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STRUCTURAL BRAIN ALTERATIONS IN SPIDER PHOBIA

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List of Abbreviations

SANLM- Spatial-Adaptive Non-Local Means

AMAP- Adaptive Maximum A Posterior

ACC- Anterior Cingulate Cortex

APP- Affine Pre-Processing

ASI- Anxiety Sensitivity Index

BAI- Beck Anxiety Inventory

BAS- Brain Aversive System

BAT- Behavioral Avoidance Test

BDI- Beck Depression Inventory

BII- Blood-Injection Injury

CAT12- Computational Anatomy Toolbox 12

CBT- Cognitive Behavioral Therapy

CeA- Central Nucleus of Amygdala

CIDI- Composite International Diagnostic Interview

CS- Conditioned Stimulus

CT- Computed Tomography

FOV- Field of View

GM- Gray Matter

GMD- Gray Matter Density

ICD-10- International Statistical Classification of Diseases and Related Health Problems 10th Edition

LA- Lateral Nucleus of Amygdala

MPRAGE- Magnetization Prepared Rapid Acquisition Gradient Echo

MRI- Magnetic Resonance Imaging

MRS- Magnetic Resonance Spectroscopy

NPSR1- Neuropeptide S Receptor 1

OCD- Obsessive Compulsive Disorder

OFC- Orbitofrontal Cortex

PD- Panic Disorder

PTSD- Posttraumatic Stress Disorder

ROI- Region of Interest

SAD- Social Anxiety Disorder

SP- Specific Phobia

SPM12- Statistical Parametric Mapping 12

SPQ- Spider Phobia Questionnaire

SSRI- Selective Serotonin Reuptake Inhibitor

TE- Echo Time

TIV- Total Intracranial Volume

TR- Repetition Time

US- Unconditioned Stimulus

WHO WMH-CIDI- World Mental Health Composite International Diagnostic Interview

1 Introduction

1.1 A short history of Specific Phobia

Specific phobias are anxiety disorders characterized by unreasonable or irrational fear of a specific object or situation (APA, 2013; Battle, 2013). Whereas the term "specific phobia" has only been used for a relatively short time, the concept of phobias has been around for centuries (Errera, 1962). *The Hippocratic Corpus*, a collection of Greek medical texts attributed to Hippocrates (470-410 B.C.E), contains one of the first written accounts of phobia (Hippocrates, cited in Crocq, 2015):

"Nicanor's affection when he went to a drinking party, was fear (Φόβος) of the flute girl. Whenever he heard the voice of the flute begin to play at a symposium, masses of terrors rose up. He said that he could hardly bear it when it was night, but if he heard it in the daytime he was not affected. Such symptoms persisted over a long period of time".

The term "phobia" was first used by a Roman doctor Celsus about 500 years later, when he used the word *Hydrophobia* to describe a condition of a person who was afraid of water (Crocq, 2015; G. Korgeski, 2009). The term *Phobia* itself was acquired from a Greek god of war, Phobos, who would frighten his enemies to get them to give up in fights (G. Korgeski, 2009).

Phobias became a separate category of psychiatric disease in 1947, when they were first included in the International Classification of Disease (ICD) (G. P. Korgeski, 2009). Shortly after that, phobias were listed as a separate diagnosis in the Diagnostic and Statistical Manual of Mental disorders in 1952, too (APA, 1952). The latest edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-5) places SPs under the category of anxiety disorders (APA, 2013). Here, SPs are further divided animal type (fear of animals, insects), natural environment type (heights, water), blood-injection-injury type (BII phobia; blood, injections and medical procedures) and situational type (driving, flying, elevators) (Antony, Brown, & Barlow, 1997; APA, 2013).

1.2 The role and importance of SPs

SPs are often considered to be widespread mental health disorders, that have little impact on the well-being of a person- the distress brought on by SPs is only limited to situations where there is a contact with the phobic stimuli, other than that, the lives of the phobic patients are seemingly unaffected by the disorder (Becker et al., 2007). This is quite different from psychiatric illnesses such as schizophrenia or depression, where virtually all aspects of life are affected, including sleep, appetite, memory and ability to experience joy. However, the distress brought on by SP could be larger than we had first anticipated. Studies have actually shown that patients with SP report rates of disease severity, impairment and associated emotional stress that do not significantly differed from those reported in other mental disorders (Becker et al., 2007; Wittchen, Nelson, & Lachner, 1998). In addition to this, SPs are second most common mental health disorders in females, being topped only major depression (Alonso et al., 2004; Wittchen & Jacobi, 2005). The lifetime prevalence of SPs in females is reported to be around twice as high as that of males, with the gender gap increasing as the patients age (Alonso et al., 2004; Kessler, Petukhova, Sampson, Zaslavsky, & Wittchen, 2012; Regier, Narrow, & Rae, 1990; Wittchen & Jacobi, 2005). It has been estimated that in Germany, 12.8% of females aged 18-24 meet the criteria for a lifetime prevalence of at least one SP, with around 10% suffering from a SP at any point in time (Becker et al., 2007). Studies have also observed that there is little difference between lifetime prevalence and 12-month prevalence of SP, meaning that SPs have a long duration and do not subside easily (Becker et al., 2007). This observation is in line with a study discovering that patients with SP had the poorest rates of recovery out of all anxiety disorders (Last, Perrin, Hersen, & Kazdin, 1996). Additionally, females also experience the fear more intensely and give higher fear ratings for the phobic subjects or situations (Fredrikson, Annas, Fischer, & Wik, 1996).

Another reason to research SP is that patients with SP are likely to suffer from additional psychiatric comorbidities. The link between SPs and other anxiety disorders is the most obvious (Becker et al., 2007; Benjet, Borges, Stein, Mendez, & Medina-Mora, 2012; Brown, Campbell, Lehman, Grisham, & Mancill, 2001;

Regier, Rae, Narrow, Kaelber, & Schatzberg, 1998). It has been shown that women with specific phobia are twice as likely to develop other anxiety disorder (Trumpf, Margraf, Vriends, Meyer, & Becker, 2010). Major depression is another disorder frequently associated with SP- the lifetime prevalence of major depression in patients with SP is 40.7% (Choy, Fyer, & Goodwin, 2007). This is a contrast to general population, where the lifetime prevalence of major depression is around 10%. Studies investigating the impact of phobia on the onset of other mental disorders have discovered that SPs are also correlated with later onset of OCD and PTSD (Becker et al., 2007; Brown et al., 2001; Lieb et al., 2016; Regier et al., 1998). Looking at these and other studies in this field, it becomes clear that there is a positive correlation between SP and most other mental health disorders (Benjet et al., 2012; Brown et al., 2001; Regier et al., 1998; Trumpf et al., 2010).

In cases of comorbid depression, the onset of SP precedes the onset of depression for about 10 years (onset at 24.5 and 13.5 years, respectively) (Regier et al., 1998). In the case of panic disorder comorbidity, the SPs clearly tended to precede PD as well, often by many years (Starcevic & Bogojevic, 1997). This means that SPs can be an indicator of a higher risk of developing other mental health disorders later in life. The increased risk of other mental health disorders in phobic patients could be due to many factors. One of the explanations is that SPs shares a common underlying diathesis with other psychiatric disorders, which means that the research dedicated to SP could also help discover the pathophysiology of other mental health disorders.

1.3 The global burden of SP and anxiety disorders

Even though we could not find any studies directly addressing the health care costs and the financial burden of SPs, several studies have addressed the immerse burden of anxiety disorders on a population. Considering that SP is a common form of anxiety disorder, and that SP is often followed by other anxiety disorders, this chapter will shortly go over the burden of anxiety disorders. According to the Global Burden of Disease (GBD) study conducted in 2010,

anxiety disorders were the sixth leading cause of disability, in both high and low income countries (Baxter, Vos, Scott, Ferrari, & Whiteford, 2014). This is higher than the numbers for severe mental health disorders such as schizophrenia (ranked 18th at global level) (Baxter et al., 2014). In 2004, it was estimated that anxiety disorders cost an excess of 41 billion Euros for the European Union (Andlin-Sobocki, 2005). This same German study found that the additional costs associated with anxiety disorders were up to €1600 per patient in 2004 (Andlin-Sobocki, 2005).

The amount of days lost from work due to anxiety disorders are comparable to those of serious somatic disorders such as diabetes (Alonso et al., 2004). In year 2010, anxiety disorders were responsible for 390 YLDs (a measure for expressing years of life lived with disability) per 100 000 persons (272 YLDs per 100 000 for males and 509 YLDs for females), which accounted for a staggering number of 27 million YLDs overall. Regarding mental health disorders, this number was only topped by YLDs attributed to major depressive disorder. To illustrate the significance of this number, anxiety accounted for six times the YLDs of all cancers combined (Baxter et al., 2014).

1.4 Neural substrates of phobia and fear

Contrary to other anxiety disorders, there is no clear model of neuroanatomical mechanisms behind SP (Linares et al., 2012). Previously, it was believed that the limbic system (e.g. amygdala, hippocampus, thalamus, cingulate cortex) was responsible for all emotional responses and that neocortex had no role in emotions and was reserved to cognitive processes (LeDoux, 2000). However, with the help of further studies, this distinction broke down and we now know that both the neocortex and the limbic system play a role in processing emotions including fear (LeDoux, 2000).

One theory proposes that phobias are learned and acquired via mechanisms of fear conditioning. This idea was first suggested in 1920, when researchers observed that an infant could be taught to fear a neutral animal (conditioned

stimulus or CS) after it was paired with a frightening stimulus (unconditioned stimulus or US) (Fyer, 1998). Because specific phobias are also described as unrealistic fear of situations that are harmless, it could be suggested that SP is a type of fear conditioning. If this is the case, we would expect to find similar structures involved in both fear conditioning and phobia.

The mechanisms behind fear conditioning are relatively simple and well-studied (Phelps & LeDoux, 2005). The conditioned fear is mediated by the transmission of US and CS to amygdala via neuronal pathways. The CS reaches the lateral nucleus of amygdala (LA) passing through either thalamus or cortical structures (Romanski & LeDoux, 1993). The connections between amygdala and cortex seem to conduct the information more slowly than the thalamic pathways. Functional neuroimaging studies have also observed that in fear conditioning, the activity in amygdala correlates with that of thalamus and not the cortex, supporting the role of a direct pathway between thalamus and amygdala in fear conduction (Quirk, Repa, & LeDoux, 1995). The importance of amygdala itself in SP is also highly supported by fMRI studies finding abnormalities in the activation of amygdala in SP patients, which we will investigate in next chapters (Del Casale et al., 2012).

After the US and CS have been paired in the amygdala with the help of neuroplasticity, the presentation of CS alone is able to cause an activation in central nucleus of amygdala (CaA), that transmits the information to brainstem through outgoing amygdala projections. The brainstem is then responsible for initiating autonomic and endocrine fear responses.

In rats, CS-US coupling comes with a phenomenon called contextual fear, where rats not only learn to fear the CS, but also have a fear response to the surroundings where the coupling took place. For this to occur, connections between amygdala and hippocampus are required (Blanchard, Blanchard, & Fial, 1970).

Other structures that have been proven to take a part in fear responses are the bed nucleus of stria terminalis, the preaqueductal gray and the hippothalamus (LeDoux, Iwata, Cicchetti, & Reis, 1988).

However, there are certain basic differences between conditioned fear and phobias that suggests the involvement of mechanisms and structures other than the ones taking part in fear conditioning. Firstly, most people with SP do not recall a certain event that caused the phobia (Fyer, 1998). This could be explained by two separate pathways being involved in fear conduction: a short pathway that goes to thalamus to amygdala and a longer pathway that includes the sensory cortex (LeDoux, 2000). The cortical pathway is the one with connections of hippocampus and the ability to form explicit memories. So, if the conditioning occurs only via the subcortical pathway, it is possible that the patient will have no memory of the conditioning event (Fyer, 1998). Secondly, there is a small number of phobic objects that are responsible for most phobia cases. For example, fear of objects such as curtains or tables is unheard of, but the fear of spiders is extremely widespread. If CS-US coupling would be the only mechanism responsible for SP, we would expect the distribution of phobic objects to be more even. This phenomenon could be explained by a process called prepared learning, in which the conditioned stimulus has a biological significance that makes the human brain particularly responsive (Mineka & Ohman, 2002). Lastly, the laboratory fear conditioning is easy to extinguish, but phobias are difficult to treat (Dunlap & Stephens, 2014). One explanation for this could be the involvement of brain structures that make the fear difficult to extinguish. Studies in rats have found that damage to medial prefrontal cortex makes fear particularly resilient, so it might be possible that a part of developing phobias has to do with functional abnormalities in prefrontal cortex (Morgan, Romanski, & LeDoux, 1993). MPFC has also been proven to have a role in risk assessment and modulation of defensive behaviors, further suggesting that this structure could be relevant in SP (LeDoux, 2000).

1.5 Voxel-based morphometry

In history, research has relied on post-mortem studies to investigate the abnormalities of brain structure. Postmortem examinations of brain lesions in patients who had exhibited certain symptoms while still being alive provided information about the functions of lesioned regions. The introduction of structural neuroimaging methods such as computer tomography (CT) and magnetic resonance imaging (MRI) we now can visualize subtle changes in brain shape and size of living patients. This can not only be used in research, to confirm the relationship between brain alterations and cognitive deficits in living patients, but also in correctly diagnosing and finding the optimal treatments for our patients (Mayberg, 2014).

Several methods have been developed to analyze MRI images. The easiest method to assess MRI images is the analysis of total intracranial volume. However, this is an unspecific measurement, as total brain volume is not only affected by pathology, but factors such as age, gender and body mass.

A common method called ROI analysis has been developed for studying brain regions that can be defined clearly (e.g. hippocampus, amygdala, cingulate cortex). This method, however, has its flaws. Firstly, protocols for ROI analysis are usually individually developed, so it can be difficult to compare the results between individual studies. Secondly, there are many studies covering the structural differences in brain regions such as hippocampi, amygdala, thalamus or ventricles (Ashburner & Friston, 2000; Focke, Trost, Paulus, Falkai, & Gruber, 2014), but, because a large part of the brain consists of structures with no sharp boundaries, structural differences in other brain regions can be overlooked (Ashburner & Friston, 2000).

These flaws can be corrected by methods that analyze the whole brain. Whole brain techniques are divided into those that account for either macroscopic differences in brain shape or the local differences disregarding the macroscopic parameters (Mechelli, Price, Friston, & Ashburner, 2005). One of the methods of the latter group is Voxel-based morphometry (VBM). VBM enables a voxel-by-voxel whole brain volume and density estimation. The term `voxel` consists of the

two words “volumetric” and “pixel” and is used to describe the three-dimensional volume elements that together make up a whole MRI image. In this study, the size of a voxel was $1mm^2$. It must be noted, however, that this method does a lot better job analysis the density of gray than white matter (Kurth, 2015). The sensitivity of white matter analysis of this method is low, because white matter consists of large regions of homogenous tissue, with only small changes in density across regions (Kurth, 2015).

VBM analysis consists of 4 basic steps: (1) spatial normalization of all MRI images, (2) segmentation or tissue classification, (3) smoothing and (4) statistical analysis (Ashburner & Friston, 2000).

(1) To conduct a comparison between brains of many patients, voxels must have the same anatomical locations for all subjects. This is ensured by a process called spatial normalization. The goal of spatial normalization is not to match every brain feature between brains, but to correct for global differences in the brains of subjects so that local differences could be computed and statistically compared later on (Mechelli et al., 2005).

(2.) Tissue classification also called segmentation is conducted by extracting gray matter (GM), white matter (WM), and cerebrospinal fluid (CSF), and non-brain background classes, that are removed (Ashburner & Friston, 1997). This is done with the help of 2 methods: a priori probability maps that provide information of the probability that a certain brain region would contain a certain tissue class and analyzing the brightness of different regions to classify the tissue. This way, images are produced where each voxel is assigned a probability ranging from 0 to 1of it containing a particular tissue type (Mechelli et al., 2005). In addition, tissue classification also evens out the differences in image brightness caused by magnetic field inhomogeneity, called bias correction (Kurth, 2015).

(3) The segmented images are then smoothed using an isotopic Gaussian kernel. Despite spatial normalization, we still cannot be sure that the anatomical location of each voxel between patients is the same. Additionally, statistical tests conducted in step 4 assume a normal distribution, and blurring of the images

helps ensure a normal distribution. The amount of smoothing (Gaussian kernel) should be chosen based on the size of the regions that are expected to produce significant results (Ashburner & Friston, 2000; Mechelli et al., 2005).

(4) The last step, the voxel-wise statistical analysis allows conduction of variety of statistical tests such as group comparisons and correlations using the general linear model. Since the results of the statistical analysis are composed of many between-voxel tests, these results are automatically corrected for multiple comparisons.

1.6 Regions with altered brain activity in SP

fMRI is the preferred method of studying functional abnormalities in psychiatric disorders, mostly because it provides a high temporal solution, it is cost-efficient, and with this method, the patients are not exposed to radioisotopes (Linares et al., 2012). In 2012, there were 36 original functional neuroimaging studies on specific phobia, that enrolled a total of 506 patients (Del Casale et al., 2012). Most of these studies (66%) were conducted using fMRI, but other functional neuroimaging methods were used as well: 5.7% of all studies used SPECT and 28.6% used PET (Del Casale et al., 2012). A metanalysis conducted in 2012 found that altered activation in patients with specific phobia were most commonly observed in amygdala (16/38), insular cortex (18/38), cingulate cortex (13/38), and prefrontal cortex (11/38) (Linares et al., 2012). Regarding the study design, most of the studies were conducted by comparing the neuronal responses in patient and control groups. Studies with this design hold a greater interest for us, too, because they investigate the differences in pathological and physiological processing of the phobic stimuli (Rauch et al., 2004). The regions found in structural and functional neuroimaging studies tend to overlap, meaning that the structural alterations could be a cause of functional changes or vice versa. Additionally, most fMRI studies are conducted using patients with spider phobia (69% of all patients), making this summary even more relevant to our study (Del Casale et al., 2012). In this chapter, the most common results of PET and fMRI studies will shortly be summarized.

Neuroimaging studies commonly find significant activation abnormalities in the executive-evaluative cortex which includes several regions of the prefrontal cortex, the orbitofrontal cortex, the cingulate gyrus and insula. The visual and associative cortices also show an abnormal activity in phobic patients (Del Casale et al., 2012).

In prefrontal cortex (PFC), the ventral PFC (VPFC), ventromedial PFC (VMPFC), dorsomedial PFC (DMPFC) and dorsolateral divisions of prefrontal cortex (DLPFC) seem to be involved. PET studies have reported reduced activity in prefrontal cortex of spider phobic patients, particularly in patients with pronounced panic symptoms (Fredrikson et al., 1993; Johanson et al., 1998). In patients, the changes in cerebral blood flow were usually observed on the non-dominant (right) side (Johanson et al., 1998). Similarly, most other studies that have reported abnormalities in activations of prefrontal cortical regions, have reported a diminished activity in DLPFC, DMPFC and VMPFC in response to phobia-relevant stimuli (Hermann et al., 2007, 2009; Ochsner & Gross, 2005). However, there have been at least two studies reporting an increased activity in areas of DLPFC and DMPFC in response to phobic visual stimuli in phobic patients (Straube, Mentzel, Glauer, & Miltner, 2004; Straube, Mentzel, & Miltner, 2006). Interestingly, fMRI studies have also found a normalization of abnormal activation in prefrontal cortex in response to CBT therapy: CBT could normalize the hyperactivity in DLPFC (Straube, Glauer, Dilger, Mentzel, & Miltner, 2006).

Orbitofrontal cortex is another cortical structure with a possible involvement. PET studies have shown, that in a fearful state caused by visual stimuli, rCBF increases significantly in the left posterior medial OFC (Rauch et al., 1995). Activation has also been found in lateral middle and inferior orbitofrontal gyri and the superior frontal gyrus. These results, however, seem to be more specific for dental phobia in comparison to other types of phobia (Lueken et al., 2011). The activity of regions of OFC cortex are also impacted by CBT: in patients undergoing CBT, the baseline hypoactivity in medial OFC becomes potentiated (Schienle, Schafer, Hermann, Rohrmann, & Vaitl, 2007; Schienle, Schafer, Stark, & Vaitl, 2009).

Both PET (Rauch et al., 1995) and fMRI (Martis, Wright, McMullin, Shin, & Rauch, 2004) studies have found an abnormal insular activation in phobia patients. In most cases, greater left insula activation in patients has been observed, interestingly, even compared to patients with other anxiety disorders (Rauch et al., 1995). Plenty of other fMRI studies have found an increased insular activation, both in left, right and bilateral insula induced by both phobia-related pictures and words (Goossens, Schruers, Peeters, Griez, & Sunaert, 2007; Straube et al., 2004; Straube, Mentzel, et al., 2006; Wendt, Lotze, Weike, Hosten, & Hamm, 2008). Interestingly, a study comparing the insular activation between different subtypes of phobia found the highest bilateral insular activation in spider phobia (Dilger et al., 2003). Similarly, as in the case of orbitofrontal cortex and prefrontal cortex, successful cognitive behavioral therapy can eliminate the hyperactivation of insula (Schienle et al., 2009; Straube, Glauer, et al., 2006; Veltman et al., 2004).

fMRI studies also point to an involvement of various parts of ACC (dorsal anterior cingulate cortex, rostral anterior cingulate cortex, posterior cingulate cortex) in the anticipatory anxiety of phobic patients. Spider phobia related words elected an increased response in the ACC and PCC. Researchers proposed that this is caused by a greater memory processing (Straube et al., 2004). This increased activation of ACC cortex structures has been confirmed in other studies: dACC (Straube, Mentzel, & Miltner, 2007) rostral ACC (Britton, Gold, Deckersbach, & Rauch, 2009). There seem to be differences in ACC modulation between diverse types of phobia. Patients with spider phobia show a significantly higher activation of the dorsal ACC than patients with blood-injection injury (BII) phobia (Caseras et al., 2010). Additionally, spider phobic patients show lower activation in the rostral ACC in comparison to patients with BII phobia and healthy controls (Fredrikson, Wik, Annas, Ericson, & Stone-Elander, 1995). As with other brain regions, CBT normalizes the activation of ACC (Straube, Glauer, et al., 2006).

Early PET studies already suggested the possible involvement of visual and associative cortices in specific phobia. In 1993, Fredrikson et al. showed an increased activity in secondary visual cortex (BA18 and BA19) in phobia-

associated visual stimulation in patients with snake phobia (Fredrikson et al., 1993). More recent studies have also pointed out the role of occipital cortex, that, similarly to ACC, is activated differently in different subtypes of specific phobia. Two fMRT studies have found a higher activation of occipital cortex in the BII phobia in response to disgust-provoking images (Schienle et al., 2003). A study conducted by Lueken et al (2011) found a phobic-stimulus induced hyperactivity in the supra-marginal and angular divisions of occipitoparietal cortex in patients with dental phobia, the dental phobic patients also having a higher activity than patients with other SPs.

Regarding the limbic system, there have been two major sites with consistent findings: the thalamus and the amygdala (Del Casale et al., 2012). The results on thalamus are heterogenous. There have been several studies showing a higher thalamic activation in SP: higher rCBF in PET (Rauch et al., 1995; Wik, Fredrikson, & Fischer, 1997), bilateral (Straube et al., 2007) and left thalamic activation in response to phobic stimuli (Martis et al., 2004). Hyperactivity has also been observed in the pulvinar of the thalamus (Goossens, Schruers, et al., 2007; Goossens, Sunaert, Peeters, Griez, & Schruers, 2007). Lueken et al (2011), however, reported a thalamic hyperactivation in snake phobia and hypoactivity in dental phobia.

Many studies have investigated the activity of amygdala, since it has a central part in fear processing. A study by Wendt et al (2008) found activation in amygdala in spider phobic patients when exposed to spider pictures, however, this activation was not specific, because amygdala was also activated during an exposure to other aversive stimuli. In contrast, plenty of fMRI studies have found an increased activation of amygdala in spider phobic patients in response to spider phobia related images (Dilger et al., 2003; Goossens, Sunaert, et al., 2007; Schweckendiek et al., 2011; Straube, Mentzel, et al., 2006), other studies have also found a bilateral amygdala activation (Wendt et al., 2008) and right medial hyperactivity (Veltman et al., 2004) in response to visual stimulation. There could also be a difference in the pattern of amygdala activation in patients in controls. During an exposure to phobic stimulus, patients show stronger but briefer

amygdala activation than controls, whose activation of amygdala was weaker but longer (Larson et al., 2006). Interestingly, a study conducted by Alpers et al (2009) found that amygdala responded to aversive stimuli in a dose-response relationship. When presented with two pictures of spiders, the biggest amygdala activity was found in spider phobic patients. A lower activity was found when presented with one picture of bird and one of spider and the activity of amygdala dropped when presented with two pictures of birds (Alpers et al., 2009). It is also worthwhile to mention, that the activation of amygdala in phobia happens regardless of attention resources, in contrast with the activation of ACC, DMPFC and insula that is dependent on the amount of attention paid (Straube, Mentzel, et al., 2006). The authors of this study suggest that amygdala could play a unique role in processing of phobic stimuli, because other brain structures are not activated during distraction tasks. They also hypothesized that many studies fail to find amygdala activation because it has a role in initial preprocessing and that after longer exposure to phobic stimuli, the amygdala activity decreases (Straube, Mentzel, et al., 2006).

1.7 Summary of previous studies on structural brain alterations in SP

Phobias are often used in research to study the neural basis of fear. For this reason, there are plenty of neurofunctional studies of specific phobia subtypes. However, studies investigating structural brain anatomy of these disorders are still scarce (Hilbert, Evens, Maslowski, Wittchen, & Lueken, 2015). From all the neuroimaging studies on SPs conducted up until 2012 (38 neuroimaging studies altogether), only two used MRI (Linares et al., 2012). However, it is important to address the structural changes to get a full picture of pathological mechanisms behind phobias. These structural alterations could potentially be the basis of functional alterations and could eventually help us find an effective treatment.

Even though many functional neuroimaging studies have found both decreased and increased activity in different cortical regions in patients with specific phobia (Del Casale et al., 2012), up to now, there have only been three studies

addressing cortical thickness in specific phobia (which have yielded different results).

The first structural brain study of SP was conducted in 2004 (Rauch et al.). Based on previous functional neuroimaging study finding, a hypothesis was formed that regional differences in cortical thickness would be found in paralimbic cortex (i.e., posterior orbitofrontal, cingulate, insular, parahippocampal and temporopolar cortex) and sensory cortex (somatosensory and visual cortex). In this study, MRI scans of 10 subjects with SP and 20 healthy controls matched by gender, age, and years of education were studied. All subjects were right-handed. Patients were diagnosed with spider phobia by using Structural Clinical Interview (SCID) (First, 1994). The same tool was used to rule out spider phobia in control subjects. SCID was also used to rule out any other Axis I diagnosis. Subjects were screened for depression and anxiety symptom severity using Beck Depression Inventory (BDI) (Beck, 1960) and Beck Anxiety Inventory (BAI) (A.T Beck, 1990). Cortical thickness was estimated using a previously developed protocol (Fischl & Dale, 2000; Rosas et al., 2002).

Surprisingly, this study found a significant difference in whole brain cortical thickness (mean cortical thickness ± SD: SP = 2.16 ± .42 mm, HC = 2.11 ± .45 mm; $t(1284) = 3.19$, $p = .001$) between patients and controls. Regarding regions of interest, the study found a statistically significant increase in cortical thickness in the paralimbic cortex (insular cortex, pregenual anterior cingulate cortex, and posterior cingulate cortex) sensory cortex (occipital and left occipitotemporal cortex). Post hoc analysis found 8 additional loci with a decreased cortical thickness in SP. Among these findings, the left middle temporal, left inferior parietal and left subparietal loci met the corrected criteria of significance. No areas of decreased cortical thickness in spider phobics were found (Rauch et al., 2004). These results were in contrast with findings of reduced cortical thickness in paralimbic regions in other psychiatric disorders such as posttraumatic stress disorder (PTSD) (Rauch et al., 2003), panic disorder (PD) (Vythilingam et al., 2000) and obsessive-compulsive disorder (OCD) (Szeszko et al., 1999). The small number of subjects was a limitation of this study.

There is not much evidence supporting cortical thickening in spider phobia. In contrary, a study conducted in 2014 with 19 spider phobic patients and 17 age, education and socioeconomic status-matched volunteers observed a cortical thinning (Linares et al., 2014). The patients were diagnosed with spider phobia using the SCID-IV. Additionally, all subjects were screened with Spider Phobia Questionnaire (SPQ) and Beck Anxiety Inventory (BAI) (Klorman, 1974). This study also included left-handed patients. The cortical thickness was estimated using *Freesurfer* software (Fischl, 2012). In statistical analysis, the results were corrected for multiple comparisons using the false discovery rate (FDR) with the significance level set at $p \leq 0.05$. Correlation analysis was conducted between magnetic resonance spectroscopy (MRS) results and SPQ and BAI, but not the results from cortical thickness analysis. The study found a cortical thinning of the right anterior cingulate cortex (ACC) in spider phobic patients: $t(34) = 3.19$, $p<0.001$. No other differences in cortical thickness between SP and HC were found (Linares et al., 2014). The study interpreted the thinning of ACC to be caused by environmental factors, instead of the thinning of ACC directly causing the phobic symptoms. As a limitation of this study, the small sample size should be mentioned (Linares et al., 2014).

A ROI study conducted in 2010 compared the insular cortical thickness and insular volume in 19 patients with fear of small animals (spiders, rodents, snakes) and 20 healthy demographically group-matched controls (Rosso et al., 2010). Subjects were assessed using SCID to make sure that patients met the criteria for animal phobia and that HC had no history of Axis I pathology. All subjects completed the Anxiety Sensitivity Index (ASI) questionnaire (Reiss S, 1986). In contrast to other studies, the participants had to be free from psychotropic medication only 4 weeks prior to participation. The study found a significant positive association between right anterior insula thickness and ASI in patients with small animal phobia ($r = 0.57$, $df = 17$, $p = .01$). A significant correlation between right anterior insula volume and ASI ($r = 0.47$, $df = 35$, $p= .003$) (Rosso et al., 2010) was also observed. However, there were no group differences in insula volume and thickness between SAP and HC (Rosso et al., 2010).

A ROI study by Fisler et al (2013) researched the possible structural alterations of amygdala in spider phobia. The association between amygdala volume (AMV) and clinical features was investigated by comparing MRI scans of twenty female spider phobic patients and twenty age matched healthy female controls. Patients were not matched for education, which could have interfered with the results. Women using contraceptive medication were excluded, as this was considered a confounding factor for the structural brain study. The diagnosis of spider phobia was based on the DSM-IV criteria, the clinical interview used was based on the Composite International Diagnostic Interview (CIDI) (Rubio-Stipec M, 1991). SKID-II questionnaire was also used to screen for possible mental disorders (Fydrich T, 1997). To assess the fear of spiders, all patients were asked to fill out the German version of SPQ (with a cut-off score of less than 21) and a questionnaire for the assessment of disgust severity (FEE) (Schienle A, 2002). A German version of State-Trait Anxiety Inventory (STAI) was used to measure anxiety (Spielberger CD, 1970). All MRI scans were acquired during the luteal phase patients' menstrual cycles. An amygdala mask to exclude the analysis of any other structures was used (Fisler et al., 2013).

Statistical analysis showed an approximately 13% smaller left AMV in patients compared to controls, $F [3, 36] = 6.39$; $p= 0.02$. There was no difference in the right amygdala volume between patients and controls. Additionally, there was a negative correlation between the SPQ score and changes in left amygdala volume ($r = -0.47$; $p = 0.005$), but no correlation between the right amygdala and SPQ score (Fisler et al., 2013). The authors suggested that the autonomic manifestations of fear response are mediated in the prefrontal cortex, and that this mediation suffers dysregulation due to an amygdala deficiency (Fisler et al., 2013). The hemispheric differences were explained by the different functioning of both hemispheres in the processing of emotions, that has been confirmed by previous studies (Fisler et al., 2013). As limitations of this study, the inclusion of only female subjects should be mentioned, which does not represent the entire population of spider phobic patients. The inconsistencies in patient and control screening is another limitation: patients were screened for other psychiatric comorbidities using SKID-II questionnaire and CIDI-based structural clinical

interview. Controls, however, were screened using SCL-90-R questionnaire, to exclude Axis-I disorders (Fisler et al., 2013).

A study by Hilbert et al (2015) compared the structural brain differences in snake phobia and blood injection injury phobia. It has previously been suggested that these phobias could have several different characteristics. For one, animal phobia might be considerably different from other subtypes of phobias. The female: male ratio is a lot higher than in other phobias (3 and above in comparison to 1.1-1.5) (Ajdacic-Gross et al., 2016). In addition to this, animal phobias start at a much younger age (range 7.7-10.1 in comparison with 15.0-18.7 for other phobias) (Ajdacic-Gross et al., 2016; Hilbert et al., 2015). In addition to epidemiological differences, different pathophysiological mechanisms exist in animal phobia and the subtype of blood injection injury phobia. Animal phobia is characterized by a fear response in more automatic response domains (neural activation) and disgust response in more controlled aspects (facial expression). Blood-Injection Injury phobia, however, appears to be associated most strongly with disgust (Cisler, Olatunji, & Lohr, 2009). Additionally, blood injection injury phobia is characterized by a vasovagal response that can cause fainting (Hamm, Cuthbert, Globisch, & Vaitl, 1997; Ost, Sterner, & Lindahl, 1984).

The researchers examined structural brain imaging data from 26 patients suffering from dental phobia, 33 snake phobia patients and 37 HC. Healthy controls were selected from subjects scoring low on snake and dental phobia questionnaires SNAQ (Hamm, 2008) and DFS (Tönnies S, 2002). This could be a limitation of the study, as it does not eliminate the possibility of other phobias (e.g. spider phobia) in control group. For exclusion of patients with other psychiatric illnesses, DSM-IV-TR diagnostic criteria and the CIDI were used. ASI and the BDI-II were used as additional measures. For MRI data analysis, Statistical Parametric Mapping-8 (SPM-8) and VBM8 toolboxes were used. Age, smoking status and sex were included as covariates in the VBM analysis. It is not mentioned if total intracranial volume (TIV) was also included as a covariate.

For gray matter (GM), both phobic groups showed increased volumes in the right subgenual anterior cingulate cortex (ACC; Brodmann area 25), left

medial orbitofrontal cortex (OFC), left precuneus, right calcarine sulcus, right fusiform gyrus and right vermis. Separate comparison of snake phobic patients versus HC showed increased GM volume in the left postcentral gyrus in snake phobic patients. The dental phobia patients showed increased GM volumes in left dorsolateral and dorsomedial prefrontal cortex, the left OFC, bilateral occipital and parietal cortices, right subgenual ACC, left insula, right fusiform gyrus, right lingual gyrus and right cerebellum. For white matter (WM), significant increased right orbitofrontal cortex (OFC) volumes were found in phobia subjects (Hilbert et al., 2015).

The discovery of different affected regions of the two phobias was explained by different procession of fear (Hilbert et al., 2015). The increased volumes of insula and ACC in dental phobia were contributed to the processing of anticipation, evaluation and phobic thread. The lack of volume differences between snake phobic patients and healthy controls were explained by the possibly similar quality of fear response in animal phobia and healthy fear reactions. In contrast, the fear in DP is associated with biphasic vasovagal response (symptomatically mediated defensive behavior) and the feelings of pain and disgust. These differences could also be associated with the different evolutionary roles of the two phobias (Hilbert et al., 2015).

The use of subjects from student population can be seen as a limitation of the study, because education might have an impact on regional brain volume. Also, patients with Blood-Injection Injury phobia scored higher on the BDI-II which poses the question if the depressive symptoms might have had an impact on the results. Other limitation is the use of a cluster-size based threshold of 60 voxels at p=0.001, but not correcting the results for multiple comparisons. A summary table of structural brain neuroimaging results in SP can be found in the appendix.

1.8 Goals of the study

Specific phobia is a common mental health disorder, with patients having high ratings of disease severity and emotional stress. Patients with SP are also more

prone to developing psychiatric comorbidities later in life. For these reasons, the discovery of pathophysiology of SP is of great importance, as this could help develop new therapy options and predict patient`s response to therapy. Many fMRI studies have already investigated differences in brain functioning in patients with specific phobia. However, there is a lack of studies investigating the structural alterations.

From the scarce number of structural studies, most investigate regions that are expected to be involved in SP (such as the amygdala or ACC) using ROI studies, that could be overseeing many regions that could unexpectedly be involved in SP. To be able to get a complete overview of structural brain differences in patients with SP, we chose a method called Voxel-based Morphometry. This method allows to conduct a between-subject comparison of grey matter density in the entire brain.

As for now there has only been one other VBM study examining SPs, and this study did not examine patients with spider phobia. There have also been no structural brain studies investigating the correlation between spider phobia severity and regional grey matter density.

The central questions of the study are:

1. Are there any regional gray matter density (GMD) differences between patients with spider phobia and healthy controls? Based on previous studies on structural and functional brain alterations in SP, we expect to find structural differences in the following regions:
    - prefrontal cortex;
    - orbitofrontal cortex;
    - anterior cingulate cortex;
    - insula;
    - visual and associative cortices.
2. Does regional GMD correlate with the severity of spider phobia?

3. Do different types of spider phobia score (SPQ and BAT) correlate with the same brain region densities?

2 Materials and methods

2.1 "Spider VR" study

The patient demographic data, MRI images and test scores used for describing the severity of spider phobia (SPQ and BAT) for this dissertation were acquired from an ongoing "Spider VR" study. "Spider VR" is a transregional study that began in 2017 and is now simultaneously taking place in *Universitätsklinikum Würzburg Klinik und Poliklinik für Psychiatrie, Psychosomatik und Psychotherapie* and Klinik *und Poliklinik für Psychiatrie und Psychotherapie Westfälische Wilhelms-Universität Münster.* This study examines the functional and structural changes in the brain of spider phobic patients treated with virtual reality exposure therapy.

2.2 Patients and controls

Patients interested in the study are contacted through telephone to check if they meet all of the inclusion criteria:

- age >18;
- sufficient knowledge of German language that are both stated by the subject and subjectively assessed by the interviewer;
- right-handedness;
- Caucasian background.

Additionally, patients cannot meet any of the following exclusion criteria:

- use of any psychopharmacological medication e.g. antidepressants and anxiolytics;
- use of opiates or any other strong painkillers;
- current or previous history of psychotherapy or psychiatric treatment;
- history of exposure or confrontation therapy;

- history of neurological disorders e.g. epilepsy, Parkinson's disease, Multiple Sclerosis, brain hemorrhage;
- current or possible pregnancy;
- suicidal thoughts.

Exclusion criteria particularly important for the acquisition of MRI data are:

- implants (including cochlear implants), stimulatory devices, vascular clips, pacemakers or medication pumps;
- tattoos on upper back, forearms, neck, head, permanent make-up or piercings;
- contraceptive coil or retainers.

All patients are subjected to WHO WMH-CIDI and SKID interviews, that are designed for assessment of mental disorders based on their ICD-10 and DSM-IV definitions (*https://www.hcp.med.harvard.edu/wmhcidi*). CIDI interview is conducted during telephone screening to check if the patients have any psychiatric comorbidities that would be an exclusion criteria. SKID interview is conducted during baseline assessment and is also used for the exclusion of patients.

Altogether, we used the data from 35 spider phobic patients. For between-group comparison, we matched these patients with 33 age, gender and education-matched controls. Subjects were matched in these parameters because it known that:

- total brain volume and ventricular volume are significantly associated with person's age. Global grey matter volume decreases with age (Hafkemeijer et al., 2014). Regions that are particularly prone to ageing include regions that take part in processing of emotion including the insula and the cingulate sulci (Erten-Lyons et al., 2013; Good et al., 2001);
- there are gender differences in whole-brain and regional brain volume and density. A meta-analysis of 126 articles addressing the sex differences in brain volumes found that males have larger intracranial

volumes (ICV), total brain volumes (TBV), grey matter, and white matter and cerebrospinal fluid volumes (Ruigrok et al., 2014). This study also found several regional volume and tissue density between-gender differences including regions that also are responsible for emotional processing such as the left amygdala, hippocampus, insular cortex and anterior cingulate gyri (Ruigrok et al., 2014). These differences could stem from multiple factors including genetics, environment, cell-to-cell communication etc. (McCarthy & Arnold, 2011);

- there could be a relationship between education and brain volume. Even though this relationship is not clear, some studies have found education-related brain differences. For example, a study conducted in 1999 by Coffey et al found a correlation between the level of education in years and CSF volume (Coffey, Saxton, Ratcliff, Bryan, & Lucke, 1999).

Descriptive and inference statistics of patient and control groups can be found in **table 1**. We found no significant between-group differences in age, gender and the level of education in patient and control groups. Calculations were done using IBM SPSS Statistics (Statistical Package for the Social Sciences) version 24.0 64-Bit-Version. For all statistical tests, we set a significance level of $p<0.05$.

Table 1. Demographic data analysis in patient and control groups.

Variable	Spider phobics;	Controls	Test	t; X^2	df	*p*
Gender	female= 29 male= 6	female= 31 male= 2	Chi Square Test	2,01	1	0,16
Age	M= 27,5 SD= 9,21	M= 28,0 SD= 6,46	t-Test	-0,24	66	0,81

2.2.1 TIV volumes

TIV volumes for each subject were acquired during VBM analysis. Descriptive and between- group comparison of these values can be found in **table 2.** We found no significant difference in TIV volumes between controls and spider phobic patients.

Table 2. Comparison of TIV volumes between patients and controls.

Variable	Descriptive statistics spider phobics	Descriptive statistics controls	Test used	t	df	p
TIV volume	M = 1569,45 SD = 132,19 min = 1333,75 max = 1825,09	M = 1555,93 SD = 130,83 min = 1272,52 max = 1967,24	t-test	0,42	66	0,67

As expected, we found a significant difference between TIV scores in males (M = 1748,25, min = 1525,55, max = 1967,24, SD = 135,18) and females (M = 1538,18, min = 1272,52, max = 1793,7, SD = 109,35), t(66) = -4,97, p < 0,001, with males having higher TIV volumes than females. Surprisingly, we also found a negative relationship between subject's age and TIV volume F(1,66) = 18,08, p<0,001, with an adjusted R^2 of 0,20.

2.3 Methods

2.3.1 Spider Phobia Questionnaire (SPQ)

SPQ was used during the baseline assessment (first patient visit) of "Spider VR" study to objectify our subjects` self-reported fear of spiders (Klorman, 1974). SPQ is the oldest and most commonly used self-assessment tool for detection of spider phobia created by Klorman (1974).

The SPQ consists of 31 yes/no questions, 9 of which are reversed. In Spider VR study, a score of 20 or higher is considered to be a sufficient confirmation of spider phobia and has to be met for the inclusion in the study. In our patient group, the average SPQ score was 23,1 (SD=2,27), which is consistent with mean SPQ score of pre-treatment spider phobics (Muris & Merckelbach, 1996). In the VBM analysis, the SPQ was used to express the severity of patient`s phobia. SPQ is also reported to be consistent with other spider phobia questionnaires, so we will safely be able to compare the results of our study with the results of studies using other measurements of spider phobia (Muris & Merckelbach, 1996).

2.3.2 In vivo Behavioral avoidance test (BAT)

During baseline assessment in “Spider VR” study, the patient has to undergo BAT, which is designed to measure the level of avoidance of spiders in spider phobic individuals. It can also be used to measure the effectiveness of treatment. This particular BAT for spider phobic individuals was developed by Muris et al and first used in 1998 to examine the exposure as a treatment method of spider phobia in children (Muris, Merckelbach, Holdrinet, & Sijsenaar, 1998). However, these forms of tests have been used even earlier. To mention a few, a study conducted in 1974 used a live snake (Bernstein) to measure the level of avoidance. Yet another study from as early as 1969 used live rats as phobic test stimulus (Levis).

At the beginning of the examination, the spider is placed in a transparent plastic box with a closed lid. The box is then placed on a movable platform. At the start of the procedure, the platform is placed 3 meters away from the patient in eye level. The patient can roll the platform closer or further away from himself. The patient is asked to slowly drag the box with the spider towards himself as close as possible. The distance between the patient and platform is closely monitored by a measuring tape glued to the side of the desk and the final distance between patient and spider is registered and used to objectively describe the extend of fear (**figure 1, figure 2, figure 3**).

Figure 1. Spider used for the BAT.

Figure 2. Setup for BAT. The spider is placed in the plastic box, which is then placed on a movable platform.

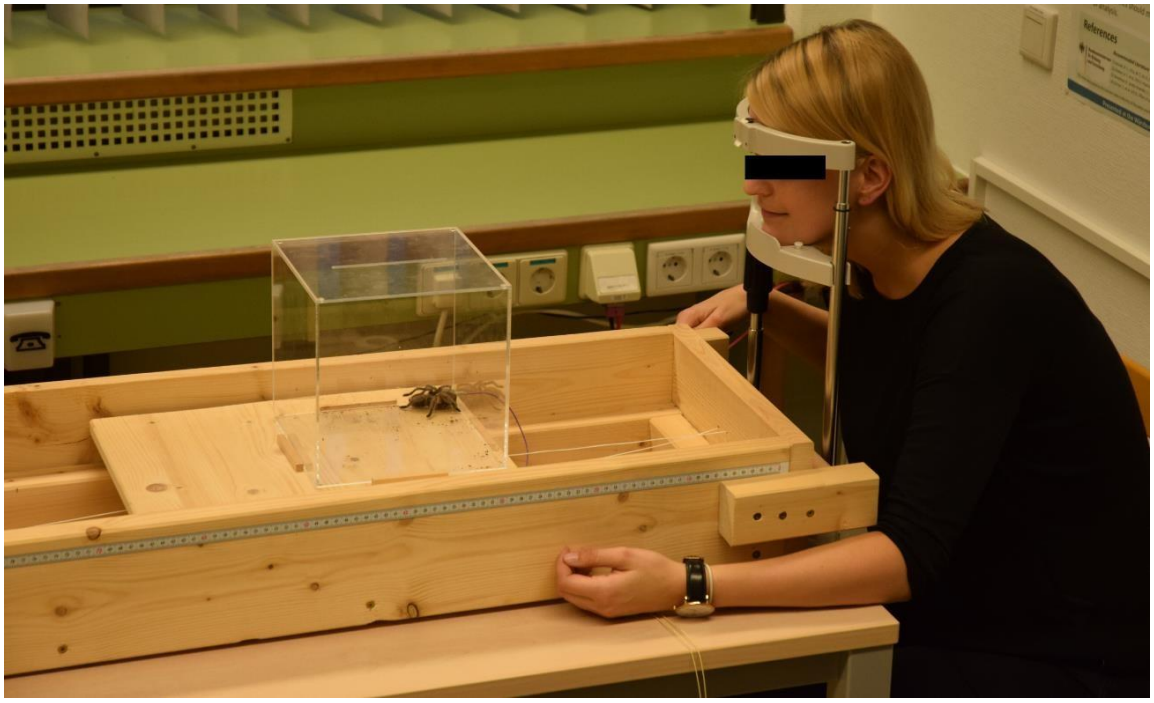

Figure 3. The subject is asked to move the box with the spider as close as possible. This can be done with a handle located on the right side of the subject, without having to touch the box itself. See appendix for permission to publish the photo

The patient is additionally asked to assess his subjective fear on a scale from 0 to 100% at several points of the test

1. before the spider is brought into the room (anticipation fear);
2. the box with the spider crosses the doorstep (fear at the door);
3. right after the patient sits down (fear beginning);
4. after the assessment of final distance (fear at the final distance);
5. at the end of test (fear at the end);
6. when the spider is taken away from the room (fear spider away).

The results of these tests were used in our study to see if there is any correlation between the extend of subjective and objective fear and the regional GMD.

2.3.3 Voxel based morphometry- preprocessing and data analysis

MRI images were acquired with Siemens Skyra Whole Body Scanner (Siemens Medical Systems, Erlangen, Germany) with a magnetic field of 3 Tesla. For the acquisition of structural scans, a standard T1 magnetization rapid gradient-echo imaging sequence (MPRAGE) was used. 176 sagittal slices were acquired for one subject with a slice thickness of 1mm; TE = 2.25 ms and TR = 1900 ms, flip angle 9˚. FOV was 256 x 256 mm, matrix- 256 x 256. For pre-processing of MRI images, a morphometric method VBM was used (Ashburner & Friston, 2000). VBM is an extension to the CAT12 toolbox (*http://dbm.neuro.uni-jena.de/cat12/)* that operates in SPM12 core program (*http://www.fil.ion.ucl.ac.uk/spm/;* released in October 2014) (Ashburner et al., 2016). All the procedures were conducted in Matlab environment (the MathWorks Inc, Natick, Massachusetts, USA, 64-bit, version 9.2) on a windows workstation.

VBM was conducted mostly based on the parameters and procedures described in CAT12 manual created by C. Gaser and F. Kurth, the developers of CAT12 (*http://www.neuro.uni-jena.de/cat12/CAT12-Manual.pdf*). Further, a more detailed description of the conducted procedures is provided.

At first, the T1 weighted MRI images were converted from their original format DICOM to NIfTI that is recognized by CAT12. This was done using SPM12 built-in converter. Before segmentation we made sure that the orientation of patient scans matched that of SPM priors. Only this way we can ensure that the normalization and segmentation run correctly. The default SPM12 tissue probability maps (TPMs) were used for spatial normalization, initial skull-stripping and as a segmentation estimate because all our subjects were grown adults. In comparison to SPM, the segmentation process in CAT12 relies on an approach that requires no previous information of tissue probabilities and tissue maps are used only as an estimate (Ashburner & Friston, 2005). We used the default settings for initial SPM12 pre-processing and CAT12 pre-processing (APP, Strength of Local Adaptive Segmentation, Skull Stripping and Final Clean Up to remove any residual unwanted tissue).

The CAT12 DARTEL IXI555_MNI152.nii template was used for spatial registration and normalization of data into the Montreal Neurological Institute (MNI) space (Ashburner, 2007). Voxel size for normalized images was 1.5. For all other writing options, the default settings mentioned in CAT12 manual were chosen.

After this, the “Display one slice for all images” option was used to view the newly written segmented and normalized data in horizontal slices. To check if any of the data were low quality we visualized the distribution of data with the help of boxplot graphs and correlation matrices with the “Check sample homogeneity” option. Additionally, previously produced xml-files were also checked for sample homogeneity. The outlier data were inspected manually. Our images contained no artefacts, so no samples were excluded.

The normalized and segmented data were then smoothened using a Gaussian smoothing algorithm with an isotopic 8mm kernel (CAT 12 Manual; http://www.neuro.uni-jena.de/cat12/CAT12-Manual.pdf). The total intracranial volume (TIV) was estimated for all subjects using the previously created xml-files. This was done so that TIV values could later be used as a covariate during statistical analysis to correct the between-subject brain size differences.

For the analysis of VBM data, two statistical models were used: a two-sample t-test and multiple regression both available in SPM12 core program. For two sample t-test, the previously yielded normalized smoothened and segmented scans were divided in two groups- patients (1) and controls (2). Data on TIV volume were uploaded as .txt files to be used for the analysis. For the t-test, the groups were considered independent. The unequal variance and centering to overall mean options were chosen from the batch editor menu. No threshold masking was used.

With the help of “Design orthogonality” option, we checked if TIV values had no correlation with any other covariates. This was done, because TIV correlation with other covariates could lead to the removal of parts of the variance between our samples (CAT 12 Manual; http://www.neuro.uni-jena.de/cat12/CAT12-

Manual.pdf). No orthogonality was found, so both parameters were included in our statistical analysis.

For the two-sample t-test, two contrasts were defined as follows:

Patients (1) > Controls (2)	1	-1
Patients (1) < Controls (2)	-1	1

For results, no masking was selected; the threshold p value was set to 0.001. At first, the extended threshold was set to 0. Additionally, for a better overview, only results reaching a familywise error rate of ≤ 0.01 were considered significant. We used the “atlas labeling” function integrated in SPM12 to identify the statistically significant brain regions. For labeling, the AAL atlas was chosen. For better visualization, images of significant clusters were saved for use with MRIcron. Additionally, to eliminate the possibility that the density changes in our regions of interest could be attributed to brain density changes associated with age, we conducted a voxel-wise correlation analysis between brain region density and patient`s age.

2.3.4 MRIcron

For better visualization of our results, we used an additional software designed for viewing neuroimaging data called MRIcron. MRIcron is a widely used free NIfTI image viewer created by Chris Rorden (https://www.nitrc.org/projects/mricron). A template called “ch2better” was used as a background. Additionally, we used the “yoke” option to synchronize the “ch2better” template with an “aal” (short for automated anatomical labeling) template, so that the brain regions would be recognized and labeled automatically.

3 Results

3.1 Voxel-wise grey matter comparison between patients and controls

Using TIV values as a covariate of no interest, we conducted a voxel-wise independent t-test in SPM12 to compare the differences in gray matter density in patients and controls. This test found no brain regions that were significantly different between patient and control groups. This was the case at both cluster and peak levels and for both contrasts (patients > controls and patients < controls).

3.2 Spider phobia score in spider phobic patients

The summary of different values evaluating patients' fear of spiders can be found in **table 3.** Judging from the BAT values, patients experienced the biggest amount of subjective fear at the point of experiment, when the spider was the closest to the patient and lowest level of fear when the spider had been taken away.

Table 3. Descriptive statistics of subjective and objective score representing the severity of spider phobia.

Score type	M	Min	Max	SD	N
SPQ	23,1	20	28	2,29	35
Bat fear at the beginning	74	15	100	23,9	35
BAT anticipation fear	56,1	5	100	24,9	35
BAT fear at the door	70,7	20	100	22,5	32

BAT fear at the final distance	79,3	30	100	20,7	35
BAT fear at the end	68,7	0	100	25,9	35
BAT fear spider away	26,6	0	90	29	32
BAT final distance (cm)	171	52	300	66,7	34

We found a positive correlation between SPQ and distance in centimeters ($r = 0,413$, $p = 0,015$) and distance in centimeters and BAT mean score ($r = 0,635$, $p < 0,001$). There was no significant correlation between BAT fear score and SPQ score. Besides, the different BAT fear values were correlated with each other. There was no significant between-gender differences. However, there was a significant negative relationship between age and SPQ score $F(1,33) = 5,92$, $p = 0,021$, with an adjusted R^2 of 0,126 and age and BAT fear score $F(1,33) = 9,19$, $p = 0,005$, with an adjusted R^2 of 0,194. Age had no predictive value on final distance in centimeters.

3.2.1 Multiple linear regression analysis between spider phobia score and regional gray matter density

In addition to independent t-test, we also conducted a regression analysis to see if there was a relationship between patients' fear of spiders and gray matter density in different brain regions. This analysis was also performed on the entire brain using TIV volumes as a variable of no interest. In contrast with the between-group t-test, we found a relationship between these scores and several brain region densities.

Firstly, there was a positive correlation between SPQ score and 3 different brain region densities: dorsal anterior cingulate cortex, left insula and left inferior parietal lobule. For SPQ, negative correlation analysis yielded no significant results. These results are illustrated in **figure 4, figure 5** and **figure 6.**

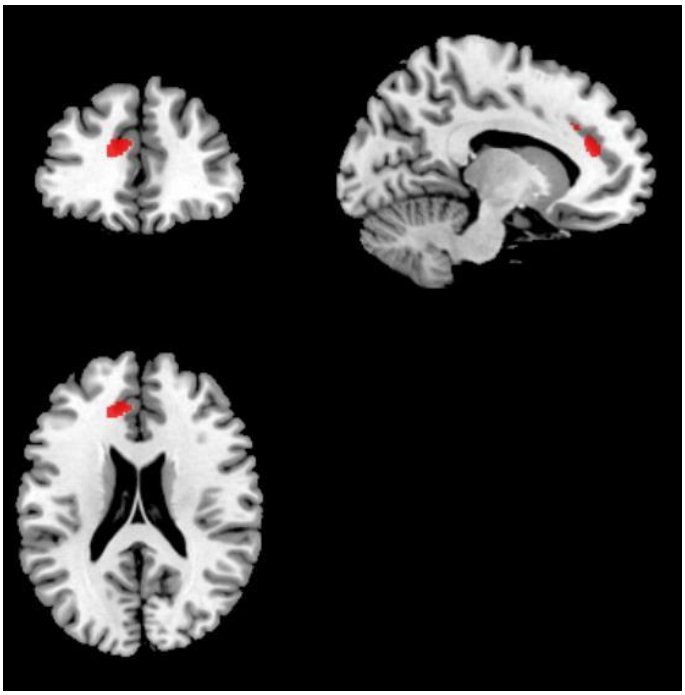

Figure 4. Correlation between SPQ score and dorsal anterior cingulate density. Peak voxel coordinates: x = -11 y = 39 z = 20. p_{uncorr} <0.001; $p_{FWE\text{-}corr}$ <0.001

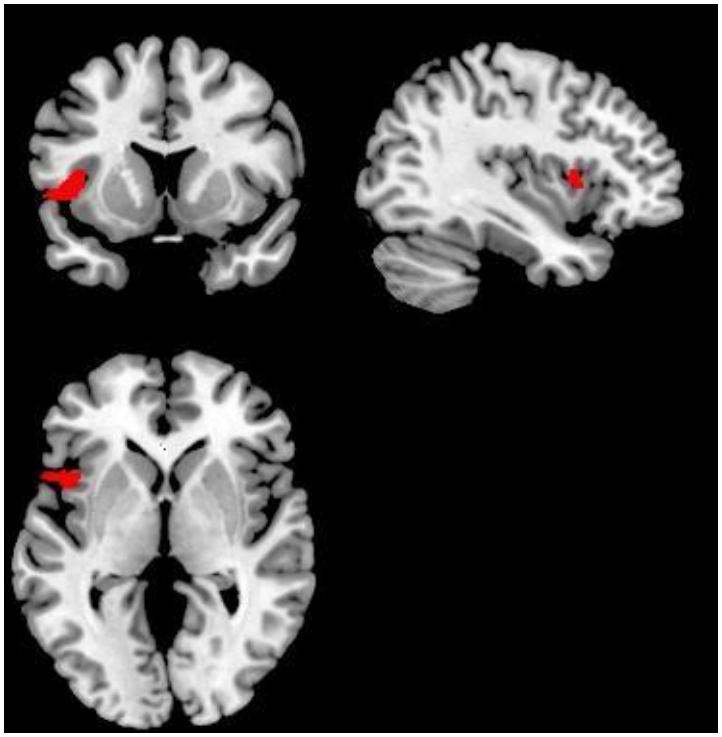

Figure 5. Correlation between SPQ score and the left insula density. Peak voxel coordinates: x = -44 y = 11 z = -6. p_{uncorr} <0.001; $p_{FWE\text{-}corr}$ =0.017

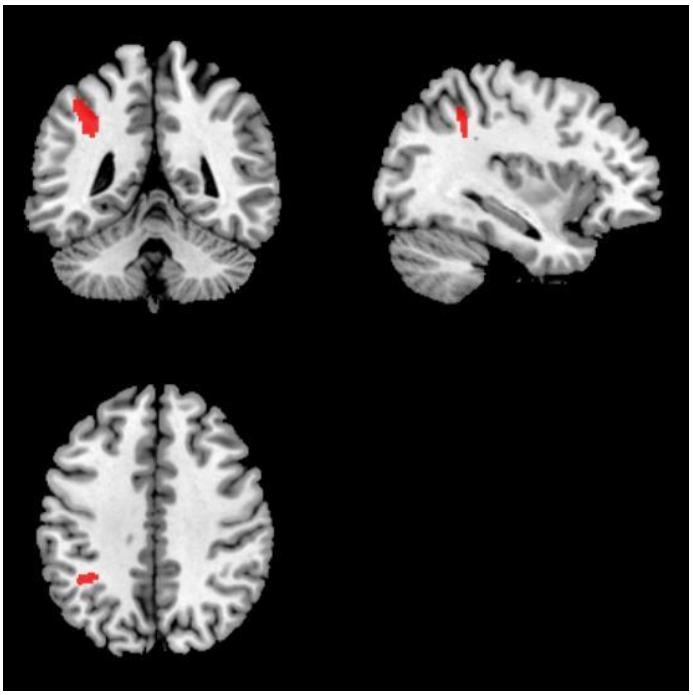

Figure 6. Correlation between SPQ score and left inferior parietal lobule density. Peak voxel coordinates: x = -44 y = -45 z = 51. p_{uncorr} <0.001; $p_{FWE\text{-}corr}$ =0.001

Next, there was a positive correlation between BAT final distance in centimeters and left superior frontal gyrus (BA 9) and right paracentral lobule densities (**figure 7 and 8**). This cluster extended to right supplementary motor area.

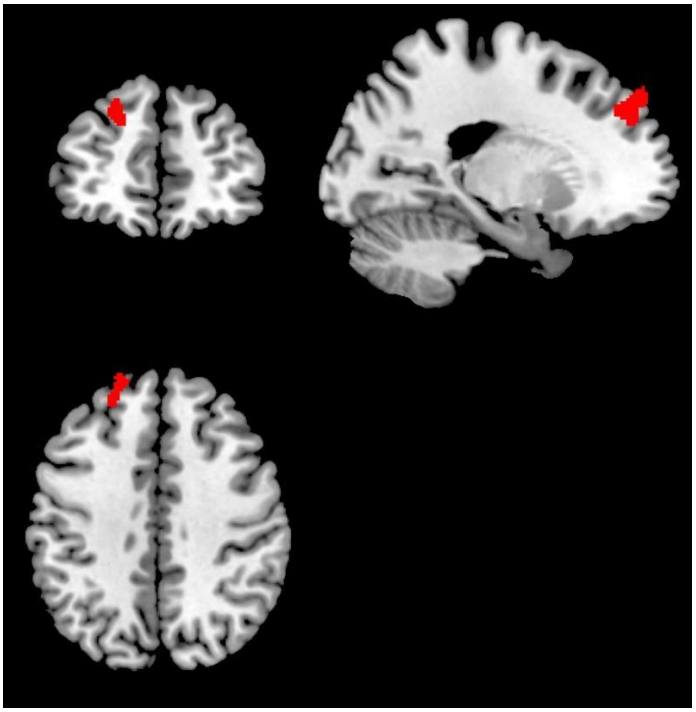

Figure 7. Correlation between final distance in centimeters and left superior frontal gyrus density. Peak voxel coordinates: x = -20 y = 45 z = 36. p_{uncorr} <0.001; $p_{FWE\text{-}corr}$ =0.01

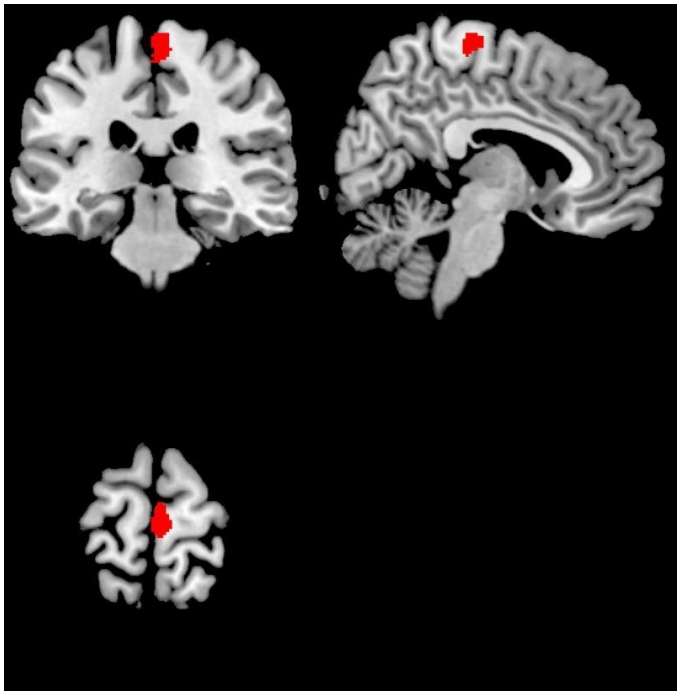

Figure 8. Correlation between final distance in centimeters and right paracentral lobule density. Peak voxel coordinates: x = 5 y = -24 z = 71. $p_{uncorr} < 0.001$; $p_{FWE-corr} = 0.003$

For several subjective fear scores recorded during different points of BAT, we found positive correlations only between fear when the spider is carried away and vermis density (**figure 9**). No negative correlations between any brain region densities were observed. An overview of all regions correlated with spider phobia can be found in **table 4.**

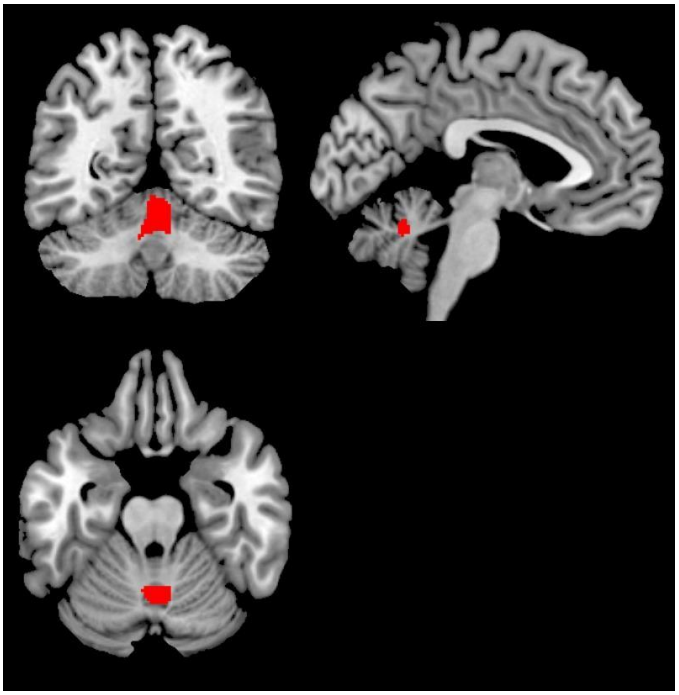

Figure 9. Correlation between BAT fear when the spider is taken away and vermis density. Peak voxel coordinates: x = 5 y = -56 z = -15. p_{uncorr} <0.001; $p_{FWE\text{-}corr}$ <0.002

Table 4. Overview of brain regions with density correlated with patient`s fear of spiders.

score	region	type	$p_{FWE\text{-}corr}$	T	cluster size
SPQ	dorsal anterior cingulate	+	<0.001	5.2	763
	right insula	+	0.017	4.83	327
	left inferior parietal lobule	+	0.001	4.26	545

Final distance in centimeters	left superior frontal gyrus	+	0.01	5.01	366
	right paracentral lobule	+	0.003	4.46	456
BAT fear spider away	vermis	+	0.002	3.97	468

The voxel-vise correlation analysis between brain region density and patient`s age showed that none of the brain regions associated with spider phobia had a significant correlation with patient`s age.

4 Discussion

4.1 Increased grey mater density in left anterior cingulate cortex

The most significant correlation ($p<0.001$) in this study was found between SPQ score and anterior cingulate cortex density. From all regions of significance, this also was the one with the largest cluster size (763 voxels; peak voxel at x = -12 y = 38 z = 21). This cluster also correlated with final distance in centimeters.

As for now, the only VBM study investigating SP also found an increased grey matter density in ACC (Hilbert et al., 2015). Interestingly, however, these changes were predominant in the dental phobia group, and the contrast between healthy subjects and patients with animal phobia (snake) showed no significant volume differences in ACC (Hilbert et al., 2015). Furthermore, this study also examined the correlation between clinical score and regional volume but found no significant correlation in ACC or any other regions, which is a contrast to our finding. Researchers of this study suggested that symptom severity might be too broad of a measure to find a correlation between these regional changes and the score (Hilbert et al., 2015). Another structural brain imaging study of SP also pointed to structural alterations in anterior cingulate cortex (Rauch et al., 2004). This ROI study found an increased cortical thickness in bilateral anterior and posterior cingulate cortex in spider phobic patients (Rauch et al., 2004). Lastly, a study on brain cortical thickness found a cortical thinning of the right anterior cingulate cortex in spider phobic patients (Linares et al., 2014). Even though it has been proven that cortical thickness is significantly correlated with grey matter volume, it is important to keep in mind that these measurements are not identical and an increased cortical thickness does not necessarily equal an increased grey matter density (Winkler et al., 2010). We should keep this in mind while comparing cortical thickness and grey matter density/volume studies.

Our findings and the findings of other structural studies are also in line with those of many fMRI studies. An increased activity in cingulate cortex in patients with specific phobia has been observed in different scenarios: patients being confronted with phobia related words, anticipation of phobia related stimuli, and

exposure to phobia related pictures and videos (Goossens, Schruers, et al., 2007; Straube et al., 2004; Straube et al., 2007). Interestingly, fMRI studies have found an increased activity in cingulate cortex during symptom provocation (Del Casale et al., 2012). These results have not been limited to spider phobia alone; the increased activation in the cingulate is also seen in other types of SP (Caseras et al., 2010).

Anatomically, cingulate cortex forms a ring or a cingulum around most of corpus callosum (Vogt, Finch, & Olson, 1992). The ACC is a part of the limbic system, specifically, the paralimbic cortex (Bush, Luu, & Posner, 2000; Vogt et al., 1992). Cingulate cortex can be divided in two parts- the anterior and posterior cingulate cortex, that are differentiated from one other by their cytoarchitecture, their functions and connections to other brain structures (Bush et al., 2000). Our findings were limited to ACC. ACC can itself be divided in two separate divisions: the executive and evaluative regions. ACC has wide connections with other brain structures- the portion of ACC responsible for cognition (evaluative) has connections to lateral prefrontal cortex, parietal cortex, premotor and supplementary motor areas (Devinsky, Morrell, & Vogt, 1995). The affective (executive) subdivision has connections to amygdala, nucleus accumbens, hypothalamus, anterior insula, hippocampus and orbitofrontal cortex, that have connections with autonomic, visceromotor and endocrine systems (Devinsky et al., 1995).

Even though cingulate cortex is usually viewed as a structure responsible for emotional experiences and social interactions, it is also responsible for non-emotional behaviors (Vogt et al., 1992). Studies implementing different methods including lesion studies, electrical stimulation PET, CT, MRI etc. have observed, that the functions of anterior cingulate cortex include the regulation of behavior in social situations, pain, maternal behavior, visceral and motor control, aggressiveness and attention (Malamud, 1967; Vogt et al., 1992). Even though these functions seem diverse, the common theme is that they are executive. Executive functions also refer to of emotional processes, as every emotion relates to its expression through autonomic, endocrine and motor systems. This is

supported by several case reports, where anterior cingulotomy and ablations to the ACC have improved the symptoms of obsessive behaviors and anxiety (Ballantine, Bouckoms, Thomas, & Giriunas, 1987; Lewin, 1961; Long, Pueschel, & Hunter, 1978). Interestingly, it has been reported that monkeys that undergo cingulectomy lose their fear of humans (Ward, 1948).

Involvement of ACC in visceral reactions might have a particular importance in the expression of SP. Regulation of autonomic systems prepare the organism for movement responsible for fight or flight responses observed in SP. Electrical stimulation to cingulate cortex have been observed to cause elevation in blood pressure, piloerection, respiratory rate and blood cortisol levels- all reactions that are characteristic for SP, and lesions in animal cingulate cortex blocks fear expression in fear conditioning (Vogt et al., 1992), which is expressed by direct connections from ACC to brainstem nuclei.

PET studies have also demonstrated that ACC exhibits a higher activity in tasks that are more cognitively demanding and require more attention (Wendt et al., 2008). For example, ACC increases when subjects are required to name the word representing a colour, when the word is written in another colour, versus when it is written in the same colour (Pardo, Pardo, Janer, & Raichle, 1990). This role of ACC in attention could explain the results of fMRI studies that observed that, when confronted with phobic stimuli, the activation of ACC was present only when the patient was paying direct attention to the stimuli, in contrast with amygdala, whose activation did not require direct attention.

Anterior cingulate cortex is reciprocally connected with amygdala, which, as discussed in introduction, has a large role in fear conditioning. For example, DTI studies have discovered that higher trait anxiety is associated with stronger connections between amygdala and dorsal anterior cingulate cortex (Greening & Mitchell, 2015).

Based on this information, it becomes apparent, that dysfunction in this structure could lead to the symptoms observed in animal phobia, including hypersensitivity to the phobic stimulus, overestimation of threat, inability to suppress emotional

responses, and visceral and autonomic responses to the phobic stimulus (Rauch et al., 2004). In comparison to other regions involved in SP, ACC is specifically related to anticipatory anxiety, threat perception and evaluation of phobic stimuli (Straube, Mentzel, et al., 2006; Straube et al., 2007).

42 Increased regional density in right insula

Secondly, SPQ score was positively correlated with left insula volume ($p = 0.01$). Cluster size was 327 and peak voxel was located at $x = -44$ $y = 14$ $z = -6$.

Similarly to ACC, these results are also in line with other structural brain studies in phobic patients. The only VBM study conducted on patients with specific phobia also found an increased left insula density in phobic patients. However, comparing patients with small animal phobia (snakes) to patients with dental phobia, the changes in insular density were only significant in dental phobia group. Just as in case of ACC density, this study did not find any correlation between right insula density and clinical severity score (Hilbert et al., 2015). A RIO analysis investigating the relationship between right insular volume and cortical thickness and ASI in patients with small animal phobia found that both right insula thickness and volume had a positive correlation with ASI score, but there was no difference in insular volume and thickness between patients and controls. The researchers proposed that insular volume and thickness were a substrate of Anxiety Sensitivity within specific phobia, and not an independent marker of phobic disorders (Rosso et al., 2010). Based on these results, it seems important to further investigate the relationship between Anxiety Sensitivity and right insular volume.

Additionally, a whole brain voxel-wise cortical thickness analysis comparing spider phobic patients and healthy controls found an increased cortical thickness in bilateral insular cortex in spider phobic patients (Rauch et al., 2004). Same as with ACC, cortical thickness does not always translate to volumetric changes in these regions.

A large number of PET and fMRI studies also point out to an increased insular activity in patients with SP (Del Casale et al., 2012; Rauch et al., 1995), with both the involvement of right and left insula being reported. Same as with ACC, the activation of insula is attention-specific (Straube, Mentzel, et al., 2006), and fMRI studies have discovered, that the activation of ACC is largely correlated with the activation of insula (Straube, Mentzel, et al., 2006).

Anatomically, the insular cortex forms an individual hidden lobe in the depth of Sylvian fissure, separating the temporal, parietal and the frontal lobe (Nieuwenhuys, 2012; Ture, Yasargil, Al-Mefty, & Yasargil, 1999). The insula can roughly be divided in anterior and posterior section, that are distinguishable by their cytoarchitectology, connections with other brain structures and functions, with the posterior regions being responsible for somatosensory, vestibular and motor functions, as it is connected to the spinal cord, brainstem and association cortices (Namkung, Kim, & Sawa, 2017). The posterior insula is thought to play a role in relaying sensory information form visual, auditory and somatosensory cortices to higher- order association cortices (Mesulam & Mufson, 1982). The anterior regions, however, are interconnected with ACC, prefrontal cortex and amygdala, and are said to be responsible for the regulation of autonomic and visceral information of emotion, cognition and motivation. Due to the strong connections and structural similarities between ACC and insula, the insula has been called “the limbic sensory area” and ACC “the limbic motor area” (Namkung et al., 2017). A variety of functions have been attributed to insula, including memory, affect, empathy, autonomic control, gustation and olfaction (Augustine, 1985). However, the full role of insula remains to be unknown.

The involvement of insula in SP could be related to its role in disgust. fMRI studies have observed, that insula is activated when subjects are exposed to disgust provoking odours or tastes, but there is no insular activation when patients are subjected to neutral or pleasant stimuli (Royet, Plailly, Delon-Martin, Kareken, & Segebarth, 2003; Wicker et al., 2003). Similarly, the activity in insula increases viewing disgusted facial expressions (Schienle et al., 2002). It has been proposed that insula is responsible for generating sensation “of being sick” that is also a

part of experiencing disgust (Penfield & Faulk, 1955). It has been reported that damage to anterior insula leads to impaired recognition of disgusting stimuli (Sprengelmeyer, Rausch, Eysel, & Przuntek, 1998) and that a Huntingtons's disease patient with damage to the insula was unable to differentiate between expressions of disgust and anger, and this did not cause problems in subjects with a healthy insula (Calder, Keane, Manes, Antoun, & Young, 2000).

Early stimulation studies of insula in monkeys found that insular stimulation produced respiratory, somatomotory and circulatory effects, including sudden fall in blood pressure, inhibition of respiratory movements, and these responses could be diminished by the section of the vagus nerve (Penfield & Faulk, 1955). This could explain why some structural MRI studies have found larger changes in insular volume in dental phobia, as dental phobia is also distinguished by vagal responses. Metabolism in the right insula correlates with bladder sensations, the feeling of breathlessness, which can also be characteristic sensations of SP (Gasquoine, 2014).

The insula is also responsible for representation of arousal, and it could support the interaction between perceived threat and bodily states observed in arousal that lead to the experience of emotion (Reiman et al., 1997). The role of insula in these processes suggests a relationship between the activity of insula, increased awareness of bodily states and the proneness to anxiety (Critchley, Wiens, Rotshtein, Ohman, & Dolan, 2004).

4.3 Increased grey matter density in left inferior parietal lobule

Lastly, SPQ score were correlated with left inferior parietal lobule density ($p<0.001$). This was the second largest cluster with cluster size of 545 voxels and peak voxel located at x = -44 y = -45 z = 51.

Changes in the function of inferior parietal lobule, or parietal regions for that matter, have not been a common finding in patients with SP. This does not, however, mean that these results are false. Structural brain studies have mostly examined regions of interest that have been suspected to be involved in

processes responsible for fear and not examining the entire brain. Additionally, ROI analysis is usually limited to well defined regions, and it is not successful in detecting changes in large homogenous structures. However, VBM analysis in patients with SP also found an increased density in the parietal cortex (Hilbert et al., 2015).

Many fMRI and PET studies have found the involvement of association cortex in SP. Patients with spider phobia show an increased activity in superior parietal lobule while viewing disgust inducing images, however, the activity in inferior parietal lobule is decreased while viewing phobia inducing pictures (Schienle, Schafer, Walter, Stark, & Vaitl, 2005). Additionally, patients with SP show an increase in right inferior parietal gyrus activity after CBT therapy (Schienle et al., 2007).

The inferior parietal lobule is a part of the association cortex, located between visual, auditory and tactile areas. Anatomically, the inferior parietal lobule consists of two areas: the supramarginal gyrus (mainly consisting of BA40), and the superior part of angular gyrus (BA39) (Gulledge, 2017). Our results were confined to BA40. Generally, functions of inferior parietal lobule revolve around the intersection of written and spoken language, with BA40 being particularly important for sound processing (Gulledge, 2017). Based on the involvement of inferior parietal cortex in language processing, it would be difficult to interpret the role of this region in SP. However, the role of the region extends to more than just that. Firstly, the parietal lobe seems to be involved in anxiety disorders: ten days of parietal lobe rTMS improve anxiety in patients with GAD (Huang et al., 2018).

Inferior parietal lobule is also involved in the recognition of fearful stimuli: patients with schizophrenia show a trend of decreased activation of inferior parietal lobule in response to fearful facial expressions. This is in contrast with healthy individuals, whose parietal lobule shows a positive activation (Radua et al., 2010). IPL also has a role in attention- unilateral ablations of the monkey IPL cause neglect in contralateral sensory stimuli (Mesulam, Van Hoesen, Pandya, & Geschwind, 1977). Besides, just like the previously mentioned structures, IPL

also has extensive connections to brain structures involved in SP, including the prefrontal cortex and the limbic lobe (amygdala, hypothalamus, ACC), with cingulate cortex having one of the highest amounts of connections to IPL (Mesulam et al., 1977).

44 Increased grey matter density in left superior frontal gyrus

Final distance in centimeters showed a positive correlation with grey matter density in left superior frontal gyrus. The cluster size was 366, and the peak voxel was in x = -20 y = 45 z = 36. This region is in BA9, which overlaps with dorsolateral prefrontal cortex. Additionally, a part of the cluster mostly located in left dorsal anterior cingulate whose density showed a correlation with SPQ score also extended to left frontal superior gyrus region.

VBM analysis of patients with snake phobia found an increased grey matter density in left superior frontal cortex (Hilbert et al., 2015). Just as in the case of parietal lobule, this is the only study examining the involvement of prefrontal regions in SP, as region of interest studies cannot detect changes in large homogenous brain regions.

However, there is a lot of evidence from fMRI studies that suggest the involvement of different areas of prefrontal regions in the pathophysiology of SP. A large number of functional studies have shown, that prefrontal areas have both reduced (Hermann et al., 2007; Johanson et al., 1998; Wik et al., 1993) and increased (Straube et al., 2004) activity in SP patients. These studies have found that different parts of prefrontal cortex are involved: DMPFC, VMPFC and DLPFC (Del Casale et al., 2012).

Even though the PFC is usually viewed as responsible for executive functions, it is also involvement in emotional regulation. For example, the extent of regional brain activation in prefrontal cortex measured by EEG could predict the ability of subjects to supress emotions- subjects with greater prefrontal activity showed an increased startle attenuation after an attempt to supress the startle response (Davidson, Putnam, & Larson, 2000). Research with humans and

primates have shown, that subjects with damage in prefrontal cortex areas have difficulty suppressing negative affect in response to a stimuli that has caused negative affect in the past (Davidson et al., 2000). It has been suggested that the suppression of negative emotions is based on inhibitory connections from prefrontal cortex to amygdala. This is supported by the finding that lesions in prefrontal cortex interferes with fear extinction in classical fear conditioning (Morgan et al., 1993), implying that PFC normally has an inhibitory effect on the amygdala, but as the PFC suffers from dysfunction, the amygdala starts lacking inhibition. Dysregulation in the activity of prefrontal cortex would be a good explanation why phobic fear is so difficult to extinguish and why there are many cases when the object causing phobia had not been paired with an unpleasant unconditioned stimulus. This would also explain why individuals with SP find it difficult to suppress their emotions, even when realizing, that they have no reason to be afraid.

The DLPFC has previously been linked to executive processes in working memory, so the role of this area in SP could also be linked to enhanced processing of phobia-related information (Phelps, 2006; Straube et al., 2004).

Or, the increased activity in DLPFC could be explained by the increased use of coping strategies aimed at the regulation of anxiety. This could also explain why, after CBT, the activity in DLPFC decreases, as the use of coping strategies also decreases (Straube, Glauer, et al., 2006).

As with inferior parietal lobule and insula, prefrontal cortex is also involved in the procession of facial expressions of disgust (Phillips & Young, 1997).

4.5 Increased grey matter density in right paracentral lobule

Unexpectedly and contrary to any other structural and functional MRI studies, the final distance in centimeters was also associated with increased grey matter density in right paracentral lobule ($p = 0.003$). Cluster size was 456 voxels, with peak voxel located at $x = 5$ $y = -24$ $z = 71$.

The paracentral lobule which is a U-shaped convolution located on the medial hemispheric surface that connects medial portions of postcentral and precentral gyri (Malobabić, 2013). It is the boundary between frontal and parietal lobes, which includes the primary motor and sensory areas for lower limbs and genitalia (Johns, 2014).

The anterior parts of PCL belong to BA4, which contains the primary motor area representing the muscles of urinary bladder, leg and foot. The posterior parts of PCL however, reflect primary somatosensory representation of leg and foot (Johns, 2014).

As we did not expect to find this area in our study, we would lean towards interpreting these results as a false positive. This could be due to any limitations in our study or limitations in VBM in general, which are described in chapters 4.9 and 4.10. Of course, further VBM studies on SP would help distinguish if this area is or is not involved in SP.

4.6 Increased grey matter density in vermis

Out of all subjective BAT fear score, only fear when the spider was taken away was correlated with grey matter density changes. This score was positively correlated with vermis density, with the cluster size of 468 and the peak voxel being located at x = 5 y = -56 z = -15.

This is in line with the other VBM study on SP (Hilbert et al., 2015), that found an increased right vermis density in phobic patients, although no functional studies have found the involvement of vermis in SP.

Mostly motor functions have been attributed to cerebellar vermis. In recent years, however, a structure called the limbic cerebellar vermis has been discovered. Some cognitive, emotional and affective responses have been attributed to the limbic cerebellar vermis and studies are showing that vermis could also play a role in neuropsychiatric disorders (Bambico et al., 2018). For example, a study found that patients with vermis pathology experience personality changes,

diminished affect and disinhibition (Schmahmann & Sherman, 1998). Other studies have shown that patients with depression have an increased blood flow to the cerebellar vermis (Liotti, Mayberg, McGinnis, Brannan, & Jerabek, 2002). Moreover, there have been some studies suggesting that vermis has a role in modulation of anxiety. A microinjection of histaminergic agonist into vermis can induce an inhibitory effect on anxiety and memory consolidation (Fernandes, Serafim, Gianlorenco, & Mattioli, 2017).

The polysynaptic projections from vermis to several limbic structures and prefrontal cortex would put vermis in an ideal position to influence emotional processes, including fear (Allen & Courchesne, 1998). The stimulation of vermis modulates the activity of limbic structures, evoking responses in ACC, amygdala, hippocampus and hypothalamus (Anand, Malhotra, Singh, & Dua, 1959).

Vermis also has a role in the regulation of cardiac responses in fear conditioning (Nisimaru, 2004). This has been discovered by observing changes in blood pressure and heart rate after the stimulation of cerebellar vermis in rabbits (Nisimaru, Yamamoto, & Shimoyama, 1984). It has been shown that lesions in the region of vermis limit the acquisition of conditioned fear responses such as bradycardia, but they do not affect the heart rate in the response to conditioned stimulus (Supple & Leaton, 1990). The inactivation of cerebellar vermis with an injection of AMPAR receptor antagonist inhibited the expression of conditioned bradycardia in mice (Kotajima-Murakami, Narumi, Yuzaki, & Yanagihara, 2016)

4.7 The influence of spider phobia severity on regional GMD- possible causes

For now, we cannot clearly explain the connection between spider phobia severity and regional grey matter density. From our study and previous studies, it is becoming clear, that most regions showing structural brain changes in SP are responsible for functions, which are closely linked to the characteristics of SP, including exaggerated assessment of threat, the ability to control anxiety responses and visceral, autonomic and attentional processes (Rauch et al., 2004). So, we can propose that the changes in volume represent functional

changes in these regions. This is also confirmed by the overlap of brain regions being found both in structural and functional neuroimaging. However, it has not been confirmed that these changes are responsible for the development of phobia. It is also possible that the abnormal regional brain volumes could be a consequence of the disorder. SP could cause an overdrive of certain brain circuits in critical stages of neuronal development, in a way that produces volume and density abnormalities (Rauch et al., 2004). Additionally, abnormalities in brain volume could represent compensatory changes caused by pathological processes in other brain regions (Rauch et al., 2004). This is supported by the fact that the number of regions affected in SP found in studies is quite large, considering the limited symptoms of SP.

In association with neurodegenerative disorders, it clear that the decrease in grey matter is directly dependent on the loss of neurons (Baron et al., 2001). In healthy subjects, however, the mechanism of volume changes in not clear. Some studies suggest that changes in grey matter are caused by increased use of certain brain regions. For example, a study discovered, that a 3 months long juggling training increased grey matter density in the hippocampus (Draganski et al., 2004), this has also been true for other physical exercise (Killgore, Olson, & Weber, 2013). Increase in grey matter density could also be induced by a 5-day rTMS (May et al., 2007). These fast changes in volume could more likely be explained by processes such as synaptic plasticity and not neurogenesis and glia genesis, because these processes take much longer (Liebau, 2010). Increase in grey matter volume can also be induced by medication. A 4-week long administration of lithium at therapeutic doses induced a significant increase in volume in ACC and dorsolateral prefrontal cortex (Monkul et al., 2007).

Additionally, we also must consider the relationship between results found in fMRI and MRI studies. Even though a lot of the same regions show up in both types of studies, the relationship between increased/decreased density and volume and functional alterations is still not examined sufficiently. A thesis study examining the relationship between changes in fMRI and MRI results after a mirror reading training found that fMRI and MRI scans both showed change in the same brain

regions (superior parietal cortex and dorsolateral occipital cortex). Interestingly, this study also showed no clear relationship between activation and volume changes. Even though superior parietal cortex activity showed a positive correlation with volume changes, dorsolateral occipital cortex activity was negatively correlated with volume changes (Liebau, 2010). There is a possibility that increased activity is responsible for decreased volume, caused by residual cells trying to manage the workload (Linares et al., 2014). This would explain why some regions that show increased density (like prefrontal regions), can show decreased activity in fMRI studies.

An increased volume could be an indicator of greater neuronal volume (caused by branching of dendrites), greater number of neurons or even an increased volume of glial tissue. It could also be explained by insufficient pruning of axons and synapses in childhood and adolescence (Landing, Shankle, Hara, Brannock, & Fallon, 2002). Synaptic pruning is the brain`s way of remodeling neural connections, and dysfunctional pruning can lead to disruptions in the processing of perception, language, consciousness and learning. The association between phobic disorders and a dysfunctional pruning is also supported by the early onset of phobic disorders (Becker et al., 2007).

It is also worth mentioning that in comparison to other types of anxiety disorders, SP usually shows decrease in cortical thickness and regional brain density, whereas other anxiety disorders are characterized by decreased density. This might be related to the unresponsiveness of SP to SSRIs (Rauch et al., 2004).

4.8 Linear regression and two sample T-test: different outcomes

Other studies in this field have found significant between-group differences in patients and control subjects. Our between-group comparison did not find any significant difference. For us, the results of significance were limited to multiple linear regression analysis of regional GMD and spider phobia score in subjects with spider phobia.

There is no clear explanation of these results. We did not screen our control group for spider phobia, so it is possible that we didn't find between group differences due to having spider phobic patients or patients with other phobias in our control group.

The score correlating with most brain region densities and showing the strongest correlation was the SPQ. SPQ can be viewed as a subjective assessment of fear. A contrast to this is the final distance in centimeters, which is rather objective, but was only correlated with one (and rather unexpected) regional GMD. The subjective fear score can be affected by different factors unrelated to the phobia itself. For example, the overall anxiety during experiment could make the subjects report higher values in SPQ. It could then be possible, that the correlated regions are not responsible for only spider phobia, but anxiety in general. Using an anxiety measure such as BAI or ASI as a covariate could provide more information on this matter.

Even though our spider phobic patients were selected carefully, and it was made sure that the fear of spiders is significant, it could be that only the patients with extreme fear of spiders develop changes in regional GMD. This could explain why the brain of the spider phobic group in general was no different from the brain of HC.

4.9 Limitations of our study

As one of the largest limitations of this study, the number of subjects must be mentioned. Even though other structural neuroimaging studies have used a similar number of subjects (10:20 (Rauch et al., 2004); 19:17 (Linares et al., 2014); 20:20 (Fisler et al., 2013); 26:33:37 (Hilbert et al., 2015)) with the same being true for fMRI studies (Del Casale et al., 2012), a larger number of spider phobic patients could help avoid any false positive results. Another large limitation is that a part of our control group was not screened for spider phobia or other types of SP, so we cannot rule out that some of our control patients suffered from SP.

Studies have shown that brain regional volumes could be impacted by Anxiety Sensitivity, which is increased in patients with specific phobia (Rosso et al., 2010). It has even been suggested that anxiety traits and not SP itself could be responsible for regional brain volume differences in SP patients (Rosso et al., 2010). Even though psychiatric exclusion criteria were used to minimize clinical presence of other psychiatric disorders, no data on Anxiety Sensitivity was included in this study. It would also be useful to use other clinical score such as BDI-II even if the patients don't qualify for a diagnosis of depression, to see if brain density changes are correlated with depressive symptoms.

It has been reported, that brain structures can change volume in females according to the phase in menstrual cycle, so conducting the brain imaging during the luteal phase of the menstrual cycle in female subjects could have yielded more accurate results (Fisler et al., 2013; Ossewaarde et al., 2011). Some studies have also reported differences in regional brain matter density in patients with different BMI, so using BMI as a covariate of no interest could also make the results more precise (Kennedy, Collins, & Luciana, 2016).

Not using FWE correction and threshold masking in our VBM statistical analysis was a rather liberal decision and could have led to inaccurate results. It is worth to mention, however, that the unidirectional nature of our results (there were no regions of decreased density) and the involvement of grey matter only argue, that our results are valid. Additionally, not using age as a covariate but conducting a separate VBM analysis to investigate the relationship between grey matter density and patient`s age would have led to different results compared to the method we used to check the influence of age.

All of the above-mentioned limitations could have caused the differences in the results of our study and other structural studies. This could also explain why we didn't find any differences between patients and controls but found a correlation between fear score and brain structure densities.

4.10 Limitations of structural and functional brain studies in the field of psychiatry

Even though studies in this field have found changes in a lot of the same regions, there is no doubt that many results cannot be replicated or are contradictory. In earlier years, the functional symptom provocation studies had no control groups, so the brain activity of only spider phobic patients was assessed. Even though this gave information about regions involved in phobia, the physiological processes could not be distinguished from the pathological processes (Rauch et al., 2004). Due to this, we tried to not compare our results to those of earlier studies.

As for structural studies in SP, all studies except two have only analyzed previously chosen regions (ROI studies), leaving out a large part of brain structures, that could possibly be involved in phobia (Rauch et al., 2004). Additionally, these ROI studies largely depend on elaborate region maps that can be imprecise. The use of a method that can analyse the whole brain can be mentioned as strength of our study. Also, two VBM studies are not enough to draw conclusions, so other VBM studies must be conducted.

Different parameters of VBM statistical analysis can also vary from study to study, making studies difficult to compare to each other. For example, even though almost all studies use a multiple comparison correction (Scarpazza, Tognin, Frisciata, Sartori, & Mechelli, 2015), the differences between studies are found in the use of threshold masking, the extend of the threshold, the use of FWE and FRW, threshold size for clusters and the Gaussian Kernel used for smoothing. Additionally, different covariates are used during the between-group comparisons (Scarpazza et al., 2015). A development of guidelines for VBM analysis could be a solution to this problem.

There has also been criticism directly addressed towards VBM. VBM results are largely dependent on the quality of MRI images used for analysis. Within different VBM studies, differences can occur due to the use of different scanners, magnetic field strength and the protocol for acquisition of the MRI data (Scarpazza et al., 2015). For this reason, it is important to use the same scanners for all subjects in

the same study. It is also extremely important that patients hold still during the acquisition of MRI images. This can be difficult during studies where an aversive stimulus is being presented. However, this should not have caused a problem in our results, as all MRI images were inspected for their quality.

Secondly, the MRI images of patients with large structural brain abnormalities could also influence the VBM results. This, however, is also irrelevant for our study, as any brain pathologies were checked for after the acquisition of MRI images. However, even a healthy brain has large between-subject variability and VBM is known for being insensitive to these regions (Good et al., 2001). It is not clear, if the changes in grey substance detected by VBM are a result of concentration or volume changes, so different studies report this aspect differently. Additionally, several studies have proven, that structural differences measured by MRI are not necessarily explained by pathological changes due to a disease. Brain structure volume can be influenced by changes in perfusion, changes in fat and water concentration in brain tissue, medication, nutrition and hormonal fluctuations (May & Gaser, 2006). For example, the ventricle size experiences changes with fluctuations in blood glucose levels (Puri, Lewis, Saeed, & Davey, 1999). VBM also has problems analyzing brain regions that cannot easily be classified as grey or white substance such as the brainstem or the thalamus. So, if the differences in brain structure in SP patients are located in any of these brain regions, it is possible that they have not been detected (Good et al., 2001).

The previous versions of VBM have been susceptible to partial volume effect. They classified each voxel in either grey or white matter. A lot of voxels in the brain, however, include several tissue classes. This is the case for grey and white matter borders and the brain tissue located around ventricles. This lead to false classification of tissue (Ashburner & Friston, 2000). This effect has been accounted for and corrected in SPM12 (http://www.neuro.uni-jena.de/cat12/CAT12-Manual.pdf).

Even though it has normally been assumed that the smoothing process is enough to ensure normal distribution of data, it still often leaves the data non-normally distributed, which can lead to false statistical analysis (Scarpazza et al., 2015).

It has also been reported, that VBM suffers from a large quantity of false positive results in general. For example, a single subject versus a matched control group analysis using an extend threshold of 10 voxels and a voxel-wise threshold of $p < 0.05$ (corrected) reported, that the chance of finding at least one significant region of abnormality in a healthy subject was 93.5% for increased brain volume and 71% for a decreased volume (Scarpazza, Sartori, De Simone, & Mechelli, 2013). The largest chance of discovering a false positive result was reported in frontal and temporal cortical regions, the lowest chance- in subcortical regions (Scarpazza et al., 2013). This is somewhat worrying, considering that our results were limited to cortical regions. This study however concentrated on single subject versus group VBM analysis, so it has not been proven that these problems are also present in group comparisons.

In contrast, a study conducted by the same study group found that false positive rates are not, in fact, typical for between-group analysis (Scarpazza et al., 2015). While comparing equally sized groups of healthy individuals, a false positive result rate of no more than 5% was reported, regardless of the sample size and smoothing applied (Scarpazza et al., 2015). Interestingly, this study also found that false positive results were not limited to frontal and temporal regions but were equally distributed across the whole brain (Scarpazza et al., 2015).

4.1.1 Future directions in structural neuroimaging in specific phobia

Considering that voxel-based morphometry has proven to be relatively reliable method for MRI data analysis and that the methods used in VBM keep developing (Scarpazza et al., 2015), further studies in this field could help paint a clearer picture of structural changes in SP.

To our knowledge, there has only been one other VBM study researching specific phobias. As this study included only snake and dental phobias, this is the first

and only analysis of structural whole-brain changes in spider phobia. To confirm the validity of our results, other studies of similar nature need to be conducted, ideally, with larger count of subjects and HC that have specifically been screened for spider phobia. In addition to this, there have not been any other studies investigating the correlation of SP intensity and brain density changes.

Up to this point, we also don`t have an answer to weather the structural changes found in SP are the cause of the disease or the disease causes changes in brain structure. Conducting an MRI study on a larger population before and after the onset of SP could answer this question. Another way to find out more about the mechanisms behind the structural brain changes would be to examine the brain on a cellular level to find out if they are associated with changes in the neuropil, the size of neurons or the branching of dendrites (May & Gaser, 2006).

It has been suggested, that the changes of grey matter density in patients with specific phobia are caused by neuronal pruning (Rauch et al., 2004). Thus, it would be worthwhile to examine, if interference with neuronal pruning in animals could lead to symptoms similar to those of SP (Rauch et al., 2004). We also have no information about the relationship between structural density alterations and the changes in functional activity. Even though regions such as ACC and insula show both higher density and higher activity, there are regions such as DLPFC, whose higher density is associated with lower activity in fMRI. So, it would be useful to conduct fMRI imaging on patients with structural changes in brain regions associated with SP, to see the direction of changes of brain activity.

A trend that has been mentioned in other studies is the relationship between brain density and response to SSRIs (Rauch et al., 2004). In other anxiety disorders (that are responsive to SSRIs), grey matter density and cortical thickness in ACC seems to be reduced (Shang et al., 2014), whereas it is increased in SP, which is also unresponsive to SSRI. So, it should be established, if cortical thickness and brain density could have a predictive effect on the success of SSRI treatment.

Another use of structural neuroimaging in the exploration of treatment effectiveness could be in the field of CBT. fMRI studies have observed that CBT

therapy can normalize brain activity in SP. So, it could be observed, if the structural changes are influenced by CBT as well or if brain structure can be used as biomarker for the success of CBT.

5 Summary

In this study, we examined the regional grey matter density in 35 spider phobic patients and 33 age, gender and education matched healthy controls. We used a method called Voxel-Based Morphometry, which allowed us to conduct a voxel-by-voxel analysis of the entire brain. We also tried to determine if there was any relationship between the severity of fear (expressed in BAT and SPQ score) and grey matter density. Based on previous findings, we expected to find structural changes in the following brain regions:

- prefrontal cortex;
- orbitofrontal cortex;
- anterior cingulate cortex;
- insula;
- visual and associative cortices.

Between-group comparison of spider phobic patients and healthy controls yielded no significant results. Additionally, and as expected, we did not find a between-group difference in TIV. Surprisingly, however, we found several brain regions whose GMD was significantly correlated with severity of spider phobia.

The score that correlated with several regions GMD and yielded the largest cluster was the SPQ. SPQ was positively correlated with dorsal anterior cingulate, right insula and left inferior parietal lobule. Final distance in centimetres was correlated with left superior frontal gyrus and right paracentral lobule densities. All correlations were observed at a cluster level and no significant results at peak level were found. Interestingly, out of all BAT fear values, only BAT when the spider was taken away had a positive correlation with GMD (vermis). There were no indications of reduced GMD in spider phobic patients.

Overall, our regions of significance were in line of those of other structural and functional neuroimaging studies in the field of specific phobia. As expected, we found GMD changes in the prefrontal cortex, ACC, insula and the associative

cortices. The functions of these regions such as processing of disgust, attention, autonomous responses, consolidation of memory and regulation of affect support the possible involvement of these structures in SP.

We did, however, also yield some unexpected results (vermis, right paracentral lobule). Interestingly and in contrast to other studies, our results were only limited to the phobic group itself- we found no regions of significance in the SP-HC between-group analysis.

In the future, more VBM studies with larger size of spider phobic subjects should be conducted, further investigating both the between-group differences and the correlation between spider phobia severity and GMD. Additionally, studies should investigate the relationship between structural changes and activation patterns observed in fMRI, find out whether brain changes precede the clinical symptoms or vice versa and see, if structural changes normalize in response to CBT the same way functional changes do.

6 References

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7 Appendix

7.1 Summary of structural brain differences in specific phobia.

Study type	Animal phobia	Dental phobia	Common
Rauch et al, 2004; cortical thickness	SPIDER PHOBIA Increased whole brain cortical thickness; t (1284) = 3.19, p = 0.001 <u>Paralimbic cortex</u> • Increased insular cortex thickness (bilateral); p <5 x 10^{-4} • Increased anterior cingulate cortical thickness (bilateral); p <5 x 10^{-4} • posterior cingulate cortex (bilateral); p <5 x 10^{-4} <u>Sensory cortex</u> • increased occipital cortex thickness; p <5 x 10^{-4} • increased occipitotemporal • cortex thickness (left); p <5 x 10^{-4}		

Linares et al, 2014; cortical thickness	SPIDER PHOBIA Cortical thinning of the rostral part of ACC; t (34) = 3.19, p = 0.001		
Rosso et al, 2010; ROI volume and cortical thickness	SMALL ANIMAL PHOBIA (SPIDERS; RODENTS, SNAKES) Positive correlation between ASI score and right anterior insula thickness in SAP subjects; r = 0.57, df = 17, p = 0.01 Positive correlation between ASI score and right anterior insula volume within the sample; r = 0.47, df = 35, p = .003		
Fisler, 2013; RIO analysis	SPIDER PHOBIA Decreased left amygdala volume; F [3, 36] = 6.39; p= 0.02 Negative correlation between SPQ score and left amygdala volume; r = -0.47; p = 0.005		
Hilbert et al, 2012; VBM grey matter analysis	SNAKE PHOBIA Postcentral gyrus (left); t = 3.71, p<0.001	Anterior cingulate gyrus	Anterior cingulate gyrus (right); t = 4.30, p<0.001

cont. Hilbert et al, 2012; VBM grey matter analysis		(right); t = 4.08, p<0.001 Superior frontal cortex (left); t = 4.09,3.89, 3.81, p<0.001 Fusiform gyrus (right); t = 4.18, p<0.001 Insula (left); t = 4.49, p<0.001 Lingual gyrus (right); t = 5.15, p<0.001 Medial occipital cortex (right); t = 4.4.56, p<0.001 Inferior occipital cortex; t(right)= 3.97, p<0.001; t(left) = 4.30, p<0.001 Orbitofrontal cortex; t(medial) = 3.65, p<0.001; t(superior) = 3.62, p<0.001 Superior parietal cortex; t(right); = 4.14,	Calcarine sulcus (right); t = 3.52, p<0.001 Fusiform gyrus (right); t = 3.74, p<0.001 Precuneus (left); t = 4.03, p<0.001 Medial orbitofrontal gyrus (left); t = 4.11, p<0.001 Vermis (right); t = 4.38, p<0.001

		p<0.001; t(left) = 3.52, p<0.001 Cerebellum (right); t = 3.73, p<0.001	