voxel size: current voxel size: 3x3x3.5mm). Secondly, the region that Boubela et al., define as the amygdala for their analyses is substantially bigger than the thresholded Harvard-Oxford mask in the current analysis which would enhance a draining-vein effect relative to the current data set. Thirdly, as they have taken coordinates from meta-analyses as a basis their warning especially pertains to published and future reports where amygdala delineation is not precise. Connectivity to the fusiform gyrus and occipital lobe around the brainstem may however be affected by this limitation, hence findings on these regions need to be considered carefully.

Another important limitation relates to medication use of the current sample. In an attempt to study a representative sample of chronic pain only patients using opioids, having other severe health problems, or patients with MRI-safety concerns were excluded from the study and consequently the sample is heterogeneous with respect to antidepressant or pain killer use. As already indicated in the methods section, it is often difficult to diagnose depression independently of chronic pain and thus patients who tend to ruminate will most likely have differences in resting-state networks. Equally, patients who are content with their pain relief from medication will likely have differences in resting-state networks compared to patients who consciously or unconsciously fear sudden pain attacks.

In this respect it is remarkable that some of the current findings were statistically significant despite of this mix as subgroups on this basis are conceivable which should be followed up with another study on a larger sample size and more advanced analyses in order to avoid excessive amounts

of tests (e.g., by discriminant analysis). The current study was an important step towards this goal as the hypothesis-free and non-exclusive study of default amygdala networks raises awareness for brain areas that may otherwise not be noted amongst other results.

Another limitation pertains to including the eight data sets that were acquired while participants were asked to have their eyes closed as opposed to the rest of the sample that was acquired with participants fixating on a cross hair. Resting-state fMRI studies that investigated whether eyes closed or open conditions affect brain activity differently have found very small but significant condition effects, mostly in the visual- motor somatosensory, and auditory areas which were more active during eyes closed-resting state (Marx et al., 2004). Another study showed that replicability of resting state networks was better if the participants had their eyes fixated on a cross or, in the case of visual areas, their eyes open without focusing on a particular spot (Patriat et al., 2013). Similarly, a related study showed that cerebral blood flow was higher when participants had their eyes open (Zou et al., 2015). This suggests that results may have been confounded by mixing these two resting states but it is thought that this confounder only marginally affects the results herein because visual, somatosensory, and auditory areas are not focused on in relation to the amygdala. Conversely, if eyes-open resting states networks are indeed more replicable then the current mix with a majority of eyes-open resting state data sets likely increased chances for type II errors, lowered effect sizes and significance levels.

It is thought that the decision to include eyes closed resting state data was a better compromise than to perform analyses on a data set that is heavily skewed towards one sex in the healthy controls. The alternative, to select a smaller but balanced data set pseudo-randomly from the entire eyes open resting state sample data set, is deemed to be worse both for power and for ethical reasons. Similarly, including resting state data from a different database and hence collected at multiple sites, is thought to be a worse compromise as scanner effects in particular to the amygdala region are conceivable. In

summary, the limitation from mixing two different resting states into the current analysis is valid but should not cast concerns over current results.

Lastly, another limitation concerns sample size and power. Whilst the current work is based on a sample that exceeds commonly published sample sizes on this type of analysis, an a-priori sample size calculation was not performed as this is a pilot study for further analyses, using all available single-site data. In this respect, a crude exemplary sample size calculation was performed on the right Amygdala-dmPFC connection of the current results as this was deemed particularly relevant for further investigation: based on extracted mean z-scores for the dmPFC cluster, an online calculator recommends n=152 data sets for each sample for 80% power[4] whilst the current data set had n=85 and thus to some degree this may mirror the 'replicability crisis' in neuroimaging literature as results are usually based on small sample sizes. It needs to be highlighted, though, that is unclear how informative such a crude sample size calculation is as it is based on an average z-score. More importantly, the calculation ignores any multiple test corrections and temporal autocorrelations which are especially relevant for functional connectivity studies and thus prohibits the use of fmripower and NeuroPower tools software. Whilst these two software are increasingly in use for sample size estimations of task related fMRI data to provide more accurate estimations, there is to date no consensus for connectivity fMRI studies. A recent paper, however, suggested a statistical approach for brain-wide connexel studies (Gong et al., 2018). As this approach is still recent, caution has to accompany discussions but it is possible that such methodology becomes a new standard in neuroimaging. Considering the paucity of literature on specific amygdala network alterations in chronic pain, however, the current work is an exploratory pilot study that aimed to identify connections for further

[4] This was calculated with the online calculator on https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html. Mean z-scores were extracted from the dmPFC with a mask centered on 6, 38,38 with 5mm sphere.

investigation, and thus type II errors are deemed to be more detrimental than type I errors.

5.4.5 Conclusion

Amygdalae networks appear to be distinct in healthy and chronic pain populations, visually with regards to connectivity to the brainstem and less so to the insula, and significantly with regards to connectivity to the dmPFC which was linked in the literature with aversion processing and more pronounced in the female subsample, and to the postcentral gyrus which has received little attention in pain literature although sometimes present in group difference tables and was only discussed in one genetic-rsfMRI study linking it to vulnerability for anxiety disorders and depression (Pagliaccio et al., 2015). The present results suggest that the common interpretation to associate coupling between amygdala-dmPFC as maladaptive (Robinson et al., 2012, 2013, 2014) rather than compensatory should be challenged and tested empirically with a more suitable paradigm. The results of the present work also make a case to investigate amygdala laterality further because significant results were found in right amygdala networks which incidentally conforms with the right hemispheric bias of the serotonin system (Fitzgerald, 2012) .

Chapter 6: Summary and outlook on future work

This thesis set out with the aim to evaluate the amygdala's role in human pain using MRI. Chapter 1 discussed the possible mechanisms how the amygdala may be involved in chronic pain, how it integrates several aspects of the biopsychosocial model of pain, and potential pitfalls of why the amygdala is not reported more frequently in human pain literature; that chapter concluded that the amygdala's role can only be evaluated properly with more studies that are carefully designed to avoid some of the constraints that affect amygdala activity more than other pain-relevant brain regions.

Consequently, study chapters 4 and 5 aimed at mitigating this gap to some extent by investigating group differences between healthy controls and pain patients in its gray matter density (in a region-of-interest analysis as well as the commonly used whole-brain vbm approach) and its functional networks (in seed-based connectivity analyses). Whilst chapter 4 demonstrated that gray matter density is not an adequate parameter for a sample where no major amygdala atrophy is expected, chapter 5 showed that amygdalae networks significantly differ between chronic pain patients and healthy controls. Specifically, decreased connectivity between right amygdala and dmPFC and between left amygdala and left postcentral gyrus was found in OA pain patients relative to healthy controls, whereby post-hoc analyses were suggestive of potential sex-effects. This chapter concluded that better understanding of altered connectivity between the amygdalae and these two brain regions may be important in understanding pain progression better: as discussed in chapter 5, the current findings contradict literature which has so far implicated the amygdala-dmPFC connection in 'aversive amplification' (Robinson et al., 2012); this could reflect that maladaptive and compensatory mechanisms of this connection have not been elucidated. Furthermore, this connection is serotonergic and was shown to be altered after only one week of antidepressant use (McCabe et al., 2011) and thus clarification of the current finding is pressing.

Similarly, the postcentral gyrus has not received much attention in human pain literature which should change given the current findings and given that this cluster was recently linked to genetic vulnerability for depression (Pagliaccio et al., 2015).

Thus, rather than providing an answer on the role of the amygdala in human pain, this thesis asks more questions, outlines amygdala functions relevant for pain progression to raise awareness for further investigations, outlines pitfalls, and suggests alternative avenues.

Selected future avenues

One such alternative avenue to amygdala morphometry is to study prevalence of extinction deficits in chronic pain patients. As described in chapter 4, there is likely a link between extinction deficits and amygdala plasticity but this insight is recent and has not been directly investigated. Thus, prevalence testing in humans could be complemented with animal work providing direct evidence for causality to strengthen (or downplay) current putative pain progression models with translational data.

Uncertainty was mentioned in this thesis as one mechanistic avenue of how the amygdala modulates pain. In particular the partial reinforced extinction effect (i.e. extinction paradigm with uncertainty in the form of inconsistency in pairing CS+ and CS-) has seen comparatively little attention in human studies as opposed to animal work, yet confirming the existence of such an effect would be highly relevant for a pain progression model based on extinction deficits.

Studies should also elucidate the amygdala's function in uncertainty further because a strong role of this structure in uncertainty has been reported in (Bornhovd et al., 2002) but for instance not in (Seidel et al., 2015), which may pertain to excessive smoothing or other methodological aspects but overall there are too few studies that investigated the amygdala in relation to uncertainty.

In parallel to outlining neural correlates of uncertainty and amygdala activity, practical solutions may need to be implemented to support patients to cope better with pain-related uncertainty; this could be as simple as providing the right piece of information at the right time. Management of uncertainty (and social factors) is not a comprehensive remedy because uncertainty regarding pain attacks and treatment prospects may still exist but it may be an adjuvant to CBT, stress reduction programmes, exposure therapy or pharmacotherapy, especially for moderate to highly uncertainty intolerant individuals. While there is a report that included patients' verbatim interview responses that clearly express uncertainty as a pressing problem, it is not known *to what degree* this impacts on the chronic pain population and which aspects of pain-related uncertainty are most bothersome.

Work in progress therefore focuses on development of a questionnaire to identify the scope and the need of such endeavours.

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