Using novel imaging approaches in affective disorders : beyond current models

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Using novel imaging approaches in affective disorders: beyond current models

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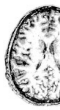
Voor mijn familie

die was, is, en nog zal zijn

Contents

Chapter 1
Introduction



Prevalence and clinical characteristics

Depression and anxiety are among the most prevalent psychiatric disorders worldwide. Whereas major depressive disorder (MDD), with lifetime prevalence estimates of over 20%, classifies as the most common disorder with the heaviest burden and disability, anxiety disorders are the most common class of disorders with a combined lifetime prevalence of 28% (Kessler *et al*., 2005a, Ressler and Mayberg, 2007). The average age of onset for MDD is in the mid-20s but the median age is 30 years; however, the disorder may occur at any age (American Psychiatric Association, 1994, Kessler *et al*., 2005a). Notably, anxiety disorders have a much earlier age of onset than MDD with a median of 11 years (Kessler *et al*., 2005a). Depression and anxiety disorders often have an onset in adolescence, and an early onset is associated with more disease severity and disability (Paus *et al*., 2008, van Noorden *et al*., 2011). Studies show that anxiety symptoms predict and often precede depression in adolescence (Wittchen *et al*., 2003, Beesdo *et al*., 2007), which is consistent with evidence of the earlier onset of anxiety than depression.

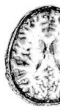
A person diagnosed with MDD will suffer from one or more major depressive episodes, which are characterised by the presence of depressed mood and/or loss of interest or pleasure over a consecutive period of two weeks, as well as five of the following: weight loss that is not a result of dieting, or weight gain; insomnia or hypersomnia; psychomotor agitation or retardation; fatigue or loss of energy; feelings of worthlessness or guilt; diminished ability to concentrate; and recurrent thoughts of death or suicide. These symptoms are not due to another psychiatric disorder, secondary to a medical condition, or accounted for by bereavement (American Psychiatric Association, 1994).

MDD is associated with high morbidity, subjective suffering, impaired social and work functioning, and high mortality. The resulting substantial disability that is caused by MDD is in turn responsible for a high economic burden (Cassano and Fava, 2002). Females have a greater risk of developing MDD than males both in adulthood and adolescence, and individuals with a first-degree relative with MDD are more likely to develop MDD than the general population. Furthermore, recurrence of depressive episodes is high and as the number of episodes an individual has experienced increases, so does the chance of having additional episodes (American Psychiatric Association, 1994, Kessler *et al*., 2005a).

Other strong predictors of recurrence and duration of the depressive episode are severity and comorbidity with other psychiatric disorders (Melartin *et al*., 2004).

Indeed, MDD frequently co-occurs with anxiety disorders (Gorman, 1996, Ressler and Mayberg, 2007). Presence of anxiety disorders in depressed patients is associated with increased symptom severity, lower response to treatment, and poorer prognosis than in patients with only one disorder, and it is even a predictor of suicide (Fawcett, 1992, Gorman, 1996). Conversely, patients with anxiety disorders often report symptoms of depression, and it is estimated that more than 30% of patients with an anxiety disorder will meet the criteria for a clinical diagnosis of depression during their illness (Gorman, 1996).

In adults, three of the most frequently co-occurring anxiety disorders with MDD are social phobia or social anxiety disorder (SAD), generalised anxiety disorder (GAD), and panic disorder (PD) (Gorman, 1996, Kessler *et al*., 1996). Social anxiety disorder is characterised by a distinct fear of social or performance situations in which embarrassment or humiliation may occur. Exposure to the feared social situation will invariably lead to an anxiety response. Individuals with SAD will typically fear that others perceive them as weak, crazy, or stupid, and that others will notice their anxiousness, for example due to trembling hands or voice. There is recognition that the fear is excessive or unreasonable. Feared situations will be avoided or otherwise endured with intense anxiety or distress, and the fear significantly interferes with the person's normal routine and daily functioning (American Psychiatric Association, 1994).
The most essential feature of generalised anxiety disorder is excessive general anxiety and worrying about a number of events, and difficulty to control the worry, which will occur more days than not for at least 6 months. Other symptoms include restlessness, being easily fatigued, difficulty concentrating, irritability, muscle tension, and disturbed sleep. Individuals with GAD report subjective distress and impaired daily functioning due to constant worry. The symptoms do not result from another psychiatric disorder or substance abuse (American Psychiatric Association, 1994).
Panic disorder is marked by recurrent unexpected panic attacks and at least one month of persistent concern about having additional attacks and their possible consequences. These panic attacks cannot be induced by substance use or be better accounted for by another mental disorder. The



unexpected panic attacks are not associated with a situational trigger, and are thus defined as spontaneous and uncued. A minimum of two attacks is required for diagnosis; however, many individuals experience more. Typical characteristics of a panic attack are a discrete period of intense fear and a number of the following symptoms: palpitations, sweating, trembling or shaking, shortness of breath, feeling of choking, chest pain, nausea, feeling dizzy or faint, derealisation (feeling of unreality) or depersonalisation (being detached from oneself), fear of losing control or going crazy, fear of dying, numbness or tingling sensation, and chills or hot flushes. Individuals with PD may or may not present with agoraphobia, which is characterised by anxiety about being in places or situations from which escape and in which getting help might be difficult, in the event of having a panic attack. Such situations are avoided or endured with marked distress (American Psychiatric Association, 1994).

Diagnostic classification

Clinical diagnosis according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) means that a disorder is either present or absent, and based on whether diagnostic criteria are met. It is of note that upon the start of the current project, the fifth edition of the DSM was still under development, and diagnoses according to the DSM in the project were based on the DSM-IV.

Initial research on depression and anxiety disorders has mostly proceeded along different tracks, reflecting the diagnostic separation between the two classes of disorders. However, with regard to the distinction between depression and anxiety, there is a longstanding and ongoing debate as they co-occur so frequently, have overlapping symptom criteria and phenomenology, respond to similar types of treatment (including pharmacotherapy with selective serotonin reuptake inhibitors (SSRIs) as well as cognitive behavioural therapy CBT)), and the same brain circuits are involved in both types of disorders (Gorman, 1996, Ressler and Mayberg, 2007). In this light, some researchers advocate that depression and anxiety disorders share the same aetiology (Ressler and Mayberg, 2007) and should be best conceptualised by a continuum (Stavrakaki and Vargo, 1986), or using a dimensional approach (Simms *et al*., 2008). The ways in which these disorders are diagnosed and classified have important implications for clinicians and patients, in terms of prognosis and treatment (Simms *et al*., 2008).

Neuroimaging

One of the approaches to study the neurobiology underlying depression and anxiety is neuroimaging, which provides the opportunity to investigate the brain in vivo in a safe and non-invasive way. This enables studying the anatomical and functional characteristics of the brain and or specific regions of the brain. Technical advances in neuroimaging methods, magnetic resonance imaging (MRI) and functional MRI (fMRI) in particular, have contributed greatly to our knowledge of the human brain. This young field of research is still rapidly expanding. Studies of healthy individuals give an ever-increasing insight in the morphology and functioning of the 'normal' brain, and facilitate comparison with the brain of persons with a (neuro)psychiatric disorder. Similarly, MRI research in healthy youngsters helps create an understanding of the normally developing brain, which provides a frame of reference for developmental difficulties that can have far-reaching consequences for psychological functioning.

Structural MRI enables the examining of the anatomical characteristics of the brain, and the structural connections between brain regions (Ashburner and Friston, 2000, Basser *et al.*, 1994). Functional MRI is based on the assumption that active brain regions require more oxygen and thus have an increased blood flow. The ratio between oxygenated and de-oxygenated haemoglobin changes with brain activation. In other words, an increased amount of oxygenated blood is delivered to active brain regions because they have a higher energy demand. With fMRI, images sensitive to changes in the concentration of de-oxygenated haemoglobin are mapped, which is known as the blood oxygenation level-dependent (BOLD). This is reflective of increased activity of the site (Ogawa *et al.*, 1990). By measuring the changes in oxygen consumption during sensory or cognitive processing using fMRI, it can be inferred what areas of the brain are activated during the presumed function. Therefore, the execution of specific sensory, cognitive and motor tasks are widely employed to investigate the function of brain regions.

More recently it was observed that the BOLD signal is not only useful for studying task-related brain activity, but it also provides information about the brain when it is not engaged in goal-directed behaviour (Biswal *et al.*, 1995, Raichle *et al.*, 2001). Technical advances have allowed the application of fMRI to the resting brain; that is, measuring brain activity whilst the individual is in the scanner, but in the absence

of stimuli or tasks. So-called resting-state fMRI has become a powerful tool to examine the unconstrained, spontaneous activity of, and connectivity between, brain areas (Biswal *et al.*, 1995, Fox and Raichle, 2007). When various brain regions simultaneously show the same spontaneous fluctuations of the BOLD signal during resting-state, they are defined as functionally connected. Such collections of correlated brain regions are known as functional connectivity networks. Studying network interactions are not only important for an understanding of the brain in general, but also for an understanding of the abnormalities that accompany mental illness. Resting-state fMRI also allows researchers to study brain connectivity as a whole rather than just the brain regions that are activated during task fMRI. Additionally, overcoming potential limitations of task-related fMRI for severely ill patients who are less capable or even unable to cope with the cognitive burden of tasks, RS-fMRI can provide an outcome in studying debilitating neuropsychiatric disorders.

Neuroimaging in depressed adults and adolescents

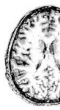
Volumetric studies indicate that several brain areas show anatomical abnormalities in MDD patients compared to healthy control subjects. Reductions in grey matter volume have been most consistently reported in the anterior cingulate cortex (ACC) and orbitofrontal cortex, as well as the hippocampus, putamen, and caudate nucleus (Koolschijn *et al.*, 2009). Structural findings from studies on adolescent depression are scarce, but point to similar brain regions and also report volume differences in the amygdala (MacMillan *et al.*, 2003, Caetano *et al.*, 2004, Rosso *et al.*, 2005). These areas are known to be involved in stress and emotion regulation.

Widely used paradigms in task fMRI studies of MDD involve the presentation of pictures, scripts of autobiographical memory, words, and faces. These stimuli have an emotional content and are used to induce a positive (happy) or negative (sad) emotion. Studies focusing on happy stimuli reported altered activation in depressed subjects of the cerebellum and various frontal, posterior and temporal cortical regions. Studies that used negative stimuli found altered activity of frontal, temporal, parietal and posterior cortical regions, the cerebellum, insula, and the amygdala (Fitzgerald *et al.*, 2008). Frequently reported resting-state functional connectivity differences in adult depression include decreases in pregenual anterior and posterior cingulate, middle frontal gyrus, insula, and

superior temporal gyrus. Areas showing hyperactivation include the thalamus, amygdala, and superior frontal and middle temporal gyrus (Fitzgerald *et al.*, 2008). Additionally, increased thalamic and subgenual ACC connectivity was reported in depressed subjects (Greicius *et al.*, 2007). Remarkably, only one preliminary resting-state study has been performed in depressed adolescents compared to healthy controls. However, in addition to a primary diagnosis of MDD most of the patients had one or more comorbid diagnosis and were treated with medication. Decreased connectivity was found in the depressed group in a subgenual ACC-based neural network including the supragenual ACC, right medial frontal cortex, left inferior and superior frontal cortex, superior frontal gyrus, and insula (Cullen *et al.*, 2009). This network is involved in emotion processing (Kober *et al.*, 2008, Cullen *et al.*, 2009).

Neuroimaging in adults and adolescents with anxiety disorders

In adult PD, structural studies pointed to a reduction of grey matter in temporal regions, particularly the hippocampus (Vythilingam *et al.*, 2000, Uchida *et al.*, 2003, Massana *et al.*, 2003a, Kent and Rauch, 2003) and amygdala (Massana *et al.*, 2003b, Hayano *et al.*, 2009). However, grey matter abnormalities were also reported in the ACC (Uchida *et al.*, 2008) and brain stem (Protopopescu *et al.*, 2006). Initial functional studies in PD have used positron emission tomography (PET) and single photon emission computerised tomography (SPECT), and most consistently indicated alterations of blood flow of hippocampal regions (Reiman *et al.*, 1986, Nordahl *et al.*, 1990, De Cristofaro *et al.*, 1993, Kent and Rauch, 2003, Sakai *et al.*, 2005). Task fMRI studies have also found altered activity in cortical regions as the anterior and posterior cingulate (Bystritsky *et al.*, 2001) and the amygdala (Pillay *et al.*, 2006). Symptom-provocation paradigms, where an anxiety state is intentionally induced through the use of pharmacologic or behavioural manipulations, point to reduced activity in widespread cortical regions including the prefrontal cortex (PFC), and increased activity in insular and motor striatal regions (Kent and Rauch, 2003). To date, no resting-state studies in PD have been done. Taken together, neuroimaging findings in PD suggest that abnormalities in the hippocampal regions may be a trait marker for the disorder. However, symptom provocation studies have also indicated the more global involvement of widespread cortical regions, including the PFC.



For SAD there is only one volumetric study, which did not report any grey matter differences between patients and healthy controls (Potts *et al.*, 1994). Functional studies in SAD, the majority of which employed emotional faces tasks or symptom provocation paradigms involving the anticipation of public speaking, consistently report increased amygdala activation and decreased frontal cortical activation (Kent and Rauch, 2003, Etkin and Wager, 2007). To date, there are only two resting-state studies in SAD. The first, using SPECT, did not report any differences in regional cerebral blood flow (Stein and Leslie, 1996). The second, more recent SPECT study reported increased resting perfusion in the frontal cortex and right cerebellum, and decreased perfusion in the pons, left cerebellum, and right precuneus (Warwick *et al.*, 2008). No resting-state fMRI studies have been done in SAD. In sum, the most prominent and consistent findings in SAD are an exaggerated amygdala response to human face stimuli or to the stress of public speaking.

There is very little neuroimaging data available for GAD. The first two volumetric studies were in a paediatric sample, reporting increased right and total amygdala volumes (De Bellis *et al.*, 2000) and larger total, white matter, and grey matter volumes of the superior temporal gyrus in children with GAD (De Bellis *et al.*, 2002b). One study in adults found increased grey matter of the centromedial nucleus of the amygdala (Etkin *et al.*, 2009). Findings of functional studies include increased activation of the ventral PFC in adolescents with GAD compared to healthy control subjects, whilst viewing angry faces (Monk *et al.*, 2006). The same group also reported that disturbed amygdala activation was associated with dysfunctional ventral PFC activation in adolescents with GAD (McClure *et al.*, 2007, Monk *et al.*, 2008). In adults, relative to healthy controls, GAD patients showed an increased response to angry versus neutral faces in the middle frontal gyrus, inferior temporal cortex, and the culmen. They also showed decreased amygdala activation during fearful versus neutral faces (Blair *et al.*, 2008b). By contrast, one study did not report differences in amygdala activity between GAD patients and healthy controls (Whalen *et al.*, 2008). One resting-state study investigated functional connectivity of subregions of the amygdala, and found less distinct connectivity patterns of the basolateral and centromedial nuclei in GAD patients, as well as increased grey matter in the centromedial nucleus. Additionally, increased connectivity was found with a frontoparietal executive control network,

and decreased connectivity with an insula- and cingulate-based salience network (Etkin *et al*., 2009). Compared to other anxiety disorders, GAD is notably understudied. However, the few existing research findings point to a disruptive activation of the amygdala and to a lesser extent of the ventral PFC.

Neurobiological models of depression

MDD is characterised by a variety of symptoms of a cognitive (e.g. attentional and concentration problems), emotional (e.g. depressed mood, anhedonia, feelings of worthlessness) and somatic (fatigue, psychomotor agitation, sleep disturbances) nature (American Psychiatric Association, 1994). It is therefore unlikely that depression is the result of dysfunction of one particular brain area. Rather, as in an influential model by Mayberg (1997), it is suggested that an array of brain regions and, more specifically, maladaptive functional interactions of cortico-limbic brain regions are involved in depression. Since such interactions are critical for the normal regulation of mood and associated processes, dysfunctional interactions are proposed to underlie depressive symptomatology (Mayberg, 1997, Seminowicz *et al*., 2004). Mayberg's model comprises three components and dysfunction of each of those is related to specific symptom sets. An effective collaboration between the three components is key to the maintenance of an intact mental state. The dorsal component, encompassing neocortical and midline limbic elements such as the PFC, dorsal ACC and striatum, is linked specifically to attentional and cognitive features of depression. A ventral component includes paralimbic (sub)cortical regions (such as the hypothalamic-pituitary-adrenal axis, insula, and subgenual cingulate) and brainstem regions. This element is hypothesised to mediate the vegetative and somatic aspects of depression. Thirdly, a rostral cingulate component is considered to have a regulatory role in the overall network by facilitating the interactions between the other two components (Mayberg, 1997) (Figure 1).

In her functional neuroanatomical model of normal emotion perception, Phillips and colleagues (2003) describe three processes that are key for emotion perception: the identification of the emotional significance of a stimulus (i.e. salience); the production of an affective state in response to the aforementioned; and regulation of the affective state. The authors

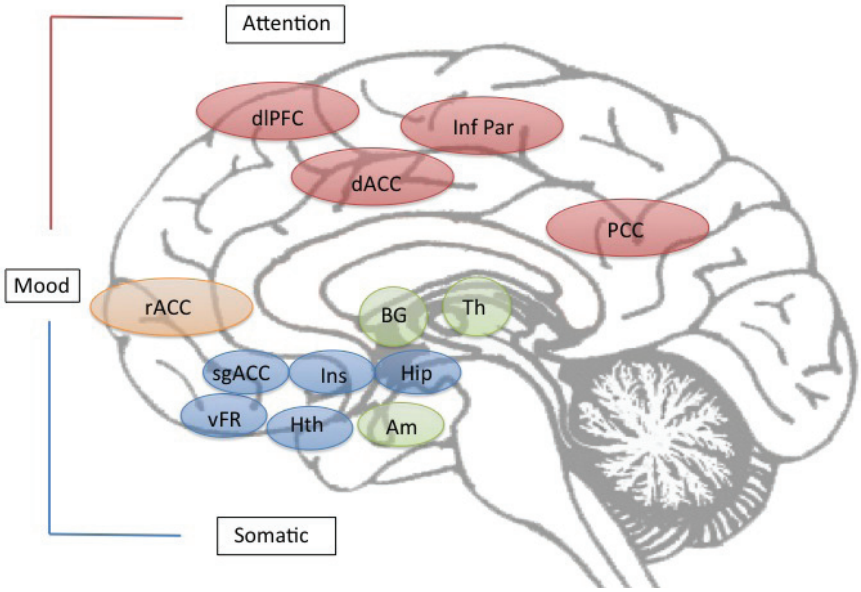


Figure 1. Representation of Mayberg's model on depression
The three systems (dorsal, red; ventral, blue; rostral, orange) are depicted. Correct regulation of each of these systems as well as an effective collaboration between them is critical for the maintenance of an intact mental state.
Abbreviations: dlPFC, dorsolateral prefrontal cortex; dACC, dorsal anterior cingulate; Inf Par, inferior parietal cortex; PCC, posterior cingulate; rACC, rostral anterior cingulate; BG, basal ganglia; Th, thalamus; sgACC, subgenual anterior cingulate; vFR, ventral frontal cortex; Ins, insula; Hth, hypothalamus; Hip, hippocampus; Am, amygdala.
Simplified schematic representation of the original model.
Adapted from: Mayberg, 1997.

further argue that two neural systems are responsible for these processes: a ventral system including the amygdala, insula, ventral striatum, and ventral regions of the ACC and PFC, which is involved in salience assessment and the production of an affective state; and a dorsal system including the hippocampus and dorsal regions of the ACC and PFC, known to play a role in higher cortical functions and a more top-down controlled regulation of affective states (Phillips *et al.*, 2003a). In a subsequent review, Phillips relates distinct patterns of structural and functional abnormalities in these systems to specific symptoms of psychiatric disorders including MDD. It is suggested that dysfunction

of particularly the ventral system, with a key role for the amygdala, leads to an identification bias towards negative emotion, resulting in the production of depressed mood and anhedonia (Phillips *et al*., 2003b).

Whilst both models emphasize the involvement of similar neural systems in depressive symptomatology, an important difference is the explicit role for the amygdala in Phillip's model. Even though Mayberg acknowledged the role of limbic structures in the pathogenesis of depressive disorders, she pointed out that their involvement was not known at the time (Mayberg, 1997) (Figure 2).

Neurobiological models of anxiety

A comprehensive and highly influential neuroanatomical model of PD was proposed by Gorman (1989), integrating biological and psychological views on the origin of the disorder (Gorman *et al*., 1989). In the original hypothesis, three components of panic disorder are discussed. The first, an acute panic attack, is proposed to originate in the brainstem, where stimulation of the locus ceruleus produces physiological and autonomic signs of panic, causing the sudden onset of an attack. The second component is anticipatory anxiety and it is suggested that limbic sites play a role in the fear of having additional panic attacks; a typical symptom of PD. The third component, phobic avoidance, involves a conscious cognitive capacity, and is linked to activation of the PFC (Gorman *et al*., 1989). A decade later, the authors revised their original hypothesis incorporating new research findings. In their updated model, Gorman and colleagues (2000) attributed a central role to the amygdala and its key function in the so-called fear network. Panic was proposed to originate from an abnormally sensitive fear network, including the amygdala, thalamus, PFC, and insula, as well as amygdalar projections to the brainstem and hypothalamus (Gorman *et al*., 2000).

There is no extensively defined neurobiological model describing the involvement of neural pathways in the symptomatology of SAD as there is for PD. However, Mathew (2001) builds upon predominantly non-human preclinical neurobiological models by reviewing pharmacological and neuroimaging research. Whilst emphasizing the many unanswered questions regarding the neurobiology of SAD, he notes impaired dopaminergic function in striatal regions as preliminary findings of

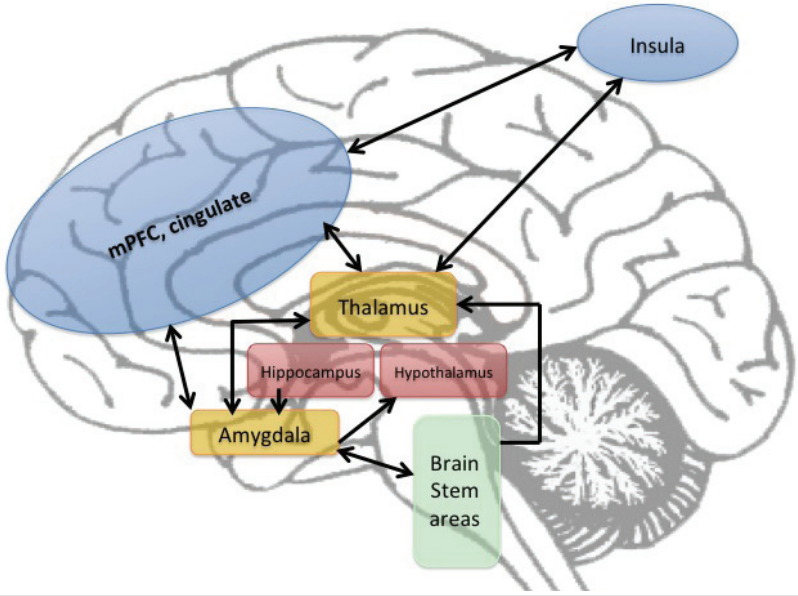


Figure 2. Representation of Gorman's neuroanatomical hypothesis of Panic Disorder
Upstream (cortical) and downstream (brain stem) sensory information is relayed to the amygdala via the thalamus. Contextual information is stored in the hippocampus and transferred directly to the amygdala, which in turn projects back to the brain stem, hypothalamus and prefrontal cortical structures.
Simplified schematic representation of the hypothesis.
Adapted from: Gorman *et al.*, 2001.

neurobiological studies on SAD. The amygdala and interactions with cortical structures and the hippocampus are also mentioned. Furthermore, the author stresses the importance of developmental studies for a better understanding of the underlying neurobiology of SAD (Mathew *et al.*, 2001).

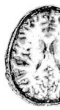
If SAD is understudied compared to PD, the paucity of available data on GAD is, as mentioned above, even more pronounced and limits the development of a comprehensive neuroanatomical model. What emerges from the few studies that do exist is that a disrupted interaction between the amygdala and regions in the PFC appear to underlie the symptomatology of GAD (McClure *et al.*, 2007, Etkin *et al.*, 2009). It is suggested that a defective coupling between these regions is consistent with cognitive theories of GAD, which suggest that the use of

compensatory cognitive strategies, such as worry, are employed to decrease the impact of emotions and regulate excessive anxiety (Etkin *et al*., 2009).

Taken together, the markedly smaller number of neurobiological studies of anxiety disorders is reflected in the limited comprehensiveness of neurobiological models of anxiety disorders compared to depression. Nonetheless, it is noteworthy that the amygdala has been given a more central role in models on anxiety than on MDD. Fear and anxiety, of course, are central features of anxiety disorders and only to a much lesser extent of MDD. The amygdala has also been implicated in other emotional states than fear as well as in the regulation of cognitive functions such as attention, perception and explicit memory. However, less is known about the circuitry involved in these functions (LeDoux, 2007).

Aim of this thesis

The constant development of new neuroimaging techniques, together with the improvement of existing methods, makes neuroimaging a highly dynamic and continuously evolving field. New ideas are generated and existing theories are adapted when new research findings point in a different direction. Consequently, neuroimaging studies have greatly contributed to the development of neurobiological models of depression and anxiety. However, it is evident that much is still unknown about the neural circuits that underlie these debilitating disorders. Various anxiety disorders are particularly understudied and have not been investigated with the newest neuroimaging approaches such as resting-state fMRI. This is surprising, since anxiety disorders are amongst the most prevalent psychiatric disorders worldwide, and it is repeatedly stressed that more research is needed. Due to its independence of externally applied stimuli or tasks, resting-state fMRI provides an unbiased research strategy that can help bridge the gaps in the knowledge of what underlies depressive and anxiety disorders. A lack of insight into the neurobiology has prevented the realisation of integral neuroanatomical models on SAD and GAD. Such models are important in providing a framework for a thorough understanding of depression and anxiety by researchers and clinicians, and inherently contribute to improved prevention and treatment strategies and the wellbeing of patients. Also to this end, insights in adolescent depression and anxiety, when the brain is still developing and malleable, are vital.



In addition, despite the ongoing discussion on whether depression and anxiety should be seen as distinct disorders or have a shared etiology, the shared and unique characteristics have not been explicitly addressed in neuroimaging studies.

The studies presented in this thesis aimed to address gaps in the current limited knowledge of the neurobiology underlying depression and anxiety in adults and adolescents, by employing novel imaging approaches. There is very little known about the network interactions between brain regions in depression and anxiety, despite the importance of such connectivity for an understanding of these disorders. Resting-state fMRI studies in PD and SAD have not been done before. Also, neither this technique nor structural MRI has been used in depressed adolescents that are free from other psychiatric comorbidities with the exception of anxiety. Finally, the shared and unique characterisations of depression and anxiety have not been studied with the use of resting-state fMRI.

The objective of this thesis is to investigate whether novel imaging approaches such as resting-state fMRI will confirm results from previous studies using other neuroimaging modalities, or uncover neural pathways implicated in depression and anxiety that have not been linked to these disorders before.

In Chapter 2, the unique characteristics of PD will be examined using resting-state fMRI in a sample of PD patients without any other psychiatric comorbidity, compared to healthy controls. In Chapter 3, resting-state functional connectivity in medication-naïve SAD patients without psychiatric comorbidity compared to control subjects will be discussed. Chapter 4 discusses the shared and unique resting-state functional connectivity characteristics of substantial groups of MDD patients without comorbidity, anxiety patients without comorbidity, and comorbid depressed and anxious patients, compared to healthy control subjects. Chapters 5 and 6 focus on developmental issues. Chapter 5 covers a resting-state fMRI study of a sample of treatment-naïve adolescents with a clinical diagnosis of depression, and pair-wise matched healthy controls. Grey matter volume in clinically depressed adolescents compared to healthy control subjects is discussed in Chapter 6. Finally, in Chapter 7, the results of Chapters 1 to 5 will be discussed in the light of existing models on depression and anxiety disorders.

Chapter 2
Aberrant limbic and salience network resting-state functional connectivity in panic disorder without comorbidity

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Abstract

Panic disorder (PD) is a prevalent and debilitating disorder but its neurobiology is still poorly understood. We investigated resting-state functional connectivity (RSFC) in PD without comorbidity in three networks that have been linked to PD before. This could provide new insights in how functional integration of brain regions involved in fear and panic might relate to the symptomatology of PD. Eleven PD patients without comorbidity and eleven pair-wise matched healthy controls underwent resting-state fMRI. We used seed regions-of-interest in the bilateral amygdala (limbic network), the bilateral dorsal anterior cingulate cortex (dACC) (salience network), and the bilateral posterior cingulate cortex (default mode network). RSFC of these areas was assessed using seed-based correlations. All results were cluster corrected for multiple comparisons ($Z > 2.3, p < .05$). Abnormalities were identified in the limbic network with increased RSFC between the right amygdala and the bilateral precuneus in PD patients. In the salience network the dACC demonstrated altered connectivity with frontal, parietal and occipital areas. The small sample size and hypothesis-driven approach could restrict finding additional group differences that may exist. Other caveats are reflected in the use of medication by two participants and the acquisition of the resting-state scan at the end of a fixed imaging protocol. We found altered RSFC in PD between areas involved in emotion regulation and emotional and somatosensory stimulus processing, as well as an area engaged in self-referential processing, not implicated in models for PD before. These findings extend existing functional neuroanatomical models of PD, as the altered RSFC may underlie increased sensitivity for bodily symptoms.

Introduction

Panic disorder (PD) patients experience recurrent unexpected panic attacks, followed by persistent concerns about having additional attacks, worrying about their consequences, and an associated change in behaviour (American Psychiatric Association, 1994). An influential neuroanatomical model of PD was proposed by Gorman and colleagues in 1989 (Gorman *et al.*, 2000). Central to their model is that panic derives from an abnormally sensitive fear network consisting of the prefrontal cortex, insula, thalamus, amygdala, and the amygdala's afferent and efferent projections from and to the hippocampus, brainstem, and hypothalamus. Further- more, a defective prefrontal cortical processing has been suggested to lead to misinterpretation of physiological triggers, ensuing in exaggerated amygdala and fear network activation, resulting in a panic attack (Gorman *et al.*, 2000, Shrestha *et al.*, 2009). Although this model has received considerable attention, the number of functional neuroimaging studies in PD is still modest (de Carvalho *et al.*, 2010). PET and SPECT studies of PD revealed decreased glucose use and/or blood flow in temporal and parietal areas, as well as in parts of the prefrontal cortex and (para)- hippocampal areas (Nordahl *et al.*, 1990, Lee *et al.*, 2006, Shin and Liberzon, 2010), while fMRI studies using a broad range of task paradigms reported activation and found altered activity in PD in cortical and limbic structures such as the anterior cingulate cortex (ACC), the amygdala, and hippocampus (de Carvalho *et al.*, 2010). The amygdala is perceived as the centre of the fear system with an important function in detecting, signalling, and learning from threat or danger (Phillips and LeDoux, 1992, LeDoux, 1998). Aberrant functioning of amygdala circuitry is thought to have a central role in the origin of PD and several other anxiety disorders (Phillips and LeDoux, 1992, LeDoux, 1998, Gorman *et al.*, 2000, de Carvalho *et al.*, 2010).

In contrast to task-evoked activity, resting-state fMRI enables examination of the brain's intrinsic functional connections in the absence of externally controlled stimuli or tasks (Biswal *et al.*, 1995, Fox and Raichle, 2007). Functional interactions between brain areas are crucial for proper functioning of the brain. This technique may therefore provide new insights in how functional integration of brain regions involved in fear and panic might relate to the symptomatology of PD (Fox and Raichle, 2007).

Consistently reported resting-state networks of potential relevance to PD include the default mode network (precuneus/posterior cingulate cortex (PCC), medial prefrontal cortex, and lateral parietal cortex), networks involving the amygdala, and the salience network (Damoiseaux *et al.*, 2006, Seeley *et al.*, 2007, Veer *et al.*, 2011). The salience network, comprising the dACC and bilateral anterior insula, is important in assessing the relevance of internal and external stimuli in order to guide behaviour (Seeley *et al.*, 2007).
Resting-state functional connectivity (RSFC) in PD has not been investigated before, in contrast to many other (neuro)psychiatric disorders (Broyd *et al.*, 2009, Greicius, 2008). In the present study we examined RSFC in patients with PD without comorbidity, using a seed-based correlation approach. Given the postulated model and the anatomical and functional abnormalities found in previous neuroimaging studies in PD, such as the frequently reported involvement of the amygdala circuitry and the ACC (Damsa *et al.*, 2009, de Carvalho *et al.*, 2010, Gorman *et al.*, 2000, Shin and Liberzon, 2010), we hypothesised that the amygdala-centred network would show altered connectivity of the amygdala with hypersensitivity of the fear circuitry and less top-down control. For instance, PD patients are known to be more aware of and to attribute a greater significance to signals coming from their own body than healthy controls. Specifically, we expected to find altered RSFC in networks involved in fear and emotion, and in distinguishing relevant from less relevant stimuli. For the salience network we expected a heightened awareness of bodily signals, i.e. increased connectivity of areas involved in somatosensory processing. As the default mode network shows altered connectivity in depression and other anxiety disorders (Broyd *et al.*, 2009, Fox and Raichle, 2007, Greicius, 2008), we also expected abnormalities in the connectivity of this network in PD.

Methods

Participants

All subjects were recruited from the MRI study from the large- scale longitudinal multi-centre cohort Netherlands Study of Depression and Anxiety (NESDA). NESDA is designed to investigate the long-term course and consequences of depression and anxiety disorders. NESDA participants were recruited from the community, through primary care and specialized mental health institutions. The rationales, methods and recruitment for NESDA have been described in detail elsewhere; for an overview of diagnostics,

inclusion and exclusion criteria see: (Penninx *et al.*, 2008, van Tol *et al.*, 2010).

After receiving written information, all subjects provided written informed consent. Participants underwent MRI in one of the three participating centres (Academic Medical Centre Amsterdam, Leiden University Medical Centre, and University Medical Centre Groningen) (van Tol *et al.*, 2010). The study was approved by the Medical Ethics Committees of all three centres.

For the present study on PD without comorbidity, resting-state fMRI data were available from 11 right-handed PD patients, and from 11 healthy controls pair-wise matched for age, gender, education, and scan-location (Table 1). All participants were new to lying in an MRI scanner. Patients were diagnosed with PD and no other psychopathology using the DSM-IV-based CIDI, lifetime version 2.1 (American Psychiatric Association, 1994). Participants were scanned within 8 weeks after the CIDI assessment. Severity of anxiety symptoms at baseline and at the time of scanning was measured with the Dutch version of the Beck Anxiety Inventory (BAI) (Beck *et al.*, 1988). Patients were excluded if they scored lower than seven on the Beck Anxiety Inventory, since they were then considered to have a 'minimal' level of anxiety and considered in remission (Beck *et al.*, 1988). Depressive symptoms on the day of scanning were rated with the Montgomery-Åsberg Depression Rating Scale (MADRS) (Montgomery and Asberg, 1979), as well as with the Inventory of Depressive Symptomatology (IDS) at baseline and at the time of scanning (Rush *et al.*, 1986).

Table 1. Demographic and Clinical Characteristics of Patients with Panic Disorder and Healthy Controls

			Panic Disorder patients(N=11) (N=11)	Healthy controls (N=11)		
Gender			1 male / 10 female	1 male / 10 female		
Scan location			3 AMC; 2 LUMC; 6 UMCG	3 AMC; 4 LUMC; 4 UMCG		
	Mean	SD	Mean	SD	*F* / *Z*	*p*
Age (years)	34.5	10.6	35.0	9.7	0.258[a]	0.974
Education (years)	12.8	3.5	14.1	2.1	-1.127[b]	0.260
BAI[†] score at scanning	14.5	5.6	1.9	2.5	-3.993[b]	0.001**
MADRS[‡] score at scanning	12.6	8.4	1.0	1.7	-3.776[b]	0.001**

AMC = Academic Medical Center Amsterdam; LUMC = Leiden University Medical Center; UMCG = University Medical Center Groningen; [†] BAI = Beck Anxiety Inventory; [‡] MADRS = Montgomery-Åsberg Depression Rating Scale; [a] *F*-value; [b] *Z*-value; ** Mann-Whitney U Test

Image data acquisition

Image acquisition took place at the three participating centres. Images were obtained on a Philips 3T magnetic resonance imaging system (Philips Healthcare, Best, The Netherlands), equipped with a SENSE-8 (Leiden University Medical Centre and University Medical Centre Groningen) or SENSE-6 (Academic Medical Centre Amsterdam) channel head coil.

As part of a fixed imaging protocol that also included task-related fMRI and structural MRI, resting-state functional MRI data were acquired for each subject using T_2^*-weighted gradient-echo echo-planar imaging with the following scan parameters in Amsterdam and Leiden: 200 whole-brain volumes; repetition time 2300 ms; echo time 30 ms; flip angle 80°; 35 transverse slices; no slice gap; field of view 220 × 220 mm; in-plane voxel size 2.3 × 2.3 mm; slice thickness 3 mm. Parameters in Groningen were identical, apart from: echo time 28 ms; 39 transverse slices; in-plane voxel size 3.45 × 3.45 mm. In the darkened MR room participants were instructed to lie still with their eyes closed and not to fall asleep. After completion of the scan, all participants confirmed wakefulness during acquisition. A sagittal 3-dimensional gradient-echo T_1-weighted image was acquired for registration purposes and grey matter analysis with the following scan parameters: repetition time 9 ms; echo time 3.5 ms; flip angle 80°; 170 sagittal slices; no slice gap; field of view 256 × 256 mm; 1 mm isotropic voxels.
No abnormalities were found upon inspection of the subjects' structural images by a neuroradiologist.

Data preprocessing

FMRI data processing was carried out using FEAT (FMRI Expert Analysis Tool) Version 5.98, part of FSL (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl) (Smith *et al.*, 2004). The following pre-statistics processing was applied: motion correction; non-brain removal; spatial smoothing using a 6 mm full-width at half- maximum Gaussian kernel; grand-mean intensity normalisation of the entire 4D dataset by a single multiplicative factor; high-pass temporal filtering (Gaussian-weighted least-squares straight line fitting, with a 0.01 Hz cut-off). Registration of the RS data to the high resolution T_1-weighted image, and the T_1 to the 2 mm isotropic MNI-152 standard space image (T_1 standard brain averaged over 152 subjects; Montreal Neurological Institute, Montréal, QC, Canada) was carried out (Jenkinson

et al., 2002). The resulting transformation matrices were then combined to obtain a native to MNI space transformation matrix and its inverse (MNI to native space).

Statistical analysis

Demographic and clinical characteristics were analysed using SPSS 17.0 (SPSS Inc, Chicago, Illinois) using an independent-samples t-test with significance set at $p < 0.05$. If data did not meet the assumptions required to perform parametric analysis, the non-parametric Mann-Whitney *U*-test was performed.

For the current study, a seed-based correlation approach was employed to investigate functional connectivity during rest (Fox and Raichle, 2007). The following seed regions-of-interest were selected: bilateral amygdala (for the limbic network), bilateral dACC (for the salience network), and bilateral PCC (for the default mode network). We created a mask in standard space for the amygdala based on the Harvard-Oxford Subcortical Structural Probability Atlas in FSL (Veer *et al*., 2011). The coordinates for the dACC seeds were obtained directly from Table 1 of the study by Margulies and colleagues (Margulies *et al*., 2007), and posterior cingulate seed coordinates were obtained from the study by Greicius and colleagues (Greicius *et al*., 2003) (Table 3). Spheres of 4 mm radius were created around these seed voxels. The resulting masks were then transformed to native space by applying the inverse transformation matrix obtained from the registration procedure, and spatially averaged time series were extracted for each seed and for each subject. For each participant, and for each network of interest, we performed a multiple regression analysis using the general linear model (GLM) (as implemented in FEAT) (Smith *et al*., 2004). The time courses that were extracted from the voxels in our seed regions were entered as a regressor in a GLM for each network separately. Apart from the two regressors describing the left and right seeds, nine nuisance regressors were included in the model: signal from the white matter, cerebrospinal fluid signal, and the global signal, as well as six motion parameters (three translations and three rotations). The global signal was included to reduce artefacts associated with physiological signal sources (i.e. cardiac and respiratory) (Fox and Raichle, 2007).

After reslicing the resulting individual connectivity maps from our seeds and their corresponding within-subject variance maps into 2 mm isotropic MNI space, these were entered into a higher level within and

between groups mixed effects analysis (one- and two-sample t-test).
As several studies have identified structural abnormalities in PD (de Carvalho *et al.*, 2010), we used grey matter density information of each subject as a voxel-dependent covariate in our higher level model to rule out the influence of any subtle grey matter density variations. By including structural information in the functional connectivity analysis, variance explained by potential differences in grey matter density and/or possible misregistrations are taken into account (Oakes *et al.*, 2007). Lower level contrasts were analysed both within and between groups using the GLM in which age and scan location were also entered as regressors. To correct for multiple comparisons, cluster correction was applied in all group analyses with significance set at a corrected $p < .05$, using an initial cluster-forming threshold of $Z > 2.3$ (Worsley, 2001).

Results

Questionnaires

At the time of scanning, PD patients had a mean score of 14.5 (SD=5.6) on the BAI and 12.6 (SD=8.4) on the MADRS, scoring higher than controls on both scales (BAI 1.9; SD=2.5 and MADRS 1.0; SD=1.7, both p's < .05) (Table 1). BAI and IDS scores did not change significantly between baseline and time of scanning for PD patients (Table 2). Two out of eleven patients used an SSRI.

Table 2. Anxiety and Depression Severity at Baseline and Time of Scanning within Panic Disorder Patients

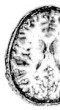
	Baseline		Scanning			
	Mean	SD	Mean	SD	*t* / *Z*	*p*
BAI[†] score	15.8	11.5	14.5	5.9	-.657[a]	.511*
IDS[‡] score	20.6	11.1	18.1	6.9	1.373[b]	.185**

[†] BAI = Beck Anxiety Inventory; [‡] IDS = Inventory of Depressive Symptomatology; [a] *Z*-value; [b] *t*-value;
* Wilcoxon Signed Rank Test; ** Paired-samples *t*-test

Table 3. MNI† Coordinates of the Seed Regions

Seed region	MNI coordinates		
	x	*y*	*z*
Amygdala	+/- 22	-6	-16
Dorsal Anterior Cingulate Cortex	+/- 6	18	28
Posterior Cinugulate Cortex/Precuneus	+/- 2	-52	26

† MNI = Montreal Neurological Institute



We first analysed amygdala RSFC. The seeds showed similar connectivity patterns in both groups encompassing the hippocampus, temporal poles, parahippocampal gyri, and the bilateral orbitofrontal cortex, consistent with previous literature (Stein *et al.*, 2007, Roy *et al.*, 2009). However, patients showed increased negative right amygdala connectivity compared to healthy controls with the bilateral precuneus and the bilateral lateral occipital cortex (Fig. 1, Supplementary Table 1). No group differences were found for the left amygdala analysis or when a contrast was made for the joint amygdala seeds.

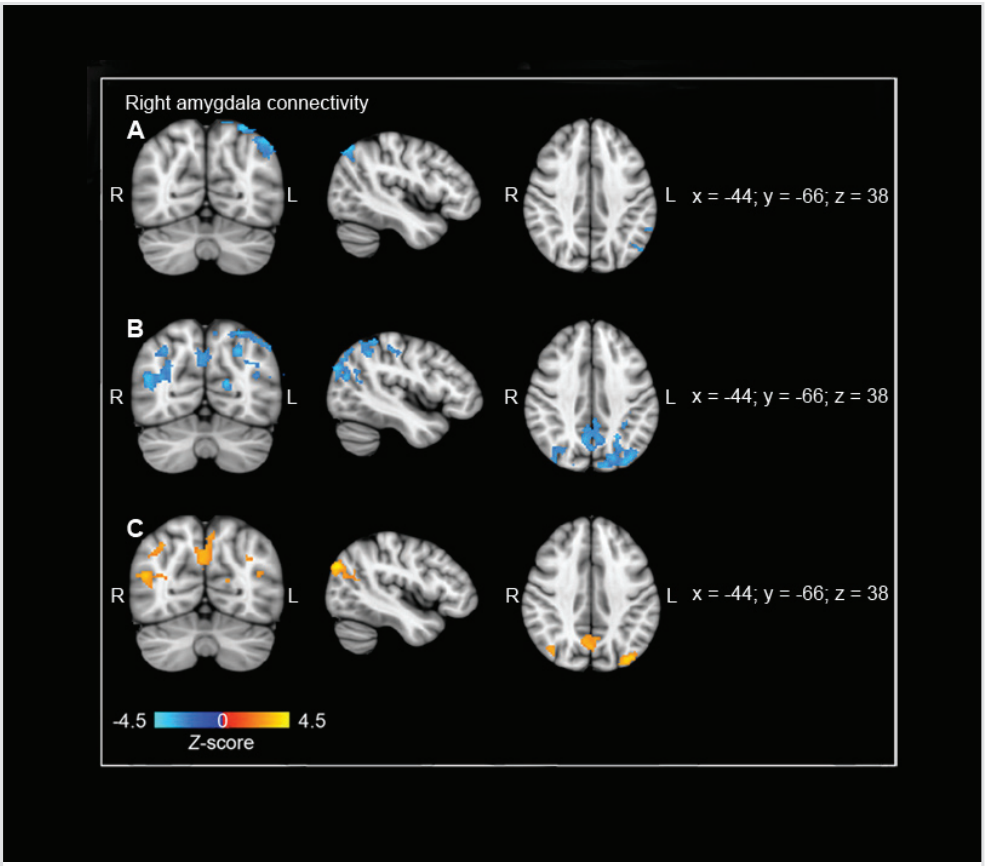


Figure 1.
Right amygdala negative connectivity. A=healthy controls; B=panic disorder patients; C=group difference: panic disorder patients > healthy controls. Results are cluster corrected at $p < .05$. Images are z-statistics, overlaid on the MNI-152 standard brain.

Next, we explored connectivity of the left and right dACC probing the salience network. Overall the seeds showed similar connectivity patterns in both groups (Figs. 2 and 3), corresponding with areas described in previous research (Margulies *et al.*, 2007). However, in PD the left dACC showed decreased connectivity with the bilateral frontal pole and superior/medial frontal gyrus compared to healthy controls.

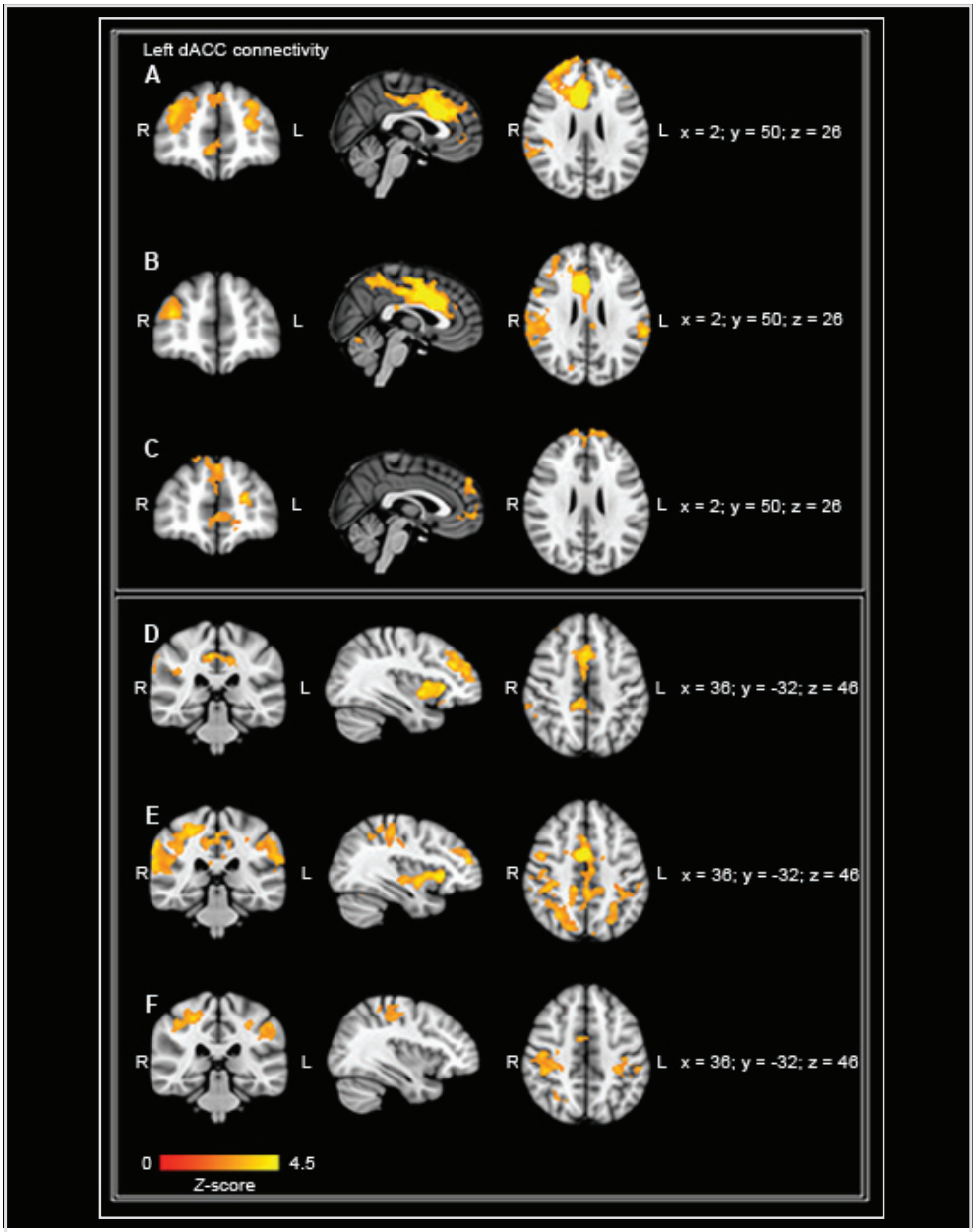


Figure 2.
Left dACC positive connectivity. dACC = dorsal anterior cingulate cortex; A = healthy controls; B = panic disorder patients; C = group difference: healthy controls > panic disorder patients D = healthy controls; E = panic disorder patients; F = panic disorder patients > healthy controls. Results are cluster corrected at $p < .05$. Images are z-statistics, overlaid on the MNI-152 standard brain.

In contrast, PD patients showed increased left dACC connectivity with the bilateral precentral and postcentral gyrus, the right supplementary motor cortex, and the right ACC (Fig. 2, Supplementary Table 2a and 2b). Finally, PD patients showed decreased right dACC connectivity with the right superior parietal lobule, the right lateral occipital cortex, the right angular gyrus, and the right central opercular cortex (Fig. 3, Supplementary Table 3). Combining the left and right dACC in one contrast did not produce any significant group differences.

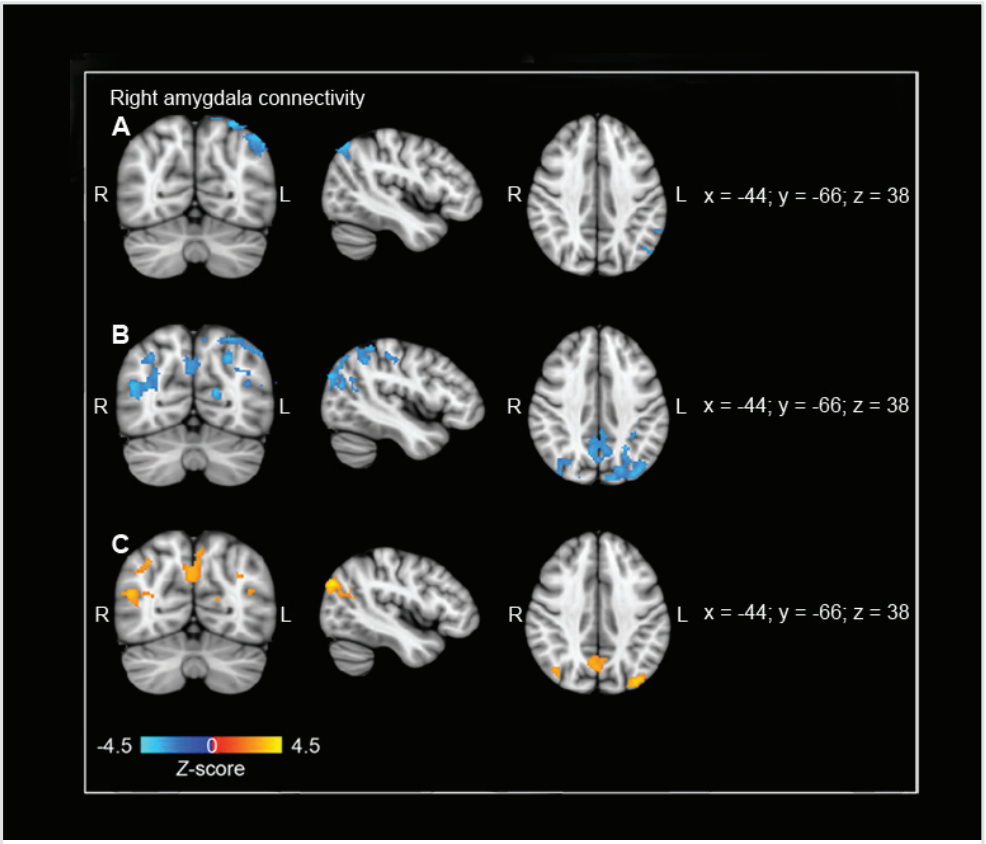


Figure 3.
Right dACC positive connectivity. dACC = dorsal anterior cingulate cortex; A = healthy controls; B = panic disorder patients; C = group difference A > B. Results are cluster corrected at $p < .05$. Images are z-statistics, overlaid on the MNI-152 standard brain.

Finally, we investigated connectivity of the default mode network with seeds in the bilateral PCC/precuneus, yet no group differences in default mode network connectivity were found.

Post-hoc, RS fMRI data were correlated with Beck Anxiety Inventory symptom scores using SPSS 17.0 (SPSS Inc., Chicago, Illinois, USA), to investigate whether the strength of connectivity was associated with symptom severity. Non-parametric tests were used if data did not meet the assumptions required for parametric testing. A mask was created of the resulting brain areas from our amygdala and dACC analyses, and the individual z-scores from these areas were calculated using Featquery, part of FSL (Smith *et al.*, 2004). No association was found between RS connectivity strength and anxiety scores.

Discussion

We investigated RSFC in PD patients without comorbidity and compared this to RSFC of pair-wise matched healthy controls with seeds in the limbic, salience and default mode network. As expected, abnormalities in connectivity were found in the limbic and salience networks, but no differences were observed in the default mode network. We found abnormal connectivity in PD patients between the right amygdala and the bilateral precuneus, as well as the bilateral lateral occipital cortex. We also found altered RSFC in patients between the left and right dACC on the one hand and frontal and more parietal and occipital areas on the other hand. Finally, we found abnormal RSFC in patients between the right anterior cingulate and the right superior parietal lobule, lateral occipital cortex, postcentral gyrus, and precentral gyrus.

Increased RSFC was found between the amygdala and precuneus in PD patients. The posterior cingulate/precuneus is thought to be involved in self-reflection and self-processing activities like mental imagery and episodic/autobiographical memory retrieval (Raichle *et al.*, 2001, Cavanna and Trimble, 2006b, Cavanna, 2007). Many of the functions of the precuneus and its connections seem of direct relevance to the phenomenology of PD. Four broad categories of cognitive-emotional functions have been assigned to the precuneus, namely visuo-spatial imagery, episodic memory retrieval, self-processing, and consciousness (Cavanna and Trimble, 2006b, Cavanna, 2007). Disturbances of self-processing and consciousness are characteristic elements of panic attacks

(American Psychiatric Association, 1994). Moreover, the precuneus and interconnected posterior cingulate and medial prefrontal cortices are constantly engaged in the gathering of information and representation of the self as well as the external world (Cavanna, 2007). The amygdala on the other hand serves as an important component of the system involved in the acquisition, storage, and expression of emotional and fear memory, playing a pivotal role in linking external stimuli to defence responses (LeDoux, 2000, LeDoux, 2003). Possibly, our results indicate that in PD patients, activation of the amygdala could lead to decreased functional connectivity with the precuneus. From the perspective of existing models, this finding might relate to decreased self-processing operations with typical symptoms such as depersonalization and loss of control experienced during a panic attack (American Psychiatric Association, 1994, Gorman *et al*., 2000, Cavanna and Trimble, 2006b). An alternative interpretation is that a disturbance in self-processing operations underlies the susceptibility to panic attacks, which would be in line with the continuously present feelings of unsteadiness, depersonalization and derealisation reported by many PD patients (American Psychiatric Association, 1994).

For the salience network seeds, the left and right dACC, we identified several differences in connectivity with frontal and occipito-parietal areas, which have been implicated in the pathophysiology of PD before (de Carvalho *et al*., 2010). These areas are involved in the processing of somatosensory information, attentional control and self-awareness (Bisley and Goldberg, 2010, Koechlin, 2011).

The left dorsal anterior cingulate showed increased positive connectivity in PD with the postcentral gyrus, known as the somatosensory cortex. This region receives proprioceptive and cutaneous input from the body (Nelson and Chen, 2008). Therefore, increased positive connectivity of the dACC with the postcentral gyrus in PD patients might be taken to reflect the increased processing of somatosensory stimuli in PD, resulting from or leading to misattribution of innocuous internal and external signals as potentially harmful. For both left and right dorsal anterior cingulate we found altered connectivity with (bilateral) superior parietal regions. Functional connectivity of the ACC with superior parietal regions has previously been reported in healthy subjects, and was suggested to be important for the maintaining of an internal representation of bodily states, a function clearly relevant to

PD (Gusnard *et al.*, 2001, Margulies *et al.*, 2007, Wolpert *et al.*, 1998). Deviations within this circuitry might contribute to an inaccurate internal representation and interpretation of the bodily state in PD patients. We also found decreased connectivity of the left dACC with the bilateral superior frontal gyrus in PD patients. These findings are in line with previous functional studies in PD, finding reduced blood flow or glucose use in the same areas (right superior frontal gyrus) (Eren *et al.*, 2003, Lee *et al.*, 2006). Interestingly, a study performed by Goldberg and colleagues (2006) showed superior frontal gyrus activity extending to the dorsal part of the anterior cingulate, when during an introspection task a sensory stimulus was slowly presented to participants after which they had to rate the emotional effect elicited by these stimuli (Goldberg *et al.*, 2006). When the task increased in speed and difficulty, no activation in the superior frontal gyrus was observed, leading the authors to conclude that the brain is able to 'switch off' self-awareness when it needs all its resources to carry out a difficult task (Goldberg *et al.*, 2006). Thus, dACC connectivity with the superior frontal gyrus could possibly play a role in the disturbed self-awareness in PD patients, who can typically experience feelings like loss of control, going crazy, derealisation, and depersonalization during panic attacks (American Psychiatric Association, 1994).

Abnormalities of the default mode network have been found in various neuropsychiatric diseases, like Alzheimer's disease, schizophrenia, epilepsy, autism, attention deficit/hyperactivity disorder, and depression (Fox and Raichle, 2007, Broyd *et al.*, 2009). The default mode network is associated with functions such as self- referential mental processing, social cognition and emotional processing (Broyd *et al.*, 2009). We did not find abnormalities in the default mode network, suggesting that processes relying on the default mode network are not affected in PD, but this might be due to our small sample size. However, even at lower thresh- olds no abnormalities in connectivity of the posterior cingulate were identified.

Limitations

There are several limitations important to note. An important notion is that RSFC data should be interpreted with caution, and any interpretation refers only to functional connectivity between brain areas as opposed to the (dys)function of a distinct brain region. Our sample size is relatively small and therefore some group differences may not have been detected.

Also, by opting for a hypothesis-driven approach and thus only exploring specific resting-state networks, abnormalities in other networks might have been overlooked. On the other hand, the use of a seed-based region-of-interest approach allows easier replication of our findings. Pooling data from different centres is another possible limitation. To account for this as much as possible, we have matched our groups based on scanning site and also added a confound regressor for site in our statistical model. A potential limitation is that patients were not diagnosed on the exact day of scanning but a few weeks earlier during the baseline interview. We did not formally assess whether additional psychopathology developed between baseline and scanning, but subjects indicated no major changes in their symptom patterns during a short clinical interview. Anxiety and depressive symptoms were again assessed on the scanning day and scores were not different from baseline. Another potential limitation is that no specific panic disorder scale was used, although the BAI predominantly contains panic-related items. Another limitation is that only one male subject in each of our groups was included, thus restricting the generalizability of the results. Furthermore, two of the included patients were using medication, so we cannot fully exclude possible confounding effects of medication use. Finally, our RS fMRI data were acquired at the end of a fixed imaging protocol (after completion of three task-related fMRI runs and the acquisition of an anatomical scan (scan sequence: Tol, word encoding, T1-weighted, word recognition, faces)), which could potentially have influenced RS connectivity (i.e. a spillover effect) with PD patients still showing aberrant connectivity while the stimuli were no longer present. Despite its limitations, this study also has some noteworthy strengths. This is the first study to investigate RSFC with MRI in PD. The major strength of this study is the inclusion of PD patients without any psychiatric comorbidity. In addition, most patients were not using psychotropic medication.

Our hypotheses regarding alterations in RSFC in PD patients were partly confirmed. As expected, aberrancies were found in amygdala and salience network RSFC. However, our results did not support the hypothesis that DMN RSFC was deviant in PD patients. In summary, our study shows altered RSFC in PD patients of areas involved in salience and emotion processing with areas engaged in self-processing and somatosensory processing, not implicated in current functional neuroanatomical models for PD. Although replication is warranted, disturbed functional connectivity in these circuits should be taken into account in future functional neuroanatomical models for PD.

Supplementary tables

Supplementary table 1. Right Amygdala Resting-State Connectivity, PD > HC

Region	Side	Z-value	p-value	MNI coordinates		
Cluster 1, 412 voxels				x	y	z
Lateral Occipital Cortex	R	3.54961	0.0004	46	-66	18
Lateral Occipital Cortex	R	3.15913	0.0016	40	-68	38
Lateral Occipital Cortex	R	3.14055	0.0017	34	-68	44
Lateral Occipital Cortex	R	3.12683	0.0018	42	-72	34
Lateral Occipital Cortex	R	2.80562	0.0050	52	-72	26
Lateral Occipital Cortex	R	2.73754	0.0062	36	-66	18
Cluster 2, 616 voxels				x	y	z
Lateral Occipital Cortex	L	4.18839	0.0001	-44	-78	32
Lateral Occipital Cortex	L	3.77343	0.0002	-34	-80	38
Lateral Occipital Cortex, BA 19	L	3.32952	0.0009	-34	-86	32
Angular Gyrus	L	2.95843	0.0031	-48	-60	20
Lateral Occipital Cortex	L	2.9051	0.0037	-48	-78	20
Lateral Occipital Cortex	L	2.88227	0.0039	-46	-72	24
Cluster 3, 1080 voxels				x	y	z
Posterior Cingulate Cortex	R	3.90197	0.0001	8	-44	14
Precuneus	L	3.62946	0.0003	-6	-52	12
Precuneus	R	3.38172	0.0007	14	-60	22
Precuneus	R	3.37847	0.0007	4	-64	34
Precuneus, BA 7	L	3.31899	0.0009	0	-68	38
Precuneus	L	3.29569	0.0010	-18	-62	12

PD = panic disorder patients; HC = healthy controls; MNI = Montreal Neurologic Institute; BA = Brodmann Area; voxel size 2 mm isotropic; z- and p-values of the local maxima, spread throughout the cluster.

Supplementary table 2a. Left dACC Resting-State Connectivity, HC > PD.

Region	Side	Z-value	p-value	MNI coordinates		
Cluster 1, 559 voxels				*x*	*y*	*z*
Frontal Pole, BA 9	L	3.5231	0.0004	-2	60	8
Frontal Pole, BA 10	L	3.59641	0.0003	-2	56	-2
Anterior Cingulate Cortex	R	3.57106	0.0004	8	42	-2
Frontal Pole	L	2.97862	0.0029	-8	66	12
Frontal Pole	L	2.82357	0.0047	-18	66	-4
Paracingulate Gyrus	R	3.3773	0.0007	0	50	2
Cluster 2, 856 voxels				*x*	*y*	*z*
Frontal Pole	L	3.84389	0.0001	-20	48	16
Frontal Pole	R	3.78664	0.0002	0	54	40
Frontal Pole	L	3.79932	0.0001	-18	58	12
Frontal Pole, BA 9	L	3.2182	0.0013	-18	62	28
Frontal Pole	L	3.30719	0.0009	-8	66	26
Frontal Pole	R	3.08149	0.0021	12	26	26

dACC = dorsal anterior cingulate cortex; HC = healthy controls; PD = panic disorder patients ; MNI = Montreal Neurologic Institute; BA = Brodmann Area; voxel size 2 mm isotropic; z- and p-values of the local maxima, spread throughout the cluster.

Supplementary table 2b. Left dACC Connectivity, PD > HC.

Region	Side	*Z*-value	*p*-value	MNI coordinates		
Cluster 1, 524 voxels				*x*	*y*	*z*
Anterior Cingulate Gyrus, BA 24	L	3.57718	0.0003	-2	0	36
Supplementary Motor Cortex, BA 6	L	3.28865	0.0010	8	-6	52
Anterior Cingulate Gyrus	R	3.50019	0.0005	2	0	38
Supplementary Motor Cortex, BA 24	R	3.22197	0.0013	6	-2	48
Anterior Cingulate Gyrus, BA 24	R	3.26338	0.0011	6	0	38
Anterior Cingulate Gyrus	R	3.1454	0.0017	8	-4	32
Cluster 2, 714 voxels				*x*	*y*	*z*
Supramarginal Gyrus, BA 40	L	3.09563	0.0020	-52	-32	44
Postcentral Gyrus	L	3.36605	0.0008	-46	-22	40
Superior Parietal Lobule	L	3.38798	0.0007	-36	-40	40
Supramarginal Gyrus	L	3.2304	0.0012	-4	-34	40
Postcentral Gyrus	L	3.36252	0.0008	-38	-22	46
Postcentral Gyrus	L	3.19629	0.0014	-36	-24	42
Cluster 3, 1477 voxels				*x*	*y*	*z*
Superior Parietal Lobule	R	4.0731	0.0001	18	-56	58
Postcentral Gyrus, BA 4	R	3.72298	0.0002	44	-16	44
Postcentral Gyrus	R	3.67095	0.0002	26	-32	52
Supramarginal Gyrus	R	3.5436	0.0004	40	-34	46
Supramarginal Gyrus	R	3.23672	0.0012	56	-22	42
Postcentral Gyrus, BA 3	R	3.27967	0.0010	32	-30	60

dACC = dorsal anterior cingulate cortex; PD = panic disorder patients; HC = healthy controls; MNI = Montreal Neurologic Institute; BA = Brodmann Area; voxel size 2 mm isotropic; *z*- and *p*-values of the local maxima, spread throughout the cluster.

Supplementary table 3. Right dACC Resting-State Connectivity, HC > PD

Region	Side	*Z*-value	*p*-value	MNI coordinates		
Cluster 1, 426 voxels				*x*	*y*	*z*
Precentral Gyrus	R	3.78293	0.0002	58	2	16
Central Opercular Cortex	R	3.37909	0.0007	52	2	4
Precentral Gyrus	R	2.78087	0.0054	52	0	16
Central Opercular Cortex, BA 43	R	3.06963	0.0021	58	-2	8
Central Opercular Cortex, BA 22	R	2.77364	0.0055	62	-4	6
Central Opercular Cortex	R	2.67973	0.0074	48	0	14
Cluster 2, 493 voxels				*x*	*y*	*z*
Superior Parietal Lobule	R	3.43418	0.0006	34	-56	52
Superior Parietal Lobule, BA 7	R	3.4361	0.0006	28	-56	54
Lateral Occipital Cortex, BA 7	R	3.27644	0.0011	20	-58	60
Angular Gyrus	R	2.89024	0.0038	38	-52	44
Superior Parietal Lobule	R	2.91071	0.0036	34	-42	56
Superior Parietal Lobule	R	3.08856	0.0020	18	-58	54

HC = healthy controls; PD = panic disorder patients; MNI = Montreal Neurologic Institute; BA = Brodmann Area; voxel size 2 mm isotropic; *z*- and *p*-values of the local maxima, spread throughout the cluster.

Chapter 3
Resting-state functional connectivity abnormalities in limbic and salience networks in social anxiety disorder without comorbidity

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Abstract

The neurobiology of social anxiety disorder (SAD) is not yet fully understood. Structural and functional neuroimaging studies in SAD have identified abnormalities in various brain areas, particularly the amygdala and elements of the salience network. This study is the first to examine resting-state functional brain connectivity in a drug-naive sample of SAD patients without psychiatric comorbidity and healthy controls, using seed regions of interest in bilateral amygdala, in bilateral dorsal anterior cingulate cortex for the salience network, and in bilateral posterior cingulate cortex for the default mode network. Twelve drug-naive SAD patients and pair-wise matched healthy controls, all drawn from the Netherlands Study of Depression and Anxiety sample, underwent resting-state fMRI. Group differences were assessed with voxel-wise grey matter density as nuisance regressor. All results were cluster corrected for multiple comparisons ($Z > 2.3$, $p < .05$). Relative to control subjects, drug-naive SAD patients demonstrated increased negative right amygdala connectivity with the left middle temporal gyrus, left supramarginal gyrus and left lateral occipital cortex. In the salience network patients showed increased positive bilateral dorsal anterior cingulate connectivity with the left precuneus and left lateral occipital cortex. Default mode network connectivity was not different between groups. These data demonstrate that drug-naive SAD patients without comorbidity show differences in functional connectivity of the amygdala, and of areas involved in self-awareness, some of which have not been implicated in SAD before.

Introduction

Social anxiety disorder (SAD) is characterized by persistent fear of social or performance situations in which judgment or scrutiny by others and embarrassment can occur. Social situations are preferably avoided or otherwise experienced with extreme anxiety and discomfort (American Psychiatric Association, 1994, Furmark, 2002). The number of neuroimaging studies investigating SAD is rather modest, but these have provided some insight in the brain circuitry involved in this disorder. Structural neuroimaging studies have been scarce, and have used different approaches, reporting inconsistent results with some studies showing no abnormalities and others decreases in volume of the amygdala, right posterior right temporal gyrus and (para)hippocampus (Potts *et al.*, 1994, Irle *et al.*, 2010, Liao *et al.*, 2011). The number of functional studies is much greater, with most of these studies focusing on the processing of emotional faces or using symptom provocation designs (Etkin and Wager, 2007, Blair *et al.*, 2008a, Blair *et al.*, 2008b, Damsa *et al.*, 2009, Gentili *et al.*, 2009, Freitas-Ferrari *et al.*, 2010, Shin and Liberzon, 2010, Blair *et al.*, 2011a). A recent systematic review on neuroimaging studies in SAD by Freitas-Ferrari *et al.* (2010), mainly focusing on functional connectivity, found the amygdala and its connections in the emotional and fear circuitry to play a key role in SAD (Freitas-Ferrari *et al.*, 2010). However, other areas such as the insula (Shah *et al.*, 2009), anterior cingulate cortex (Amir *et al.*, 2005, Blair *et al.*, 2011a) and ventromedial prefrontal cortex have also been reported to be involved in SAD (Etkin and Wager, 2007, Damsa *et al.*, 2009, Freitas-Ferrari *et al.*, 2010).

Resting-state fMRI

Resting-state fMRI (RS-fMRI) enables the monitoring of brain activity and connectivity in the absence of externally controlled tasks or stimuli (Fox and Raichle, 2007). Consistently reported RS networks of functionally interconnected brain regions include the default mode network (DMN) (precuneus/posterior cingulate cortex, medial prefrontal cortex, and lateral parietal cortex) (Raichle *et al.*, 2001), and the salience network (Seeley *et al.*, 2007, Biswal *et al.*, 2010). The salience network is important in assessing the relevance of internal and external stimuli in order to guide behaviour (Seeley *et al.*, 2007). Its principal areas are the bilateral anterior insula and bilateral dorsal anterior cingulate cortex, two areas frequently

reported to be involved in SAD (Freitas-Ferrari *et al.*, 2010). So far, five RS-fMRI connectivity studies in SAD have been published, four of which were conducted by the same research group within overlapping cohorts of SAD patients (Liao *et al.*, 2010a, Liao *et al.*, 2010b, Hahn *et al.*, 2011, Ding *et al.*, 2011, Liao *et al.*, 2011). Using independent component analysis, Liao *et al.* (2010a) revealed altered connectivity in SAD patients in seven out of the eight RS networks resulting from their analysis, including the DMN and a self-referential network. Noteworthy, a limbic network was not among these net- works. Subsequently, Liao *et al.* (2010b) used a seed-based region of interest analysis and demonstrated altered amygdala RS connectivity in social anxiety patients (Liao *et al.*, 2010b). In their third study, Liao *et al.* (2011) combined voxel-based morphometry, RS functional connectivity (RSFC), and diffusion tensor imaging (Liao *et al.*, 2011), and found decreased volumes and altered connectivity of the right inferior frontal gyrus and hippocampal areas. Finally, a whole-brain resting-state analysis was also per- formed, showing abnormal connectivity of frontal and occipital lobes (Ding *et al.*, 2011).

The group of Hahn *et al.* (2011) examined in one study RSFC in SAD using a seed-based region of interest approach and found altered left amygdala connectivity. However, the investigated sample was heterogeneous, consisting of SAD patients as well as patients with comorbid panic disorder, and one panic disorder patient (Hahn *et al.*, 2011). It is therefore difficult to ascertain whether the differences were solely attributable to SAD. Additionally, in four out of the five published RS studies patients were not using medication at the time of research, but were not drug- naive, thus possible effects of recent pharmacotherapy cannot be ruled out. Since the effect of anti-anxiety medication on neuronal activity has been demonstrated in SAD patients (Van der Linden *et al.*, 2000, Furmark *et al.*, 2005), a drug-naive sample should preferably be used to rule out such effects.

Here, we set out to investigate RSFC in drug-naive SAD patients without psychiatric comorbidity using a seed-based correlation approach. Given the anatomical and functional abnormalities found in previous MRI studies in SAD, suggesting a key role for the amygdala (Kent and Rauch, 2003, Shin and Liberzon, 2010, Freitas-Ferrari *et al.*, 2010), and its key role in emotion generation, fear and anxiety (LeDoux, 2003), we hypothesised abnormalities in amygdala RSFC. Additionally, based on previous reports

of involvement of the dorsal anterior cingulate cortex in SAD and based on the functions of the salience network (Seeley *et al.*, 2007) and its relevance for anxiety disorders (Amir *et al.*, 2005, Etkin *et al.*, 2009, Etkin *et al.*, 2010, Freitas-Ferrari *et al.*, 2010, Blair *et al.*, 2011a) we also expected abnormalities in the connectivity of the salience network. As the DMN showed altered connectivity in many neuropsychiatric disorders such as depression (Greicius *et al.*, 2007, Broyd *et al.*, 2009), epilepsy, autism, attention deficit/hyperactivity disorder, anxiety disorders (Broyd *et al.*, 2009), and perhaps also in SAD where it is thought to play a role in social cognition (Gentili *et al.*, 2009, Liao *et al.*, 2010a), we also hypothesised abnormalities in the connectivity of the DMN in social anxiety patients.

Experimental procedures

Participants

Subjects were selected from the MRI study within the large-scale longitudinal multi-centre cohort Netherlands Study of Depression and Anxiety (NESDA). NESDA is designed to investigate the long-term course and consequences of depression and anxiety disorders. Participants in NESDA were recruited from the community, and through primary care and specialized mental health institutions. The rationales, methods and recruitment have been described in detail else- where; for an overview of diagnostics, inclusion and exclusion criteria see: (Penninx *et al.*, 2008, van Tol *et al.*, 2010).

After receiving written information, all subjects provided written informed consent. Participants underwent MR imaging in one of the three participating centres (Academic Medical Centre Amsterdam, Leiden University Medical Centre, and University Medical Centre Groningen) (van Tol *et al.*, 2010). The study was approved by the Medical Ethics Committees of all three centres and con- ducted in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

For the present study on SAD patients without comorbidity, MRI data were available from 12 right-handed SAD patients (5 male) and from 12 healthy controls matched for age, gender, education, handedness, and scan-location (Table 1). All subjects were new to this specific scanning situation. Patients were diagnosed with SAD and no other psychopathology using the DSM-IV-based Composite International Diagnostic Interview, lifetime version 2.1 (American Psychiatric Association, 1994). At the time

of scanning, anxiety and phobic symptoms were also assessed using the Fear Questionnaire (FQ) (Marks and Mathews, 1979) and depressive symptoms were rated with the Montgomery–Åsberg Depression Rating Scale (Montgomery and Asberg, 1979). Demographic and clinical characteristics were analysed using SPSS 17.0 (SPSS Inc, Chicago, Illinois) with significance set at $p<0.05$.

Table 1. Demographic and Clinical Characteristics of Patients with Social Anxiety Disorder and Healthy Controls

	Social Anxiety Disorder patients (N=12)		Healthy controls (N=12)		
Gender	5 male / 7 female		5 male / 7 female		
Scan location	2 AMC; 5 LUMC; 5 UMCG		3 AMC; 5 LUMC; 4 UMCG		
	Mean	SD	Mean	SD	*p*
Age	34.8	8.8 (24-53 years)	34.0	7.2 (21-47 years)	0.821
Education	11.8	3.2 (6-18 years)	13.7	2.4 (10-18 years)	0.123
FQ social phobia score	20.8	8.2 (8-36)	4.5	4.3 (0-13)	0.001**
MADRS score at scanning	7.8	8.6 (0-25)	1.2	2.0 (0-5)	0.001**

SD = standard deviation; AMC = Academic Medical Center Amsterdam; LUMC = Leiden University Medical Center; UMCG = University Medical Center Groningen; FQ = Fear Questionnaire; MADRS = Montgomery-Åsberg Depression Rating Scale.
** Mann-Whitney U Test.

Image data acquisition

Image acquisition took place at the Academic Medical Centre Amsterdam, the Leiden University Medical Centre, and the University Medical Centre Groningen. Images were obtained on Philips 3T magnetic resonance imaging systems (Philips Medical Systems, Best, The Netherlands), equipped with a SENSE-8 (Leiden University Medical Centre and University Medical Centre Groningen) or SENSE- 6 (Academic Medical Centre Amsterdam) channel head coil.

As part of a fixed imaging protocol, resting-state functional MRI data were acquired for each subject using T2*-weighted gradient- echo echo-planar imaging with the following scan parameters in Amsterdam and Leiden: 200 whole-brain volumes; repetition time 2300 ms; echo time 30 ms; flip angle 80°; 35 transverse slices; no slice gap; field of view 220 × 220 mm; in-plane voxel size 2.3 × 2.3 mm; slice thickness 3 mm; duration 7.51 min. Parameters in Groningen were identical, apart from: echo time 28 ms; 39 transverse slices; in-plane voxel size 3.45 × 3.45mm. In the darkened MR room participants were instructed to lie still with their eyes closed and not to fall asleep. After completion of the scan, subjects confirmed wakefulness during acquisition. A sagittal 3-dimensional

gradient-echo T1-weighted image was acquired for registration purposes and grey matter analysis with the following scan parameters: repetition time 9 ms; echo time 3.5 ms; flip angle 80°; 170 sagittal slices; no slice gap; field of view 256 × 256 mm; 1 mm isotropic voxels; duration 4.5 min.

No abnormalities were found upon inspection of the subjects' structural images by a neuroradiologist.

Preprocessing

FMRI data preprocessing and statistics were carried out using FEAT (FMRI Expert Analysis Tool) Version 5.98, part of FSL (FMRIB's Software Library, http://www.fmrib.ox.ac.uk/fsl) (Smith *et al.*, 2004). The following pre-statistics processing was applied: motion correction; non-brain removal; spatial smoothing using a 6 mm full-width at half-maximum Gaussian kernel; grand-mean intensity normalisation of the entire 4D dataset by a single multiplicative factor; high-pass temporal filtering (Gaussian-weighted least-squares straight line fitting, with a 0.01 Hz cut-off). Registration of the RS data to high resolution T1-weighted, and the T1 to the 2 mm isotropic MNI-152 standard space image (T1 standard brain averaged over 152 subjects; Montreal Neurological Institute, Montréal, QC, Canada) was carried out (Smith *et al.*, 2004). The resulting transformation matrices were then combined to obtain a native to MNI space transformation matrix and its inverse (MNI to native space).

Statistical analysis

For the current study, a seed-based correlation approach (Fox and Raichle, 2007) was employed to study functional connectivity during rest (Roy *et al.*, 2009). The following seed regions-of-interest were selected: bilateral amygdala, bilateral dorsal anterior cingulate cortex (dACC) (for the salience network), and bilateral posterior cingulate cortex (PCC) (for the DMN). We created a mask in standard space for the amygdala based on the Harvard–Oxford Subcortical Structural Probability Atlas in FSL (Veer *et al.*, 2011). The coordinates for the dACC seeds were obtained directly from Table 1 of the study by Margulies *et al.* (2007), and PCC seed region coordinates were obtained from a study by Greicius *et al.* (2003). Table 2 contains the coordinates of the seed voxels. Spheres of 4 mm radius were created around the seed voxels. They were then transformed to native space by applying the inverse transformation matrix obtained from the

registration procedure, and spatially averaged time series were extracted for each seed and for each subject.

Table 2. MNI Coordinates of the Seed Regions

Seed region	MNI coordinates		
	x	*y*	*z*
Amygdala	+/- 22	-6	-16
dACC	+/- 6	18	28
PCC/precuneus	+/- 2	-52	26

MNI = Montreal Neurological Institute; dACC = dorsal anterior cingulate cortex; PCC = posterior cingulate cortex.

For each participant, and for each network of interest, we performed a multiple regression analysis using the general linear model (GLM) (as implemented in FEAT) (Smith *et al*., 2004). The time courses that were extracted from the voxels in all of our seed regions were entered as a regressor in a GLM for each network. Apart from the two regressors describing the left and right seeds, nine nuisance regressors were included in the model: signal from the white matter, cerebrospinal fluid signal, and the global signal, as well as six motion parameters (three translations and three rotations). The global signal was included to reduce the influence of artefacts caused by physiological signal sources (i.e., cardiac and respiratory) on the results (Fox and Raichle, 2007).

After reslicing the resulting parameter estimate maps and their corresponding within-subject variance maps into 2 mm isotropic MNI space, they were entered into a higher level within and between groups mixed effects analysis (one- and two-sample t-test).

As structural studies have indicated abnormalities in SAD, we used grey matter density information of each subject as a voxel-dependent covariate in our higher level model. By including structural information in the functional connectivity analysis, variance explained by potential differences in grey matter density and/or possible misregistrations are taken into account (Oakes *et al*., 2007). Lower level contrasts were analysed both within and between groups were analysed using the general linear model in which age and scan location were also entered as regressors. To correct for multiple comparisons, cluster correction was applied in all group analyses with significance set at a corrected $p<.05$ and an initial cluster-forming threshold of $Z>2.3$ (Worsley, 2001).

Results

Questionnaires

At the time of scanning, SAD patients scored significantly higher than controls on the FQ social phobia sub-scores, and on the Montgomery–Åsberg Depression Rating Scale (Table 1).

RSFC analysis

We first analysed amygdala RSFC. Within both groups the connectivity pattern largely overlapped with areas described to have functional and anatomical connections with the amygdala in previous studies (Stein *et al.*, 2007, Roy *et al.*, 2009). Areas showing positive connectivity with the amygdalae included the hippocampus, parahippocampal gyrus, insula, putamen, pallidum, thalamus, temporal pole, frontal orbital cortex, planum temporale, superior temporal gyrus, temporal fusiform gyrus, and brainstem. Areas showing a negative resting-state connectivity were the precentral gyrus, middle frontal gyrus, PCC/precuneus, supramarginal gyrus, angular gyrus, lateral occipital cortex, and cuneus.

In SAD patients we found increased negative right amygdala connectivity with the left middle temporal gyrus, left supramarginal gyrus and left lateral occipital cortex (Figure 1; Table 3). No group differences were found on left amygdala RSFC or when a contrast was made for the left and right amygdala combined.

Next, we explored RSFC of the dACC, probing the salience network. The connectivity pattern for this seed in both groups also corresponded with areas described in previous research (Margulies *et al.*, 2007). Areas showing positive connectivity in rest with the dACC included other parts of the ACC, the frontal pole, paracingulate gyrus, PCC, precentral gyrus, supramarginal gyrus, parietal operculum cortex, central operculum cortex, inferior frontal gyrus, and middle frontal gyrus. Areas showing negative connectivity in rest included the subcallosal cortex, precuneus, cuneus, lateral occipital cortex, occipital fusiform cortex, parahippocampal gyrus, and lingual gyrus.

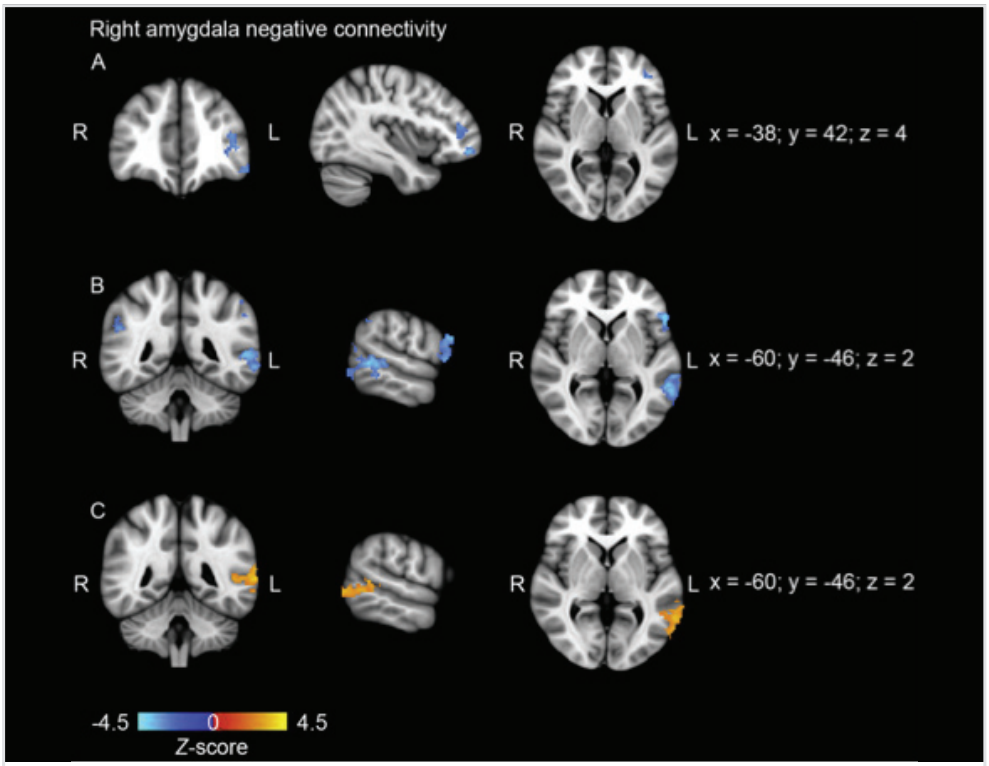

Figure 1.
Right amygdala negative connectivity. A = healthy controls; B = social anxiety disorder patients; C = group difference B > A. Results are cluster corrected at $p < .05$. Images are z-statistics, overlaid on the MNI-152 standard brain.

Table 3. Right Amygdala Resting-State Connectivity: Group Difference

MNI[a] coordinates			Side	*z*-value	*p*-value	Brain region
x	*y*	*z*				Cluster size: 536 voxels
-66	-44	4	L	3.78675	0.0002	Middle Temporal Gyrus, BA[b] 22
-62	-54	2	L	3.55479	0.0004	Middle Temporal Gyrus
-58	-68	0	L	3.50324	0.0005	Lateral Occipital Cortex, BA 19
-56	-48	8	L	3.42287	0.0006	Middle Temporal Gyrus
-62	-48	10	L	3.39525	0.0007	Supramarginal Gyrus, BA 21
-66	-46	14	L	3.32415	0.0009	Supramarginal Gyrus

[a]MNI = Montreal Neurologic Institute, coordinates of most significant voxels in cluster; [b]BA = Brodmann Area; voxel size 2 mm isotropic; *z*- and *p*-values of most significant voxel, cluster corrected.

The combined bilateral dACC showed increased connectivity in SAD patients with the left precuneus and left lateral occipital cortex (Figure 2; Table 4). Separate investigations of the left and right dACC did not produce any significant group differences.

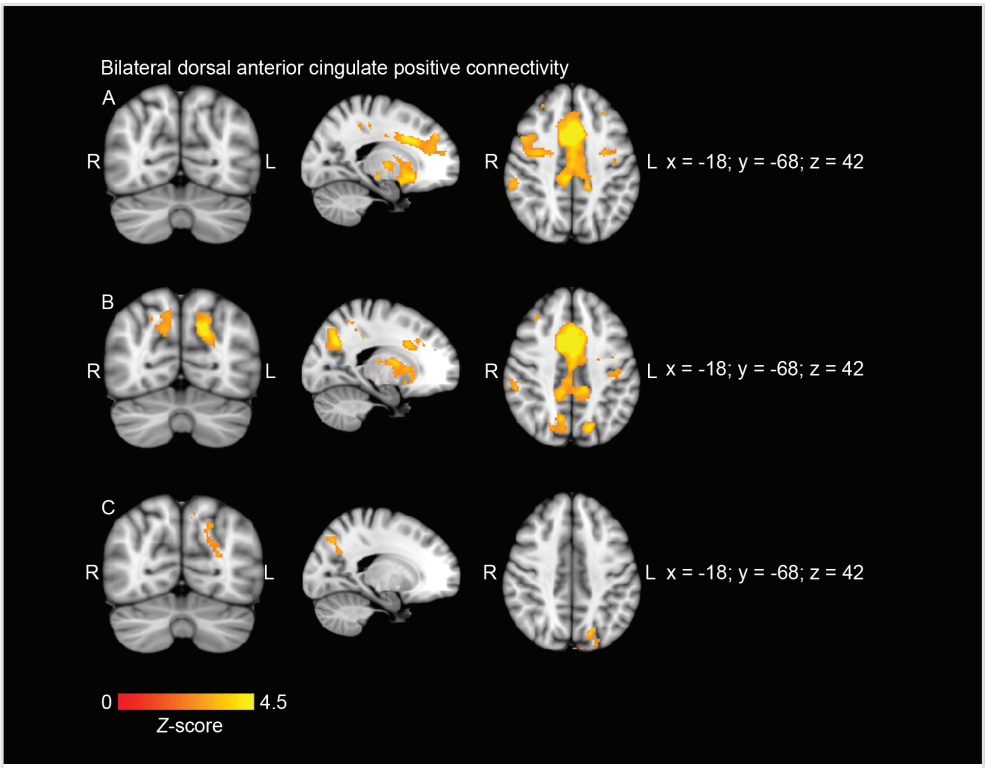

Figure 2.
Bilateral dorsal anterior cingulate cortex positive connectivity. A = healthy controls; B = social anxiety disorder patients; C = group difference: B > A. Results are cluster corrected at $p < .05$. Images are z-statistics, overlaid on the MNI-152 standard brain.

Table 4. Bilateral Dorsal Anterior Cingulate Cortex Resting-State Connectivity: Group Difference

MNI[a] coordinates			Side	*z*-value	*p*-value	Brain region
x	*y*	*z*				Cluster size: 410 voxels
-16	-72	40	L	3.64896	0.0003	Precuneus
-16	-64	32	L	3.34503	0.0008	Precuneus
-22	-84	40	L	3.28251	0.0010	Lateral Occipital Cortex
-26	-68	20	L	3.1207	0.0018	Lateral Occipital Cortex
-8	-88	40	L	3.0777	0.0021	Lateral Occipital Cortex
-12	-74	50	L	2.94138	0.0033	Lateral Occipital Cortex

[a]MNI = Montreal Neurologic Institute, coordinates of most significant voxels in cluster; voxel size 2 mm isotropic; *z*- and *p*-values of most significant voxel, cluster corrected.

We investigated DMN connectivity with a seed in the left and right PCC/precuneus. No group differences were found in DMN connectivity (Figure 3).

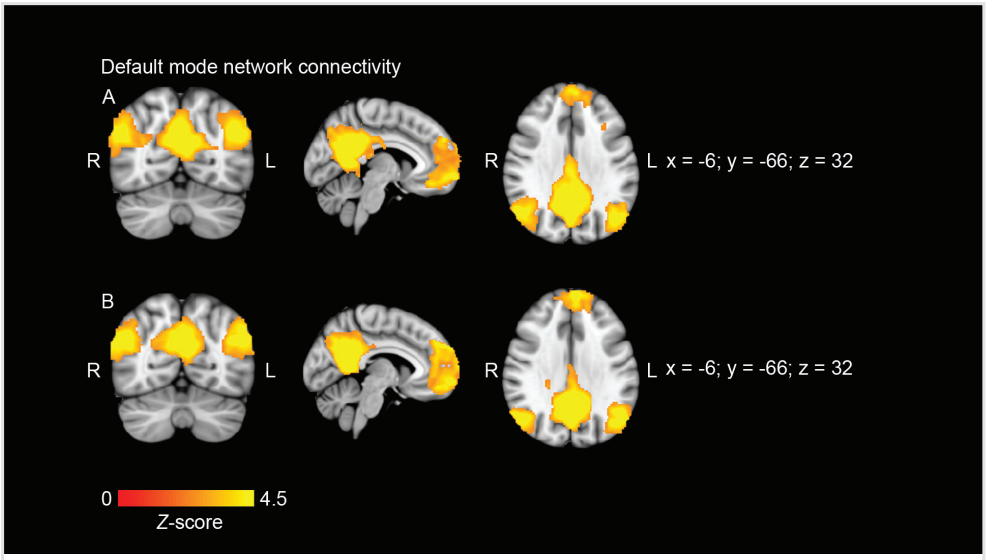

Figure 3.
Default mode network connectivity. A = healthy controls; B = social anxiety disorder patients. Results are cluster corrected at $p < .05$. Images are z-statistics, overlaid on the MNI-152 standard brain.

Correlation of clinical and RS fMRI data

Post-hoc, fMRI data were correlated with FQ scores using SPSS 17.0 (SPSS Inc., Chicago, Illinois, USA). If data did not meet the assumptions required to perform parametric analysis, and log-transforming did not resolve this problem, the appropriate non-parametric test was performed. The FQ contains a social phobia subscale that specifically focuses on feared situations that are associated with SAD. We created a mask of the resulting areas from our right amygdala analysis (the left middle temporal gyrus, left supramarginal gyrus and left lateral occipital cortex), and calculated the individual z-scores from these areas using Featquery, part of FSL (Smith *et al.*, 2004). Using non-parametric correlational analysis, we examined whether the strength of the functional connections was related to anxiety scores as measured with social phobia sub-scores on the FQ in the SAD group. We followed the same approach for the resulting areas showing aberrant connectivity in our bilateral dACC analysis (i.e., the left precuneus and lateral occipital cortex). No association was found between strength of the functional connectivity and anxiety scores in any of the identified regions.

Discussion

We investigated RSFC in drug-naive SAD patients without comorbidity and pair-wise matched healthy controls, using seed regions in the amygdala, the dACC and the PCC/ precuneus. As expected, we found abnormalities in SAD in RS connectivity for the amygdala seeds and the seeds in the salience network, but we found no differences in connectivity between patients and controls for the PCC/precuneus seeds probing the DMN. Specifically, in SAD we found altered RS connectivity between the right amygdala and the left middle temporal gyrus, left supramarginal gyrus, and left lateral occipital cortex. Increased connectivity between the bilateral dACC seeds (salience network) with both the left precuneus and left lateral occipital cortex was also found.

Our results are generally in agreement with previous findings of altered amygdala and anterior cingulate functional connectivity in SAD patients (Freitas-Ferrari *et al.*, 2010, Liao *et al.*, 2010b); however, our findings are not identical to the abnormalities in connectivity patterns found in other RS studies. Our results show some overlap with a previous study in finding involvement of the supramarginal gyrus (Liao

et al., 2010a) and middle temporal gyrus (Liao *et al.*, 2011), although these abnormalities concerned different networks. Whereas a previous functional MRI study (Gentili *et al.*, 2009) and a RS study (Liao *et al.*, 2011) in SAD have reported abnormalities in the DMN, we did not. These discrepancies could be explained by the different analysis method used (i.e., model-free analysis versus seed-based region-of-interest) and/or the drug-naive cohort without comorbidity that we used in the current study. Additionally, our relatively small sample size may be of influence, although lowering the threshold showed no significant group differences between social anxiety patients and healthy controls in DMN connectivity.

Another, admittedly speculative, explanation for the discrepancy in findings between our study and previous research is the difference in cultural backgrounds of the samples that were studied. As mentioned before, four of the five RSFC studies in SAD published to date used overlapping samples of subjects with an Asian cultural background. Several studies have compared the influence of cultural backgrounds in SAD and have found similarities as well as important differences in the phenomenology of social anxiety across Asian and Western cultures (Stein and Matsunaga, 2001, Furmark, 2002, Rapee *et al.*, 2011). Where Asian societies attribute a greater importance to concerns about offending others, the emphasis seems to be on embarrassing oneself in Western communities (Stein and Matsunaga, 2001).

We first analysed amygdala RSFC, using seeds in the bilateral amygdala. We found increased RSFC between the right amygdala, an area involved in fear and emotion processing, fear in particular (LeDoux, 2003), and the left middle temporal gyrus, left supramarginal gyrus, and left lateral occipital cortex, areas that have been linked to facial perception (Puce *et al.*, 1995, Grill-Spector *et al.*, 2004). Abnormal amygdala responses evoked by emotional faces have frequently been reported in SAD (Blair *et al.*, 2011a, Blair *et al.*, 2008b, Freitas-Ferrari *et al.*, 2010, Shin and Liberzon, 2010). The heightened RSFC of the amygdala in social anxiety patients may reflect an increased predisposition for the inaccurate interpretation of others' facial expressions.

We subsequently investigated bilateral dACC connectivity, probing the salience network. The salience network, comprising the bilateral dACC and bilateral anterior insula, is purportedly involved in identifying the most relevant internal and external stimuli, in order

to guide behaviour (Seeley *et al.*, 2007). We found increased positive dACC seed connectivity in SAD patients with the left precuneus and left lateral occipital cortex. The anterior cingulate, one of the structures of the salience network has, apart from its role in the salience network, also been associated with self- focused attention (Lemogne *et al.*, 2010). The precuneus is thought to play a central role in self-reflection and self-processing features, like mental imagery and episodic/ autobiographic memory retrieval (Cavanna and Trimble, 2006b, Cavanna, 2007). Another proposed function of the precuneus is the gathering of information on and representation of the self and the external world (Cavanna, 2007). Strong self-focus has been previously reported in depression, generalized anxiety disorder and SAD, specifically attributing a role to public self-focus in the latter (Lemogne *et al.*, 2010). The heightened connectivity between the bilateral dACC and the precuneus found in our study could underlie this raised awareness of the self and the environment, in particular of other people. More- over, since the salience network is thought to be essential in identifying the relevance of stimuli, a stronger connectivity between the dACC and the precuneus could reflect the tendency of social anxiety patients to attribute an exaggerated significance to possible self-relevant stimuli from the external world (Blair *et al.*, 2008a).

We observed that the strength of the RS functional connectivity was not related to anxiety scores as measured with the social phobia sub-scores of the FQ. Previous reports did show associations between abnormalities in connectivity and alterations in anxiety scores, as measured with the Liebowitz Social Anxiety Scale (Liao *et al.*, 2010a, Liao *et al.*, 2010b, Ding *et al.*, 2011, Liao *et al.*, 2011) and with the Spielberger State and Trait Anxiety Scores (Hahn *et al.*, 2011). Possibly, we did not find correlations due to methodological issues such as the characteristics of our sample, the sample size, or the use of the FQ social phobia sub- scores.

Overall, our results are in line with existing models of SAD. Stein (1998) reviewed several animal and human models of SAD and concluded that the neural circuitries of anxiety and self-consciousness were likely to be involved in the pathophysiology the disorder (Stein, 1998). Negative self-appraisal had been linked to SAD before in two models suggesting an abnormal tendency of SAD patients to retrospectively ruminate and to appraise themselves in a negative manner (Clark and Wells, 1995, Rapee and Heimberg, 1997). More recently, the role of circuitry underlying self-

referential processing has also been emphasized by Blair *et al*. (2008a), who found that SAD patients show amygdala and dorsal medial prefrontal cortex hyperresponsiveness to negative, self-referential comments (Blair *et al*., 2008a). This heightened propensity to focus on the self could possibly also be represented in the increased RSFC that we found between the bilateral dACC and the left pre- cuneus. Clearly, the interpretation of abnormalities in RSFC is more speculative and should be done with caution, as the relation between abnormalities in RFSC and abnormalities in task related functional connectivity in SAD has not been directly studied yet.

Our study has several strengths. This is the first study investigating RS functional connectivity in a group of drug- naive social anxiety patients without psychiatric comorbidity. There are also some noteworthy limitations to our study. Our sample size is relatively small and replication in other, larger samples is desirable in order to enable more conclusive interpretation. It should also be noted that subjects may engage in different forms of cognitive action during the resting-state condition, which can influence resting-state activity patterns. We emphasize that RSFC data should be interpreted cautiously, and stress that any interpretation refers only to functional connectivity between brain areas as opposed to the (dys)function of a distinct brain region. Also, by opting for a hypothesis-driven approach and thus only exploring specific networks, abnormalities in other networks might have been missed. On the other hand, the use of a seed-based region-of-interest approach allows easier replication of our findings. Another limitation of the current study was the possible influence of between-group differences in heart rate variability and breathing on the results. Since physiological activity was not monitored in the current study, it remains unclear if any difference between the two groups has influenced the results, although previous research shows that heart rate variability did not differ between anxiety patients and healthy controls in the larger NESDA sample (Licht *et al*., 2009). Additionally, regressing out global signal changes has shown to at least partly filter out the effects of cardiac and respiratory fluctuations (Birn *et al*., 2006, Fox and Raichle, 2007). Pooling data from different centres is another possible limitation. To account for this as much as possible, we have matched our groups based on scanning site and also added a confound regressor for site in our statistical model. A further limitation is the absence of a specific questionnaire for the assessment of social anxiety. Use of a specific social anxiety scale instead of

the more generalised Fear Questionnaire might prove to be more sensitive to identify an association between altered functional connectivity and extent of SAD symptoms. Finally, our RS fMRI data were acquired at the end of a fixed imaging protocol, including a facial expression task, which could potentially have influenced RS connectivity (i.e., a spillover effect) with SAD patients still showing aberrant connectivity in areas involved in the processing of emotional faces while the facial stimulus was no longer present.

In summary, we found altered RS connectivity in drug- naive SAD patients without comorbidity, between areas involved in the processing of fear and emotion and areas that contribute to facial perception. Additionally, we found aberrant connectivity between a key region of the salience network and areas involved in the processing of self-relevant stimuli, which has become a recent focus of research in SAD.

Chapter 4
Investigating shared and unique abnormalities of resting-state functional connectivity in depression, anxiety, and their comorbid states

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Abstract

Depression and anxiety disorders are highly comorbid and share neurobiological characteristics. However, this is usually not explicitly addressed in studies on intrinsic brain functioning in these disorders. We investigated resting-state functional connectivity (RSFC) in medication-free patients with depression, anxiety, comorbid depression and anxiety, and a healthy control group. RSFC was investigated in 140 medication-free subjects: 37 major depressive disorder patients (MDD), 30 patients with one or more anxiety disorders (ANX), 25 patients with MDD and one or more anxiety disorders (COM), and 48 healthy controls (HC). RSFC networks were calculated using a probabilistic independent component analysis. Using a dual regression approach, individuals' timecourses were extracted and regressed to obtain subjects-specific spatial maps, which were used for group comparisons in four networks of interest (limbic, default mode, salience and sensory-motor networks). When compared to HC, the COM group showed increased RSFC of the limbic network with a cluster containing the bilateral precuneus, intracalcarine cortex, lingual gyrus, and posterior cingulate, and with a cluster including the right precentral gyrus, inferior frontal gyrus, and middle frontal gyrus. This effect was specific for comorbid depression and anxiety. No abnormal RSFC of other networks or in the MDD and ANX groups was observed. No association was found between strength of RSFC and symptom severity. These results indicate that altered RSFC of regions in a limbic network could be specific for comorbid depression and anxiety.

Introduction

Major depressive disorder (MDD) is one of the most prevalent of all psychiatric disorders, and according to the National Comorbidity Study, 27.1% of all MDD patients presented with comorbid social anxiety disorder (SAD), 17.2% with comorbid generalised anxiety disorder (GAD), and 9.9% with comorbid panic disorder (PD) (Gorman, 1996, Kessler *et al.*, 1996). Comorbid depression and anxiety are associated with a greater societal burden, and with worse outcome (less response to treatment, longer illness duration, greater risk of suicide) (Gorman, 1996, Kessler *et al.*, 2005b).
MDD and anxiety patients not only show frequent comorbidity; they also respond to the same treatment strategies and it has been suggested that they share a similar etiology (Ressler and Mayberg, 2007). However, comorbidity of depression and anxiety disorders is usually not explicitly addressed in studies examining the underlying neurobiological characteristics of MDD and anxiety disorders. Instead, the separate disorders have been the focus of neurobiological studies and as a result have received considerable attention in recent years.

In depression, neuroanatomical and functional abnormalities have been reported for a range of brain regions including (subregions of) the anterior cingulate cortex (ACC), prefrontal cortex (PFC), hippocampus, amygdala, posterior cingulate cortex (PCC), thalamus, striatum, pallidum, and temporal cortical areas. Additionally, abnormalities have been reported in neural circuits such as limbic, prefrontal, sensory, motor, and default mode networks; for a review, see Drevets *et al.* (2008). In SAD, PD and GAD, the amygdala is the brain area most commonly reported to show abnormalities compared to healthy controls (Damsa *et al.*, 2009, Freitas-Ferrari *et al.*, 2010). Other brain areas that were frequently found to show aberrancies in anxiety include the PFC, ACC, insula, striatum, superior temporal gyrus, thalamus, and hippocampus (Damsa *et al.*, 2009). Although it is apparent that many brain areas and circuits are implicated in both depression and anxiety, their distinct and common roles in the disorders are still unclear.

So far, only five neuroimaging studies investigated the unique and shared neural properties of depression and anxiety in a design comparing three clinical groups of individuals with MDD only, anxiety only, and comorbid MDD and anxiety, with a group of healthy controls. In one task functional MRI (fMRI) study, depression and GAD were investigated in their unique as

well as comorbid states (Etkin and Schatzberg, 2011). The other four studies were conducted within the context of the Netherlands Study of Depression and Anxiety (NESDA), a large multi-centre longitudinal cohort designed to chart the long-term course and consequences of depression and anxiety (Penninx *et al.*, 2008). The anxiety disorders included in NESDA are the common anxiety disorders PD, SAD, and GAD. In one neuroanatomical and three task fMRI studies in the NESDA cohort, the unique and common profiles of depression and anxiety were investigated (Demenescu *et al.*, 2011, van Tol *et al.*, 2010, van Tol *et al.*, 2011, van Tol *et al.*, 2012). The results of these five studies point to shared as well as unique contributions of depression and anxiety to aberrancies in brain structure and function, with roles for the ACC, PFC, insula, amygdala, hippocampus, and the inferior, temporal and superior frontal gyri.

Functional MRI is not only widely employed to study functional activity and connectivity of brain areas within the context of task paradigms, but is also used to examine connectivity during the so-called resting-state, i.e. in the absence of externally controlled stimuli or tasks (Biswal *et al.*, 1995, Gusnard *et al.*, 2001). Coherent fluctuations in resting-state have consistently been identified across subjects and sessions, and are viewed as functional resting-state networks (Beckmann *et al.*, 2005, Damoiseaux *et al.*, 2006). Abnormalities in resting-state functional connectivity (RSFC) have been found in various neuropsychiatric disorders known to involve disturbed emotion regulation and self-processing, including depression and anxiety (Broyd *et al.*, 2009, Pannekoek *et al.*, 2013b, Pannekoek *et al.*, 2013c, Veer *et al.*, 2010, Wang *et al.*, 2012).

The default mode network (DMN) is a resting-state network containing the precuneus cortex, PCC, medial PFC (mPFC), lateral and inferior parietal cortex and ventral anterior cingulate cortex (vACC) (Greicius *et al.*, 2003, Gusnard *et al.*, 2001). Due to its critical role in self-referential processing (Broyd *et al.*, 2009, Gusnard *et al.*, 2001), this network has received substantial attention in research on depression, and abnormalities in depression were found (Broyd *et al.*, 2009, Wang *et al.*, 2012). Greicius *et al.* (2007) were among the first to investigate DMN RSFC with fMRI in depressed patients, finding greater connectivity with areas associated with depression including the subgenual ACC, thalamus, mPFC, and cuneus/precuneus. In addition, the effect in the subgenual ACC

correlated positively with length of the depressive episode (Greicius *et al.*, 2007). Various subsequent studies have also reported altered DMN RSFC, with areas such as the caudate (Bluhm *et al.*, 2009, Kenny *et al.*, 2010), precuneus (Andreescu *et al.*, 2011, Kenny *et al.*, 2010, Zhou *et al.*, 2010) and frontal cortical areas as the ACC (Andreescu *et al.*, 2011, Zhou *et al.*, 2010). In addition to altered RSFC of the DMN, aberrant RSFC of other brain networks has also been reported in MDD, such as altered cortico-limbic connectivity, especially between the PFC and the amygdala in an affective network (Anand *et al.*, 2005, Wang *et al.*, 2012, Veer *et al.*, 2010). Furthermore, altered RSFC of the frontal pole and of the lingual gyrus (Veer *et al.*, 2010), precuneus-caudate (Bluhm *et al.*, 2009), sgACC-insula (Cullen *et al.*, 2009, Horn *et al.*, 2010), thalamus (Greicius *et al.*, 2007, Kenny *et al.*, 2010), inferior frontal gyrus (Zhou *et al.*, 2010), dorsomedial PFC-precuneus (van Tol *et al.*, 2013), and cerebellum (Liu *et al.*, 2010) has been reported in MDD.

In anxiety disorders, RSFC abnormalities have been reported for a variety of networks, such as a limbic network and the salience network in PD (Pannekoek *et al.*, 2013b), and in a variety of networks in SAD (Liao *et al.*, 2010a, Pannekoek *et al.*, 2013c). Functional connectivity in the DMN was shown to be stronger between the PCC and mPFC in older GAD subjects relative to younger patients (Andreescu *et al.*, 2013). The presence of GAD, longer illness duration and more severe worrying were related to greater differences in DMN connectivity.

Evidence from neuroimaging literature indicates that many brain regions show similar abnormalities in MDD and in anxiety disorders. This is in agreement with a "common-disorder" model of depression and anxiety (Etkin and Schatzberg, 2011), although the few task fMRI studies and the neuroanatomical study that have addressed comorbidity also report unique characteristics of depression and anxiety (Demenescu *et al.*, 2011, Etkin and Schatzberg, 2011, van Tol *et al.*, 2012, van Tol *et al.*, 2011, van Tol *et al.*, 2010). In the present study, we aimed to investigate RSFC in depression, anxiety and their comorbid states by employing RS fMRI in three clinical groups and compare these with a healthy control group: MDD patients, anxiety patients (PD and/or SAD and/or GAD), and comorbid MDD and anxiety patients (MDD and PD and/or SAD and/or GAD), compared to healthy controls. Based on the current neurobiological models of depression and anxiety and the available literature, we expected abnormalities of RSFC of four networks

of interest previously associated with disturbances of emotion processing: the default mode network, the sensory-motor network, the salience network and a limbic network (containing brain regions such as the amygdala and hippocampus).

Method

Participants

Participants were recruited from a longitudinal, large-scale, multi-centre, observational cohort study: the Netherlands Study of Depression and Anxiety (NESDA) (Penninx *et al.*, 2008). This study was designed to chart the long-term course and consequences of depressive and anxiety disorders, including participants from different health care settings (i.e. the community, through primary care and specialized mental health institutions) and various developmental stages of illness.

The NESDA main sample consisted of 2981 participants, aged between 18 and 65 years. Participants aged between 18 and 57 years old were invited to participate in the NESDA neuroimaging study if they met the DSM-IV criteria for a half-year diagnosis of MDD and/or anxiety disorder (PD, SAD, and/or GAD), or no lifetime DSM-IV diagnosis (i.e. healthy controls). Participants were not screened for personality disorders, but individuals with a known personality disorder based on information from clinics or self-report were not included in the study. Patients were excluded based on the following criteria: presence of axis-I disorders other than MDD, PD, SAD, or GAD, and any use of psychotropic medication other than stable use of selective serotonin reuptake inhibitors (SSRIs) or infrequent benzodiazepine use (i.e. equivalent to 2 doses of 10 mg of oxazepam 2 times per week, or use within 48 hours prior to scanning).

Controls were currently free of, and had never met criteria for, depressive or anxiety disorders or any other axis-I disorder. They were not taking any psychotropic drugs. Overall, participants were excluded based on the following criteria: presence or history of major internal or neurological disorder, dependence or recent alcohol and/or drug abuse (past year), hypertension, and general MRI contraindications. The Composite International Diagnostic Interview (CIDI) lifetime version 2.1, administered by a trained interviewer, was used to diagnose depressive and anxiety disorders according to DSM-IV algorithms.

Overall, 301 native Dutch-speaking participants (233 patients and 68

controls) were included in the NESDA neuroimaging study and underwent MR imaging in one of the three participating centres (Academic Medical Centre Amsterdam, Leiden University Medical Centre, and University Medical Centre Groningen). After receiving written information, all subjects provided written informed consent. The study was approved by the Medical Ethics Committees of all three centres and conducted in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

For the present study, RS-fMRI data were available from 229 subjects. Ten patients were removed from the sample due to excessive head motion during scan acquisition (> 3 mm in any of the acquired volumes). Of the remaining 219 subjects, we only included participants that were medication-free at the time of the study (N = 140 in total, of which N = 114 medication-naïve). Our final sample consisted of 140 medication-free subjects: 37 patients with MDD, 30 patients with one or more anxiety disorders (PD, SAD and/or GAD) but no MDD (ANX group), 25 patients with comorbid MDD and one or more anxiety disorders (COM group: MDD and PD and/or SAD and/or GAD), and 48 healthy controls (HC group).

Image acquisition

Image acquisition took place at one of the three participating centres within on average 8 weeks after completion of the NESDA baseline interview (Penninx *et al.*, 2008). RS-fMRI data were acquired at the end of the fixed imaging protocol: after completion of three task-related functional MRI runs and the acquisition of an anatomical scan (scan sequence: Tower of London, word encoding, T1-weighted scan, word recognition, perception of facial expression, resting-state). Images were obtained on Philips 3T magnetic resonance imaging systems (Philips Medical Systems, Best, The Netherlands), equipped with a SENSE-8 (Leiden University Medical Centre and University Medical Centre Groningen) or SENSE- 6 (Academic Medical Centre Amsterdam) channel head coil.

RS-fMRI data were acquired for each subject using T_2^*-weighted gradient-echo echo-planar imaging with the following scan parameters in Amsterdam and Leiden: 200 whole-brain volumes; repetition time 2300 ms; echo time 30 ms; flip angle 80°; 35 transverse slices; no slice gap; field of view 220 × 220 mm; in-plane voxel size 2.3 × 2.3 mm; slice thickness 3 mm; duration 7.51 min. Parameters in Groningen were identical, apart from: echo time 28 ms; 39 transverse slices; in-plane voxel size 3.45 × 3.45mm. In the

darkened MR room participants were instructed to lie still with their eyes closed and not to fall asleep. After completion of the scan, subjects confirmed wakefulness during acquisition. A sagittal 3-dimensional gradient-echo T1-weighted image was acquired for registration purposes and grey matter analysis with the following scan parameters: repetition time 9 ms; echo time 3.5 ms; flip angle 80°; 170 sagittal slices; no slice gap; field of view 256 × 256 mm; 1 mm isotropic voxels; duration 4.5 min.

Data preprocessing

The preprocessing of RS-fMRI images was carried out using FEAT (FMRI Expert Analysis Tool) Version 5.98, part of FSL (FMRIB's Software Library) (Smith *et al*., 2004). The following processing steps were applied: motion correction (Jenkinson *et al*., 2002), removal of non-brain tissue, spatial smoothing using a Gaussian kernel of 6-mm full-width at half-maximum, grand-mean intensity normalization of the entire 4D dataset by a single multiplicative factor, high-pass temporal filtering (Gaussian-weighted least-squares straight line fitting with a 0.01 Hz cut-off) and registration to the high resolution T1 and MNI-152 standard space images (T1 standard brain averaged over 152 subjects; Montreal Neurological Institute, Montreal, QC, Canada) (Jenkinson *et al*., 2002). Normalized 4D data sets were then resampled to 4-mm isotropic voxels to reduce computational burden in the following analysis steps.

Statistical analysis

Motion parameters (Table 1) did not differ significantly between groups for absolute displacement nor relative displacement. Standard group independent component analysis (ICA) was carried out using probabilistic ICA (PICA) (Beckmann and Smith, 2004) as implemented in FSL's Multivariate Exploratory Linear Decomposition into Independent Components (MELODIC). ICA attempts to split the 4D functional data into a set of spatial maps, each with an associated timecourse, by performing a linear decomposition of the original data. Time series of all participants were temporally concatenated into a single 4D time series, which was separated in 20 components using ICA in MELODIC. We selected three components based on spatial similarity to functional networks consistently described before (Beckmann *et al*., 2005, Damoiseaux *et al*., 2006): the default mode network; the sensory-motor system; and the salience network. In addition,

we selected one other component that was relevant for the present study: a limbic network (containing brain regions such as the amygdala and hippocampus).

Next, the subject-specific component maps were identified by extracting individual time series for each component, using the 20 component maps in a spatial regression against the individual data. In other words, the set of spatial maps from the group-average analysis was used to generate subject-specific versions of the spatial maps, and associated time series, using dual regression (Beckmann *et al.*, 2009). First, for each subject, the group-average set of spatial maps was regressed (as spatial regressors in a multiple regression) into the subject's 4D space-time dataset. This resulted in a set of subject-specific time series, one per group-level spatial map. Next, those time series were regressed (as temporal regressors, again in a multiple regression) against the same 4D dataset, resulting in a set of subject-specific spatial maps, one per group-level spatial map.

We segmented the 4 mm standard brain into grey matter, white matter, and cerebrospinal fluid (CSF) and created a mask of grey and white matter only. This mask was applied to the mask derived from the dual regression to ensure that the voxels tested in the voxel-wise nonparametric permutation test were all situated solely in brain tissue and not in the CSF.

For the main comparison, we performed a factorial analysis with one factor (group) at four levels (MDD, ANX, COM, and HC). We then used two-sample t-tests to investigate group differences for each of the selected functional networks. These tests were performed using voxel-wise nonparametric permutation in FSL (randomise; 5000 permutations) (Nichols and Holmes, 2002), including grey matter density information from each participant as a voxel-dependent covariate. This step was repeated without a voxel-dependent covariate to examine the potential influence of grey matter differences. By including structural information in the functional connectivity analysis, variance explained by potential differences in grey matter density and/or possible misregistrations are taken into account (Oakes *et al.*, 2007).

Depression- and anxiety-related grey matter abnormalities were previously reported by our group in the NESDA neuroimaging sample (van Tol *et al.*, 2010). Three nuisance regressors describing scanner location and gender and age were added to the model, in addition to modelling regressors for each of the two groups. The resulting statistical maps for each

RS network were corrected for family-wise error using threshold-free cluster enhancement (TFCE) at a threshold of $p < .05$ (Smith and Nichols, 2009).

For between-groups effects present only in the contrast COM-HC, we planned post-hoc analyses with the contrasts COM-MDD and COM-ANX to further assess specificity for the comorbid depression and anxiety group. For these contrasts, we compared the RSFC in masks based on the effects found in COM-HC, using the FSL Randomise tool and TFCE corrected with a threshold of $p < 0.05$.

Demographic and clinical data were analysed using SPSS 20.0 (SPSS Inc, Chicago, Illinois). If data did not meet the assumptions required to perform parametric analysis, a non-parametric alternative was used. Significance was set at $p < .05$, and post hoc paired tests were Bonferroni corrected for multiple comparisons.

Results

Sample descriptives

Table 1 lists the clinical characteristics of the sample. The four groups were matched for age, sex, scan location, and handedness. However, they did differ on education as shown by the Kruskal-Wallis Test (Table 1); further exploration using the Mann-Whitney U-Test revealed that HC had more years of education than MDD patients ($U = 498$; $p < .001$), ANX patients ($U = 460$; $p = .006$), and COM patients ($U = 276$; $p < .001$).

As expected, HC showed lower scores on the Beck Anxiety Inventory (BAI) than MDD ($U = 1322$; $p < .001$), ANX ($U = 1288.5$; $p < .001$), and COM ($U = 1129.5$; $p < .001$). They also reported lower scores on the Montgomery-Åsberg Depression Rating Scale (MADRS) than MDD ($U = 1549$; $p < .001$), ANX ($U = 1270.5$; $p < .001$), and COM ($U = 1164.5$; $p < .001$). Furthermore, HC showed lower scores on the Inventory of Depressive Symptomatology (IDS) than MDD ($U = 1466.5$; $p < .001$), ANX ($U = 1288.5$; $p < .001$), and COM ($U = 1161.5$; $p < .001$). Additionally, COM scored significantly higher on the BAI compared to MDD ($U = 703.5$; $p < .001$), and on the MADRS compared to ANX ($U = 554.5$; $p = .002$). All these results survived Bonferroni correction for multiple testing.

Table 1. Demographic and Clinical Characteristics of the Sample (*N* = 140).

Characteristic	MDD	ANX[a]	COM[b]	HC	*H*	χ^2	*df*	*p*
Sample, *N*	37	30	25	48				
Sex, *N* (%)								
Male	19 (51.4)	8 (26.7)	6 (24)	18 (37.6)		6.48	3	.09
Female	18 (48.6)	22 (73.3)	19 (76)	30 (62.5)				
Age (years), Mean (SD)	35.7 (10.11)	33.1 (8.39)	34.8 (10.54)	40.0 (9.43)	9.89	3	.019	
Education (years), Mean (SD)	5.84 (1.44)	6.07 (1.84)	5.16 (2.01)	6.96 (1.64)	21.53	3	<.001	
Scan location, *N*								
AMC	7	6	5	18		10.93	6	.09
LUMC	15	9	10	21				
UMCG	15	15	10	9				
Handedness								
Left	4	2	2	4		0.39	3	.94
Right	33	28	23	44				
BAI score at scanning, Mean (SD)	8.78 (9.72)	14.17 (10.59)	16.8 (8.67)	2.09 (2.44)	63.89		3	<.001
Range	0–50	0–42	1–36	0–10				
MADRS score at scanning, Mean (SD)	11.73 (10.54)	10.4 (8.54)	19 (10.33)	1.11 (1.94)	73.97		3	<.001
Range	0–39	0–35	2–43	0–7				
IDS score at scanning, Mean (SD)	18.09 (12.41)	18.71 (10.67)	28 (11.54)	3.83 (3.8)75.83			3	<.001
Range	1–57	4–49	8–55	0–17				
Motion parameters in millimetres								
Absolute displacement, Mean (SD)	.28 (.213)	.28 (.218)	.32 (.249)	.27 (.173)				.823
Relative displacement, Mean (SD)	.09 (.038)	.08 (.030)	.09 (.060)	.08 (.034)				.572

Legend table 1

Abbreviations: MDD, major depressive disorder; ANX, anxiety disorder; COM, comorbid depression and anxiety; HC, healthy controls AMC, Academic Medical Centre Amsterdam; LUMC, Leiden University Medical Centre; UMCG, University Medical Centre Groningen; BAI; Beck Anxiety Inventory; MADRS, Montgomery-Åsberg Depression Rating Scale; IDS, Inventory of Depressive Symptomatology; *H*, Kruskal-Wallis non-parametric multiple independent-samples test; χ^2, chi-square test; *df*, degrees of freedom.

[a] 9 patients had panic disorder (PD); 12 patients had social anxiety disorder (SAD); 6 patients had PD and SAD; 2 patients had PD, SAD, and generalized anxiety disorder (GAD); 1 patient had PD and GAD.

[b] 8 patients had MDD and PD; 2 patients had MDD and SAD; 3 patients had MDD, PD, and SAD; 4 patients had MDD and GAD; 4 patients had MDD, SAD, and GAD; 4 patients had MDD, PD, and GAD.

Resting-state functional connectivity results

Twenty functional connectivity networks were generated during the independent component analysis and entered into a dual regression; four of which were selected for further analysis (Figure 1). These networks have been previously described in studies using similar analysis techniques, showing stable spatial similarity across participants and over time (Beckmann et al., 2005, Damoiseaux et al., 2006). All functional networks were present in ANX, COM, HC and MDD, all $p < .05$, family-wise corrected, based on the TFCE statistic image.

Between-group differences were examined by contrasting HC against the three clinical groups (i.e. HC versus ANX, HC versus COM, and HC versus MDD). Therefore, an additional correction for multiple comparisons was applied with a more stringent $p < .017$ (.05 / 3). Between-group differences were only revealed for the limbic network, for which patients with comorbid depression and anxiety (the COM group) showed increased RSFC of the limbic network with a cluster containing the bilateral precuneus, intracalcarine cortex, lingual gyrus, and posterior cingulate, as well as with a cluster including the right precentral gyrus, inferior frontal gyrus, and middle frontal gyrus compared to HC (Figure 2). This effect was not found in the ANX and MDD groups when compared to HC.

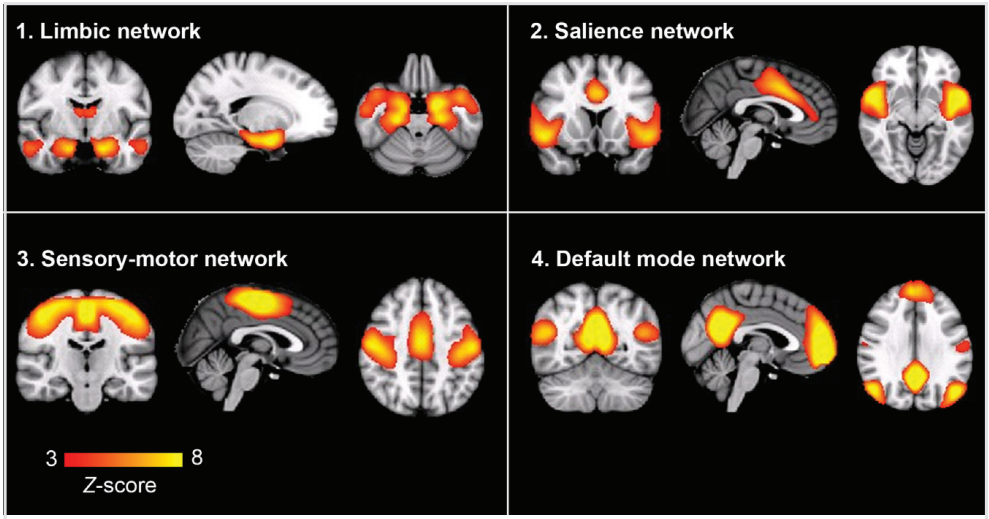

Figure 1. Independent component analysis functionally relevant resting-state networks.
Depicted are the four functional resting-state networks that were selected for further analysis, resulting from the group probabilistic independent component analysis that was carried out on the concatenated datasets from all groups (ANX, COM, MDD, HC). Images are z-statistics, ranging from 3 to 8, overlaid on the MNI-152 standard brain. The left hemisphere of the brain corresponds to the right side in this image.

Post-hoc, we investigated whether this specific increase in RSFC found in COM in comparison to HC was shared with DEP and ANX, or whether it was indeed unique to COM. Within the effect for the limbic network (i.e. increased RSFC with a more posterior and a more frontal cluster), COM showed significantly stronger RSFC compared to ANX as well as MDD, indicating that the increased RSFC of the limbic network with these two clusters was indeed specific for patients with comorbid depression and anxiety (Figure 3).

Given the grey matter differences found in the structural NESDA MRI study (van Tol *et al.*, 2010), we investigated whether the observed functional connectivity differences were influenced by grey matter density. We therefore repeated the analysis without a voxel-dependent covariate, which yielded the same results as analysis with the correction.

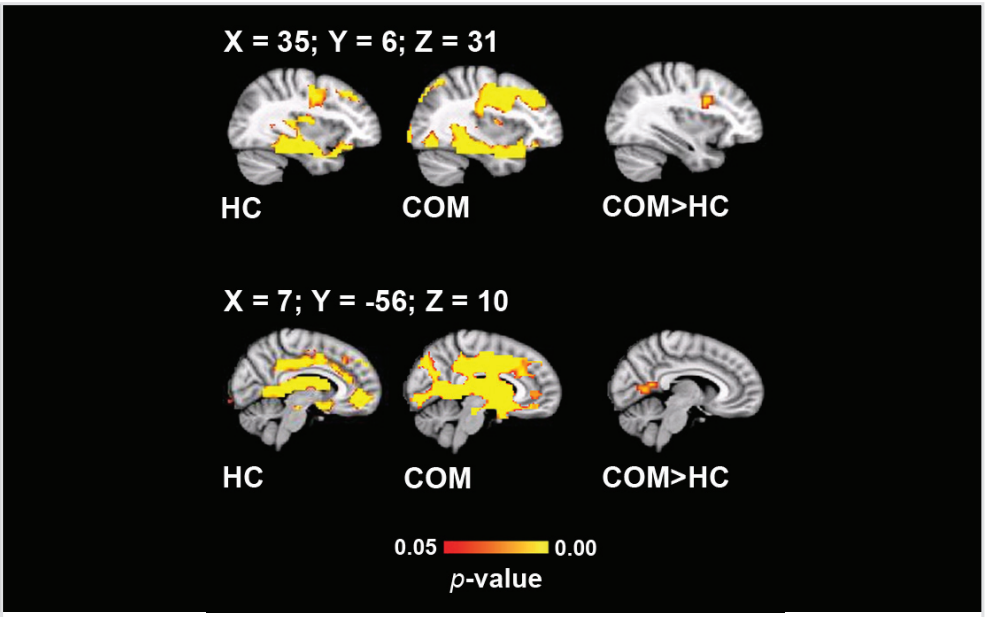

Figure 2. Group main effects and between-group effects of COM and HC. Depicted on the left side are the group main effects and the between-group difference of COM>HC for the limbic network. The top images show the group difference in an anterior cluster including the right precentral gyrus, inferior frontal gyrus, and middle frontal gyrus. The bottom images show the group difference in a posterior cluster containing the right precentral gyrus, inferior frontal gyrus, and middle frontal gyrus. Images are threshold-free cluster enhancement corrected *p*-statistics with $p < .05$, overlaid on the MNI-152 standard brain.
COM, patients with comorbid depression and anxiety; HC, healthy controls. The left hemisphere of the brain corresponds to the right side in this image.

To investigate the relation of clinical scores on the BAI, MADRS, and IDS with the aberrant functional connectivity in the COM group, a hierarchical multiple regression was performed while controlling for age and gender. Following previous work of our group in which 'mood/cognition' and 'anxiety/arousal' factors of the IDS were identified (Wardenaar *et al.*, 2010), these two IDS subscales were also addressed separately in post hoc tests in addition to the IDS total score. As a measure of the functional connectivity strength, the individual z-scores obtained from the affected areas within the corresponding individual component maps were extracted and exported to SPSS. No association was found between strength of RSFC and symptom severity.

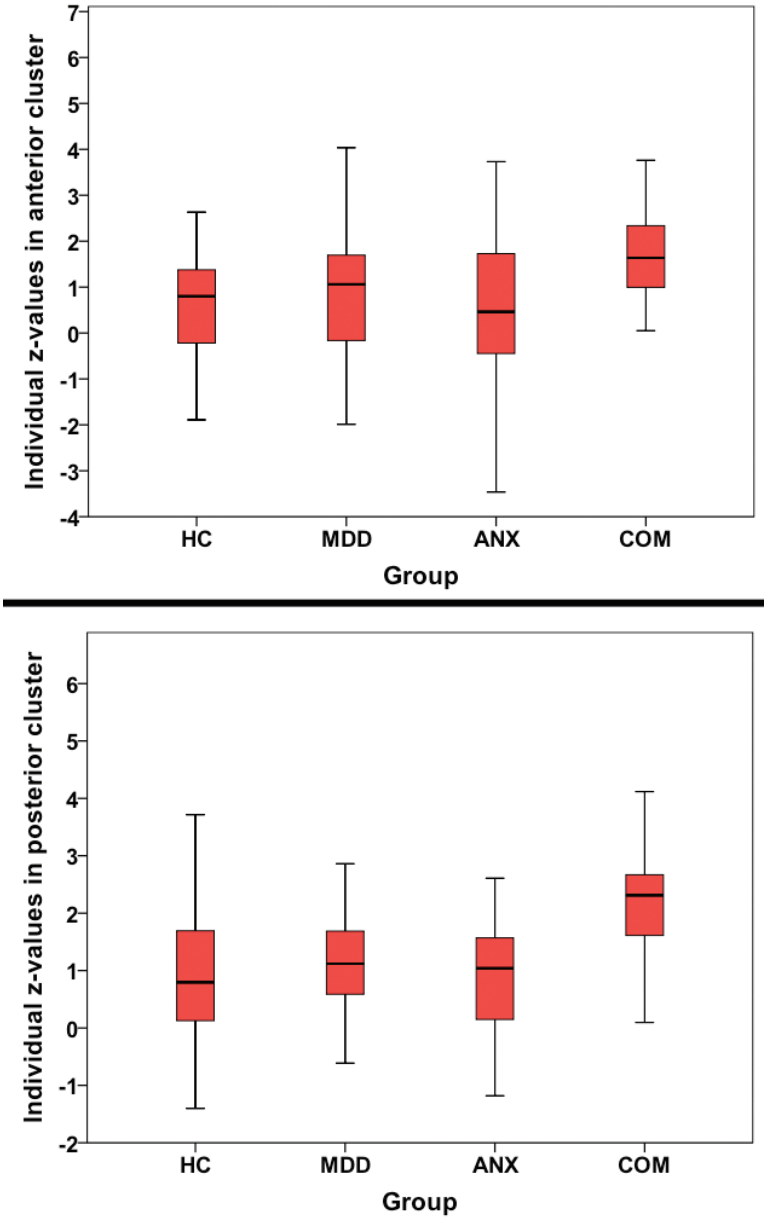

Figure 3. Graphic representation of the individual z-scores for all four groups. Displayed are the distributions for all groups of the mean individual z-scores of the effect in the anterior cluster (top plot), and of the effect in the posterior cluster (bottom plot). HC, healthy controls; MDD, patients with major depressive disorder; ANX, patients with anxiety; COM, patients with comorbid depression and anxiety.

Discussion

In this study we investigated RSFC in medication-free patients with depression, anxiety, and comorbid depression and anxiety compared to healthy controls. We used a data-driven dual regression approach to examine four resting-state networks of interest. In the comorbid group, we found increased RSFC of the limbic network with the bilateral precuneus, intracalcarine cortex, lingual gyrus, and posterior cingulate, as well as with the right precentral gyrus, inferior frontal gyrus, and middle frontal gyrus, compared to healthy controls. Post-hoc analyses showed that this effect was unique to the comorbid state of depression and anxiety. Contrary to our hypothesis, we found no abnormalities in the other networks of interest.

Recent RS studies have indicated involvement of the PCC/precuneus in depression (Greicius *et al.*, 2007) as well as in anxiety disorders (Pannekoek *et al.*, 2013b, Pannekoek *et al.*, 2013c, Strawn *et al.*, 2012). Using a seed-based region-of-interest approach, our group found increased RSFC between the limbic network and the bilateral precuneus in PD patients without comorbidity, using the amygdala as a seed (Pannekoek *et al.*, 2013b). As the precuneus has been related to a range of cognitive functions, namely visuo-spatial imagery, episodic memory retrieval, self-awareness, and consciousness (Cavanna and Trimble, 2006a), the increased RSFC of the precuneus with limbic regions in PD patients could be related to anxiety symptoms such as depersonalization and loss of control, for example during panic attacks (Pannekoek *et al.*, 2013b). In another seed-based study, in SAD patients without comorbidity, we observed an increased RSFC between the dACC and the precuneus within the context of the salience network, which could be associated with heightened self-awareness in SAD (Pannekoek *et al.*, 2013c). In depression, increased RSFC with the precuneus was also reported, albeit within the context of the DMN (Greicius *et al.*, 2007). In contrast to these RSFC findings, Strawn and colleagues (2012) demonstrated that adolescents with GAD exhibited a decrease in functional connectivity between the left amygdala and the precuneus during an attentional task involving strong negative emotional and neutral emotional distractors (Strawn *et al.*, 2012). Differences with the RSFC findings may be due to the task-related design and the adolescent sample.

The right precentral gyrus and inferior frontal gyrus also showed increased RSFC with the limbic network in COM patients compared to healthy controls. Reduced inferior frontal gyrus volumes were previously reported in MDD patients by our group, and it was suggested that this could represent a neuroanatomical basis for MDD (van Tol *et al.*, 2010). Also, in a group of clinically depressed adolescents, most of which presented with comorbid anxiety, increased RSFC was found between the amygdala and inferior frontal gyrus using seeds probing the limbic network (Pannekoek *et al.*, 2014a). In addition, increased activation of the right inferior frontal gyrus was found during an emotional word recognition task in anxiety patients (van Tol *et al.*, 2012). One study investigated emotion regulation of social situations in healthy subjects, and showed increased activation of the inferior frontal gyrus during negative appraisal of others' intentions. This region was part of a circuit acting as a modulator in socially induced emotions (Grecucci *et al.*, 2013). The inferior frontal gyrus has been implicated in coping with distracting emotions and amygdala-inferior frontal gyrus connectivity has been described as a system involved in emotion detection (Dolcos *et al.*, 2006), which is clearly relevant to mood and anxiety disorders.

We did not find an association between symptom severity scores and strength of RSFC. A possible explanation could be that the RSFC abnormalities are more trait than state dependent. However, an alternative explanation may be that the included patients mostly presented with mild to moderate symptomatology, as NESDA participants were recruited through general practitioners and outpatient clinics.

To the best of our knowledge, this is the first study to investigate RSFC in medication-free MDD patients, anxiety patients, and patients with comorbid depression and anxiety compared to healthy controls. We were able to include a large sample of patients who were all medication-free or even medication-naïve. We were therefore able to rule out any possible influence of current medication use on resting-state fMRI. However, several limitations are of note. The interpretation of abnormalities in RSFC in a cross-sectional observational design should be done with caution, and any interpretation of the current results refers to functional connectivity between brain areas and does not allude to dysfunction of specific brain regions. Additionally, no associations were found between strength of RSFC and depression and

anxiety scores as measured with the MADRS, IDS, and BAI. This suggests that there is no relation with symptomatology, but we did not use a specific instrument to assess severity of comorbidity. Although the differences did not reach statistical significance, the HC group was slightly older than the patient groups. We controlled for potential effects caused by age by including this variable as a covariate in our analyses. However, we cannot rule out that the absence of effects could be due to age differences between the HC and patient groups.

It is of note that results from previous resting-state research based on monodiagnostic subsamples of the NESDA cohort were not replicated in the present study (Pannekoek *et al.*, 2013b, Pannekoek *et al.*, 2013c, Veer *et al.*, 2010). One explanation could lie in the composition of the samples selected for each previous study. PD without comorbidity (Pannekoek *et al.*, 2013b) and SAD without comorbidity (Pannekoek *et al.*, 2013c) were investigated in smaller samples in former studies, but the current, larger sample of anxiety patients consisted of patients with PD, SAD, and GAD, or any combination of these disorders. Similarly, although there was overlap, the composition of the MDD group in the previous study by our group (Veer *et al.*, 2010) was also different from the larger MDD group in this study. In contrast to the present study, in the previous studies healthy controls were pair-wise matched for age and scanning location. In our current study, the distribution of scanning centre was different between groups, although not statistically significant. Also, the healthy controls were slightly older than the patients in the other groups and we therefore controlled for age in our analyses. However, by controlling for age, we could also have regressed out variance that was related to both group and age,

Taken together, the present RSFC study on patients with depression, anxiety, comorbid depression and anxiety, and healthy controls demonstrated an increased RSFC of a limbic network with the bilateral precuneus and the right precentral gyrus only in patients with comorbid depression and anxiety compared to healthy controls, which therefore may be specific to the presentation of comorbid depression and anxiety. This finding adds to literature suggesting that a distinctive pathophysiology is involved in comorbid depression and anxiety (Craske *et al.*, 2009, Etkin and Schatzberg, 2011). Our findings also further implicate connectivity of the limbic network with the precuneus in the pathophysiology of different affective disorders.

Chapter 5
Aberrant resting-state functional connectivity in limbic and salience networks in treatment-naïve clinically depressed adolescents

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Abstract

Depression is prevalent and typically has its onset in adolescence. Resting-state fMRI could help create a better understanding of the underlying neurobiological mechanisms during this critical period. In this study, examines resting-state functional connectivity (RSFC) is examined using seed regions-of-interest (ROIs) associated with three networks: the limbic network, the default mode network (DMN) and the salience network. 26 treatment-naïve, clinically depressed adolescents of whom 18 had comorbid anxiety, and 26 pair-wise matched healthy controls underwent resting-state fMRI. The three networks were investigated using a seed-based ROI approach with seeds in the bilateral amygdala (limbic network), bilateral dorsal anterior cingulate cortex (dACC; salience network) and bilateral posterior cingulate cortex (default mode network). Compared to healthy controls, clinically depressed adolescents showed increased RSFC of the left amygdala with right parietal cortical areas, and decreased right amygdala RSFC with left frontal cortical areas including the ACC, as well as with right occipito-parietal areas. The bilateral dACC showed decreased RSFC with the right middle frontal gyrus, frontal pole, and inferior frontal gyrus in clinically depressed adolescents. No abnormalities in DMN RSFC were found, and differences in RSFC did not correlate with clinical measures. The aberrant RSFC of the amygdala network and the dACC network may be related to altered emotion processing and regulation in depressed adolescents. Our results provide new insights into RSFC in clinically depressed adolescents and future models on adolescent depression may include abnormalities in the connectivity of salience network.

Introduction

Depression is a common mental health disorder in childhood and adolescence with a prevalence of 4–5% (Thapar *et al.*, 2012). Adolescent depression leads to social and educational impairment, and also constitutes an increased risk of obesity, smoking and substance misuse, and suicide (Thapar *et al.*, 2012). Anxiety frequently co-occurs with depression, especially in youth (Simms *et al.*, 2012) and a double diagnosis accounts for more impairment, more severe internalising symptoms (Beesdo *et al.*, 2009), and a more severe emotional disturbance (Kessler *et al.*, 2012) than either diagnosis alone. Since depression and anxiety typically have their onset in adolescence, this period may be critical in terms of the underlying neurobiological events (Blakemore, 2012).

Recent years have witnessed an increase in the number of neuroimaging studies in adolescent/juvenile depression and anxiety, with the majority of the functional MRI (fMRI) studies using task paradigms (Thomas *et al.*, 2001, Pine, 2007, Hulvershorn *et al.*, 2011). Amygdala as well as ventrolateral prefrontal cortex hyperactivation was frequently found in anxiety disorders (Pine, 2007). Similarly, altered amygdala activation was also linked to adolescent depression (Hulvershorn *et al.*, 2011). While task fMRI studies have provided important insights into altered neural processing within individual brain areas, models of depression emphasize the importance of functional connections between brain regions (Mayberg, 1997).

Resting-state fMRI allows researchers to investigate such functional connections without using externally controlled task paradigms, focusing on spontaneous fluctuations in brain activity (Biswal *et al.*, 1995, Fox and Raichle, 2007). The number of resting-state functional connectivity (RSFC) studies in juvenile depression and anxiety is small (Pine, 2007, Hulvershorn *et al.*, 2011) with one recent study in anxiety disorders (Roy *et al.*, 2013) and seven in depression. These studies typically focus on just one specific network of interest. Furthermore, the variance in medication history among subjects could be a significant confounding factor (Wang, 2012). Aberrant RSFC was reported for the subgenual anterior cingulate cortex (ACC), the limbic-striatal system, the dorsolateral, medial and inferior prefrontal cortex (PFC), and the amygdala, insula and temporal cortices. These studies have suggested aberrancies in salience attribution

and executive control related to subgenual ACC connectivity (Connolly *et al.*, 2013), in emotion processing related to subgenual ACC connectivity with frontotemporal cortical areas (Cullen *et al.*, 2009) and a range of frontal and temporal cortical areas as well as the amygdala (Jin *et al.*, 2011). Additionally, abnormalities have been suggested in cognitive emotional control, related to subgenual ACC connectivity with thalamic and parietal regions (Gaffrey *et al.*, 2010) and fronto-subcortical circuits (Jiao *et al.*, 2011), and in emotion regulation related to DMN connectivity (Gaffrey *et al.*, 2012) and the amygdala (Luking *et al.*, 2011). Despite the regularity with which the involvement of the ACC, amygdala, and medial PFC is reported in depression, inconsistency remains in terms of increased or decreased connectivity and correlations with symptom severity.

The amygdala, which is central in the fear system with an important function in detecting, signalling, and learning from threat or danger (Phelps and LeDoux, 2005), has been described as crucial in emotional reactivity (Pine, 2007). The amygdala has been deemed the critical brain region of this limbic system, for driving these processes (Robinson *et al.*, 2010). In their successful effort to find a well-fitting model of limbic circuitry, Stein *et al.* (2007) found the amygdala to be interconnected with the parahippocampal gyrus, subgenual cingulate, orbitofrontal cortex, posterior cingulate, and supragenual cingulate (Stein *et al.*, 2007). In another study, areas showing connectivity with the amygdala included medial prefrontal regions, insula, thalamus, striatum, and dorsal and posterior regions (Roy *et al.*, 2009). The dorsal ACC (dACC), along with the anterior insular cortices, constitutes an important element of the salience network, which serves to evaluate the relevance of internal and external stimuli in order to generate appropriate responses and guide behaviour (Seeley *et al.*, 2007). This network has not been studied in adolescent depression and anxiety before. This is remarkable, given the relevance of regions of the ACC (such as the pregenual and rostral ACC) to depression and anxiety in adults (Freitas-Ferrari *et al.*, 2010, Etkin *et al.*, 2010) as well as in youth (the subgenual ACC) (McClure *et al.*, 2007, Cullen *et al.*, 2009, Gaffrey *et al.*, 2010, Jiao *et al.*, 2011, Jin *et al.*, 2011, Connolly *et al.*, 2013). RSFC abnormalities of the default mode network (DMN) have been found in various neuropsychiatric disorders, including depression and anxiety, in adults (Broyd *et al.*, 2009, Sylvester *et al.*, 2012). A recent RSFC study in children with preschool onset depression also

found aberrancies in DMN connectivity, reporting decreased connectivity between the posterior cingulate cortex (PCC) and temporal and parietal cortical areas as well as the cerebellum, and increased connectivity between the PCC and subgenual anterior cortical areas (Gaffrey *et al.*, 2012).

Studies investigating the developmental course of resting-state connectivity networks observed a change in connectivity patterns of these networks over time. More diffuse patterns were reported in childhood with greater connectivity between regions that were anatomically close to one another, and cohesive and more integrated networks were shown in adulthood with increased connectivity between areas that were spatially remote (Fair *et al.*, 2007, Fair *et al.*, 2008, Fair *et al.*, 2009, Kelly *et al.*, 2009, Supekar *et al.*, 2009, Jolles *et al.*, 2011). Adolescents showed an intermediate pattern of functional connectivity. Noting that the greatest developmental effects occur in networks associated with social and emotional functions, the importance of understanding maturational neural processes in young psychiatric populations was again highlighted (Kelly *et al.*, 2009).

In the present study we set out to investigate RSFC in a sample of treatment-naïve, clinically depressed adolescents and pair-wise matched controls. We opted for a seed-based correlation approach. As opposed to previous RSFC studies, we aimed to investigate RSFC not just in one, but in several networks putatively involved in depression and anxiety in concert: the limbic network, the DMN, and the salience network. Based on reports of functional connections between the amygdala and prefrontal areas (Roy *et al.*, 2009) and altered activity patterns in adolescent depression and anxiety (Pine, 2007, Hulvershorn *et al.*, 2011, Roy *et al.*, 2013), we hypothesised decreased RSFC for the limbic network, in particular between the amygdala and the prefrontal cortex, especially the ACC. Furthermore, previous task fMRI research has indicated an attentional bias to negative stimuli in depressed individuals (Maalouf *et al.*, 2012), which in turn has been linked to involvement of the ACC (Anand *et al.*, 2005, van Tol *et al.*, 2012). Decreased coupling between the dACC and the orbitofrontal cortex (OFC) has been reported in depressed adults, which was proposed to have an association with a negative processing bias in depression (Frodl *et al.*, 2010). We therefore hypothesised abnormalities of RSFC for the salience network, particularly decreased connectivity with the OFC. Based on previous literature, we also hypothesised abnormalities in DMN RSFC

in our sample of clinically depressed adolescents, especially increased connectivity with the subgenual ACC.
In addition, we expected greater aberrancies in RSFC to be associated with greater severity of depressive and anxiety symptoms.

Method

Participants

Fifty-two adolescents (26 clinically depressed, 26 controls) were selected as part of the EPISCA study (Emotional Pathways' Imaging Study in Clinical Adolescents). EPISCA is a longitudinal MRI study in which adolescents with clinical depression and healthy controls were followed over a six-month period (January 2010 till August 2012) (also see (Aghajani *et al.*, 2013)). The current study reports on cross-sectional data from both groups that were collected prior to treatment.

Inclusion criteria for the depressed group were: having clinical depression as assessed by categorical and dimensional measures of DSM-IV depressive and anxiety disorders (see below), no current and prior use of antidepressants, and being referred for CBT at an outpatient care unit of two child and adolescent psychiatric institutes. The clinically depressed adolescents were included within two weeks after initial screening. None of the participants had started any form of treatment prior to inclusion, and therefore none used medication. Inclusion criteria for the control group were: no current or past DSM-IV classifications, no clinical scores on validated mood and behavioural questionnaires, no history of traumatic experiences, and no current psychotherapeutic and/or psychopharmacological intervention of any kind. Exclusion criteria for all participants were: primary DSM-IV clinical diagnosis of ADHD, pervasive developmental disorders, post-traumatic stress disorder, Tourette's syndrome, obsessive-compulsive disorder, bipolar disorder, and psychotic disorders; current use of psychotropic medication; current substance abuse; history of neurological disorders or severe head injury; age <12 or >21 years; pregnancy; left-handedness; a full-scale intelligence score (FIQ) <80; and general MRI contraindications. Clinically depressed adolescents and controls were pair-wise matched by age, gender and FIQ.
From the original total group of 59 adolescents (29 clinically depressed, 30 controls), four participants (two depressed, two controls) were excluded after preprocessing (see below) from further analysis due to movement

>3 mm, two (one depressed, one control) because of anomalies found on their anatomical scan after inspection of all subjects' structural images by a neuroradiologist, and one control was excluded because he could not be pair-wise matched. Consequently, 52 participants were included in this study: 26 clinically depressed, treatment-naïve adolescents (mean age 15.4 ± 1.5) and 26 healthy controls (mean age 14.7 ± 1.5). Participants were scanned within two weeks of initial screening, and all were new to MRI scanning procedures.
The study was approved by the Medical Ethics Committees of the Leiden University Medical Centre and written informed consent was obtained from the participants and their parents.

Clinical measures

For all participants, several clinical measures were used for dimensional and categorical assessment of DSM-IV disorders. After the clinical assessment by child and adolescent psychiatrists, categorical DSM-IV diagnoses were further assessed with the child and parent versions of the Anxiety Disorders Interview Schedule (ADIS) (Silverman and Albano, 1996). The ADIS is a semi-structured interview to obtain DSM-IV-based classifications of anxiety and related disorders in children and adolescents, which has excellent reliability for combined diagnoses with kappa values spanning from .82 - .90 (Silverman *et al.*, 2001), as well as excellent interrater agreement for principal diagnosis ($\kappa = .92$) and individual anxiety disorders ($\kappa = .80 – 1.0$) (Lyneham *et al.*, 2007). Additionally, the Children's Depression Inventory (CDI) (Kovacs, 1992b), the Revised Child Anxiety and Depression Scale (RCADS) (Chorpita *et al.*, 2000), the Youth Self Report (YSR) (Achenbach, 1991d), and its parent version the Child Behaviour Check List (CBCL) (Achenbach, 1991b) were used to dimensionally assess the severity of symptoms. For a detailed description of these instruments, see Appendix 1. All participants were tested with Dutch versions of the Wechsler Intelligence scales for children (Wechsler, 1991b) or adults (Wechsler, 1997b). For the controls, the same instruments were used. Controls were excluded when they fulfilled criteria for a DSM-IV disorder on the ADIS, or when they had clinical or high subclinical scores on the questionnaires.

Image data acquisition

Image acquisition took place at the Leiden University Medical Centre.

Images were obtained on Philips 3T magnetic resonance imaging systems (Philips Healthcare, Best, The Netherlands), equipped with a SENSE-8 head coil. Prior to scanning, all participants were introduced to the scanning situation by lying in a dummy scanner and hearing scanner sounds. Resting-state functional MRI data were acquired at the beginning of a fixed imaging protocol for each subject, using T_2^*-weighted gradient-echo echo-planar imaging with the following scan parameters: 160 whole-brain volumes; repetition time 2200 ms; echo time 30 ms; flip angle 80°; 38 transverse slices; no slice gap; field of view 220 mm; in-plane voxel size 2.75 × 2.75 mm; slice thickness 2.72 mm; total duration of the resting-state run 6 minutes. In the darkened MR room participants were instructed to lie still with their eyes closed and not to fall asleep. After completion of the scan, subjects confirmed wakefulness during acquisition. A sagittal 3-dimensional gradient-echo T_1-weighted image was acquired for registration purposes and grey matter analysis with the following scan parameters: repetition time 9.8 ms; echo time 4.6 ms; flip angle 80°; 140 sagittal slices; no slice gap; field of view 224 mm; 1 mm isotropic voxels; duration 4:56 minutes.

Preprocessing

FMRI data preprocessing and statistics were carried out using FEAT (FMRI Expert Analysis Tool) Version 5.98, part of FSL (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl) (Smith *et al.*, 2004) and were similar to previous studies by our group (Pannekoek *et al.*, 2013c, van der Werff *et al.*, 2013). The following pre-statistics processing was applied: motion correction using MCFLIRT, resulting in an estimation of the rigid-body motion at each time point relative to the reference volume chosen from the middle time point of the series; non-brain removal; spatial smoothing using a 6 mm full-width at half-maximum Gaussian kernel; grand mean scaling of the entire 4D dataset by a single multiplicative factor; high-pass temporal filtering (Gaussian-weighted least-squares straight line fitting, with a 0.01 Hz cut-off). Registration of the resting-state data to high resolution T_1-weighted, and the T_1 to the 2mm isotropic MNI-152 standard space image (T_1 standard brain averaged over 152 subjects; Montreal Neurological Institute, Montréal, QC, Canada) was carried out. The resulting transformation matrices were then combined to obtain a native to MNI space transformation matrix and its inverse (MNI to native space).

Statistical analysis

Demographic and clinical characteristics were analysed using SPSS 20.0 (SPSS Inc., Chicago, Illinois) using an independent-samples t-test with significance set at $p<0.05$. Head movement in the scanner can have an impact on the time courses of RSFC (Satterthwaite *et al.*, 2012, Power *et al.*, 2012, Satterthwaite *et al.*, 2013), especially in youth and patient samples (Van Dijk *et al.*, 2012). Motion in the scanner was therefore compared between groups by analysing the mean absolute and relative displacement in mm in SPSS, which were obtained for each participant during preprocessing through MCFLIRT (Jenkinson *et al.*, 2002).

Based on previous literature, the following seed regions-of-interest were selected: bilateral amygdala (for the limbic network), bilateral dACC (for the salience network), and bilateral posterior cingulate cortex (PCC) (for the DMN). A mask was created in standard space for the amygdala based on the Harvard-Oxford Subcortical Structural Probability Atlas in FSL (Veer *et al.*, 2011). A study on mapping the functional connectivity of the ACC showed that the connectivity pattern of the salience network can be generated using a seed in the dACC (Margulies *et al.*, 2007). The coordinates for the dACC seeds were obtained from table 1 of this study. The voxel most reliably located in a given region make the greatest contribution to its signal (Roy *et al.*, 2009). In the pioneering study investigating DMN connectivity in major depression, Greicius *et al* (2003) demonstrated that a seed region-of-interest in the PCC generated the DMN (Greicius *et al.*, 2003). We therefore chose the peak voxel of the PCC in the DMN in their study as the seed for our DMN analysis. The coordinates for all seeds are displayed in Table 2. Spheres of 4 mm radius were created around the seed voxels. They were then transformed to native space by applying the inverse transformation matrix obtained from the registration procedure, and spatially averaged time series were extracted for each seed and for each subject. For each participant, and for each network of interest, we performed a multiple regression analysis using the general linear model (GLM) (as implemented in FEAT (Smith *et al.*, 2004)). The time courses that were extracted from the voxels in our seed regions were entered as a regressor in a GLM for each network. Apart from two regressors describing the left and right seeds, nine nuisance regressors were included in the model: signal from the white matter, cerebrospinal fluid signal and the global signal, as well as six motion parameters (three translations and three rotations). The global signal was included to reduce

the influence of artefacts caused by physiological signal sources (i.e. cardiac and respiratory) (Fox and Raichle, 2007).

Table 2. MNI Coordinates of the Seed Regions

Seed region	MNI coordinates		
	x	*y*	*z*
Amygdala	+/- 22	-6	-16
dACC	+/- 6	18	28
PCC/precuneus	+/- 2	-52	26

MNI = Montreal Neurological Institute; dACC = dorsal anterior cingulate cortex; PCC = posterior cingulate cortex

After reslicing the resulting parameter estimate maps and their corresponding within-subject variance maps into 2 mm isotropic MNI space, they were entered into a higher level within and between groups mixed effects analysis (one- and two-sample t-test). Adolescence is a period of continued neural development, making this a highly sensitive time that is characterized by major as well as minor developmental changes in the brain, which have been demonstrated in grey matter, white matter, and functional activation (Blakemore, 2012). Therefore, age was entered as a regressor. For each subject grey matter density maps were derived from the anatomical scans using FSL. As studies have indicated structural abnormalities in childhood anxiety and depression (Pine, 2007, Hulvershorn *et al.*, 2011), we used grey matter density information of each subject as a voxel-dependent covariate in our higher level model. Additionally, a previous study by our group in this sample indicated grey matter differences between the clinically depressed adolescents and healthy controls (Pannekoek *et al.*, 2014b). By including structural information in the functional connectivity analysis, variance explained by potential differences in grey matter density and/or possible misregistrations are taken into account (Oakes *et al.*, 2007). To correct for multiple comparisons, cluster correction was applied in all group analyses with significance set at a corrected $p<.05$ and an initial cluster-forming threshold of $Z>2.3$ (Worsley, 2001).

Table 1. Demographic and Clinical Characteristics of the Sample

Characteristic	Depressed adolescents *N* = 26	Controls *N* = 26
Age (Mean ± SD)	15.4 ± 1.5	14.7 ± 1.5
Sex (*N* male/*N* female)	3/23	3/23
IQ (Mean ± SD)	104.2 ± 8.7	106.6 ± 7.8
CDI [a] (Mean ± SD)	18.6 ± 9.5**	4.6 ± 3.4**
RCADS—Depression[b] (Mean ± SD)	11.2 ± 5.7**	3.9 ± 3.0**
RCADS—Anxiety[b] (Mean ± SD)	32.7 ± 14.6**	14.8 ± 10.8**
YSR—Internalising (Mean ± SD)	24.0 ± 8.7*	8.3 ± 6.3*
YSR—Externalising (Mean ± SD)	12.5 ± 6.7**	6.7 ± 5.8**
CBCL—Internalising (Mean ± SD)	19.3 ± 7.5**	3.9 ± 3.6**
CBCL—Externalising[b] (Mean ± SD)	10.7 ± 9.3*	3.5 ± 4.0*
- Anxiety disorder	23	0

Because less than 20% of the items in CDI, RCADS, YSR and CBCL were missing, expectation maximizatio regression method was used to calculate the scale scores. IQ = Intelligence quotient; CDI = Children's Depres Inventory; RCADS = Revised Child Anxiety and Depression Scale; YSR = Youth Self Report; CBCL = Child Behav Check List.

[a] One patient did not complete the questionnaire

[b] Three clinically depressed adolescents and their parents/primary caregivers did not complete the question

* $p<0.05$; ** $p<0.001$

Results

Demographics and clinical characteristics

The 26 clinically depressed adolescents all had clinical depression scores on the categorical and/or dimensional measurements, with 18 having a comorbid anxiety disorder. All were treatment-naïve for pharmacotherapy and psychotherapy. The groups did not differ on age (U= 438.0; p= .062) and FIQ (t= 1.026; p= .310). Clinically depressed adolescents showed significantly higher scores on CDI, CBCL, and RCADS depression and total anxiety scales (p<.001), and on YSR scores (p< .05) (Table 1). The groups did not differ on mean absolute displacement (p= .392) and mean relative displacement (p=.570) as measures for head motion in the scanner. Information on motion is displayed in Supplementary Table S1.

RSFC analysis

Analysis of amygdala RSFC showed that the connectivity pattern within both groups largely comprised areas described to have functional and anatomical connections with the amygdalae in previous work (Stein *et al*., 2007, Roy *et al*., 2009). Areas showing positive connectivity with the amygdala seeds included the hippocampus, parahippocampal gyrus, middle temporal gyrus, thalamus, putamen, insular cortex, frontal pole and the brain stem. Areas showing negative RSFC included the cuneus, precuneus, PCC, angular gyrus, supramarginal gyrus, and lateral occipital cortex.

With respect to between-group analysis for the limbic network, we found increased positive left amygdala RSFC in the depressed group with the right middle frontal gyrus, inferior frontal gyrus, precentral gyrus (bordering white matter), and the postcentral gyrus relative to controls (Figure 1A; Table S2). We also found decreased negative right amygdala RSFC in depressed adolescents with the left frontal pole, right pregenual ACC, right paracingulate gyrus, and the right superior frontal gyrus, as well as with the left angular gyrus, left lateral occipital cortex, and the left supramarginal gyrus (Figure 1B; Table S2).

No group differences were found for the contrast with both left and right amygdala combined.

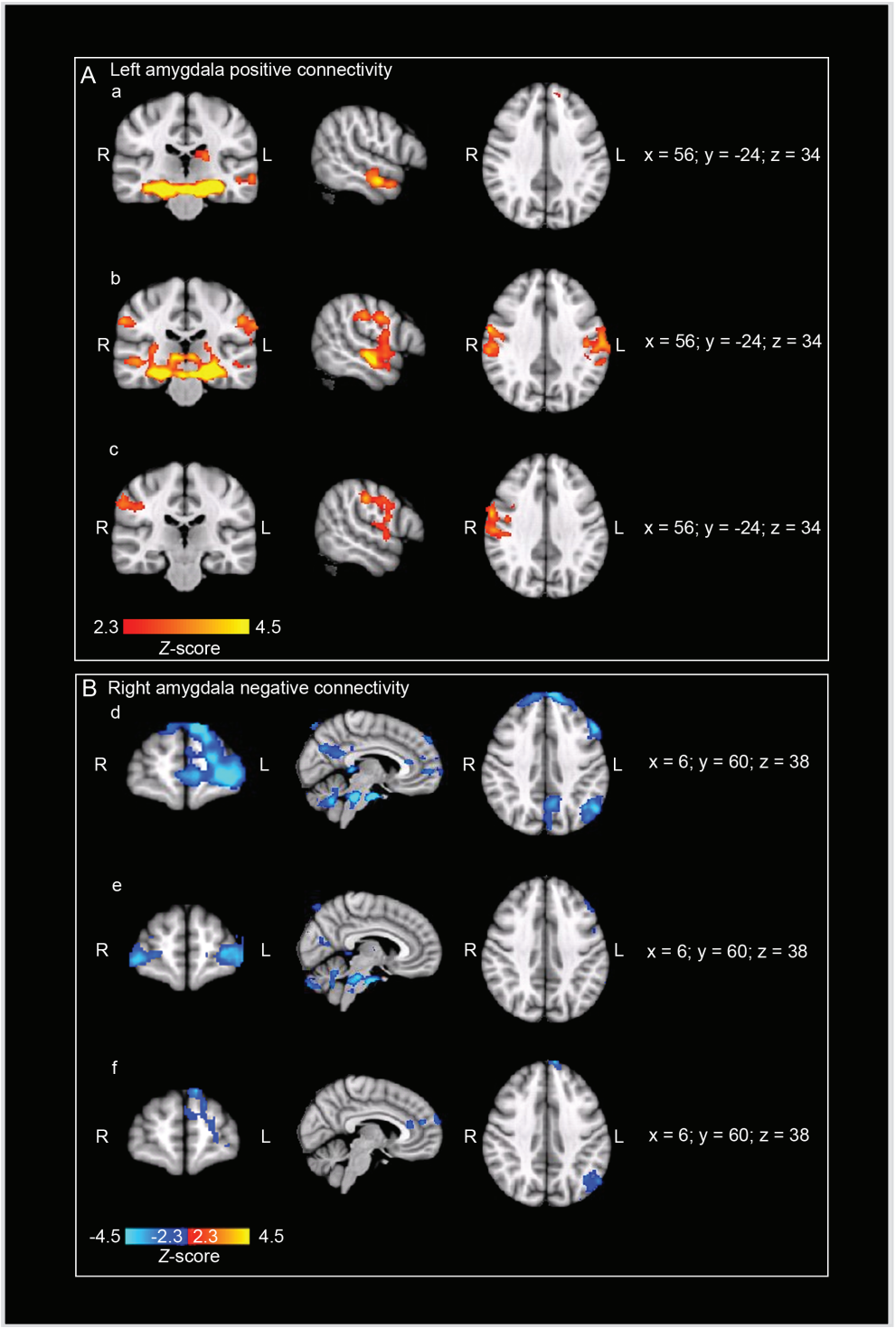

A Left amygdala positive connectivity
a
R
L
R
L
x = 56; y = -24; z = 34
b
R
L
R
L
x = 56; y = -24; z = 34
c
R
L
R
L
x = 56; y = -24; z = 34
2.3
4.5
Z-score
B Right amygdala negative connectivity
d
R
L
R
L
x = 6; y = 60; z = 38
e
R
L
R
L
x = 6; y = 60; z = 38
f
R
L
R
L
x = 6; y = 60; z = 38
-4.5
-2.3
2.3
4.5
Z-score

Figure 1.
Left (A) and right (B) amygdala connectivity. A (a) main effect for the control group in left amygdala positive connectivity; (b) main effect for the clinically depressed group in left amygdala positive connectivity; (c) the difference in left amygdala positive connectivity between the two groups. B (d) main effect for the control group in right amygdala negative connectivity; (e) main effect for the clinically depressed group in right amygdala negative connectivity; (f) the difference in right amygdala negative connectivity between the two groups. Images are z-statistics, overlaid on the MNI-152 standard brain. Cold colors indicate negative connectivity, warm colors indicate positive connectivity. The left hemisphere of the brain corresponds with the right side of the images. MNI coordinates displayed at the right side of the images correspond with the coordinates of the displayed slices.

The dACC seeds probing the salience network showed similar connectivity patterns in both groups, corresponding with previous literature (Margulies *et al.*, 2007). Areas showing positive RSFC included other parts of the ACC, the paracingulate gyrus, frontal pole, insular cortex, temporal pole, precentral gyrus, supramarginal gyrus, middle frontal gyrus, and inferior frontal gyrus. Areas showing negative connectivity at rest included the PCC, precuneus, angular gyrus, lateral occipital cortex, middle frontal gyrus, superior frontal gyrus, inferior temporal gyrus, and the frontal pole. In the between-group analyses, the combined bilateral dACC in a single contrast showed decreased positive connectivity in clinically depressed adolescents with the right middle frontal gyrus, frontal pole, and inferior frontal gyrus (Figure 2; Table S3). Separate investigations of the left and right dACC did not produce significant group differences.

We investigated DMN activity with seeds in the left and right PCC/precuneus. Similar connectivity patterns in both groups were observed with the following areas showing positive RSFC: the PCC/precuneus, cuneus, superior lateral occipital cortex, angular gyrus, supramarginal gyrus, frontal pole, superior frontal gyrus, paracingulate gyrus, ventral ACC, and the subcallosal cortex. Negative RSFC patterns included the dACC, supplementary motor cortex, precentral gyrus, postcentral gyrus, central opercular cortex, insular cortex, inferior lateral occipital cortex, and the occipital pole (Figure S1). No group difference was found in DMN

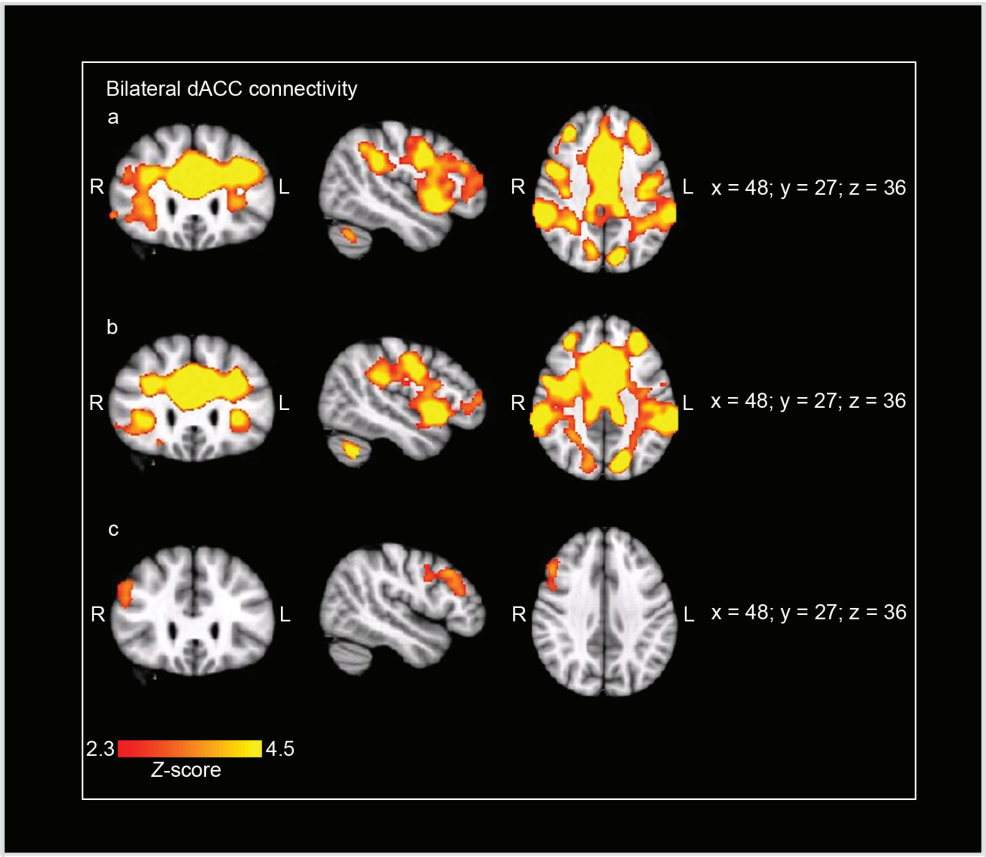


Figure 2.
Bilateral dorsal anterior cingulate cortex connectivity. (A) main effect for the control group in bilateral dorsal anterior cingulate positive connectivity; (B) main effect for the clinically depressed group in bilateral dorsal anterior cingulate positive connectivity; (C) the difference in bilateral dorsal anterior cingulate positive connectivity between the two groups. Images are z-statistics, overlaid on the MNI-152 standard brain. The left hemisphere of the brain corresponds with the right side of the images. Yellow to red are z-values, ranging from 2.3 to 4.5. MNI coordinates displayed at the right side of the images correspond with the coordinates of the displayed slices.

connectivity.

Additional analyses were carried out to determine whether the observed differences in RSFC were unchanged after removing the grey matter covariate. Analyses without correcting for grey matter differences yielded the same results as the analyses including the voxel-dependent covariate.

Symptom severity and RSFC

To test our hypothesis about an association between severity of depressive and anxiety symptomatology and RSFC abnormalities in the depressed group, RSFC data were correlated with depression and total anxiety scores on the RCADS using SPSS 20.0 (SPSS Inc., Chicago, Illinois, USA). Data points three or more standard deviations from the mean were considered outliers. One outlier was excluded from the analyses with total anxiety scores. We generated the mean z-values of the clusters resulting from each of our seed-based resting-state analyses; i.e. the individual connection between the seed and the regions of difference. For example, we created a mask of the areas resulting from our bilateral dACC analysis, and calculated the individual mean Z-scores from these areas using Featquery, part of FSL (Smith *et al.*, 2004). Hierarchical multiple regressions were used, corrected for age, IQ and gender across all subjects. To correct for multiple comparisons in the regression analyses, a Bonferroni-corrected p-value was generated based on the two RCADS scales used (depression and the total of all anxiety scales) as well as the number of regions in this analysis. We followed the same approach for the areas showing aberrant connectivity in our left and right amygdala analyses. We found a positive correlation between connectivity strength and depression scores on the RCADS in the areas that showed decreased RSFC with the bilateral dACC in depressive participants compared to typically developing adolescents (unstandardized b = 3.006, $t(21)$ = 3.253, p = .004). No significant associations were found between connectivity of any of the other regions showing abnormal RSFC and depression and anxiety severity scores in the clinically depressed group (Table S4). Similar analyses within the controls did not yield any associations between RSFC and severity scores.

Discussion

In this study of clinically depressed treatment-naïve adolescents and pair-wise matched controls, RSFC was examined in limbic, salience and default mode networks. We also investigated whether RSFC deviations were associated with depression and anxiety scores in the depressed group. As hypothesised, clinically depressed adolescents showed RSFC differences in the limbic and salience networks, but contrary to expectation not in the DMN. Greater abnormalities in RSFC were not associated with higher clinical ratings of depression and anxiety.

Our findings are partly in line with other RSFC studies investigating adolescent depression, showing abnormalities in connectivity in networks involving the amygdala, ACC, inferior frontal areas, and prefrontal areas (Jiao *et al.*, 2011, Jin *et al.*, 2011, Luking *et al.*, 2011). These brain regions were also implicated in task studies investigating depression and social anxiety in children and adolescents (Guyer *et al.*, 2008, Halari *et al.*, 2009). A novel finding for the limbic network in clinically depressed adolescents is the increased left amygdala RSFC with right cortical areas, including the middle frontal gyrus, inferior frontal gyrus, precentral gyrus, and postcentral gyrus. It is known that these areas are involved in emotion (Grecucci *et al.*, 2013), and we offer a cautious interpretation. Interestingly, building on prior findings that suggested left and right amygdala involvement in different functions (Phelps *et al.*, 2001, Goldin *et al.*, 2008), a study by Dyck *et al.* (2011) found evidence for lateralization of functions of the amygdala using visual and audiovisual mood induction paradigms, supporting a left-lateralized cognitive and intentional control of mood. The findings of their study implicated that responses of the left amygdala are modulated by subjective experience, and may reflect variations in the regulation of mood (Dyck *et al.*, 2011). Relationships between cognitive emotion regulation strategies and depressive symptoms in adolescent samples have been previously reported (Garnefski and Kraaij, 2006). In our study of clinically depressed adolescents, an increased RSFC between the left amygdala and the right cortical areas could reflect or underlie an altered involvement of the left amygdala in conscious and cognitively controlled emotion processing in adolescent depression. However, this interpretation remains speculative, as no measures of emotion regulation were used in the present study.

In agreement with prior studies and our hypothesis, we also found decreased RSFC of the right amygdala with prefrontal areas, notably the pregenual ACC (Etkin *et al.*, 2010, Carballedo *et al.*, 2011). This is in line with previous literature linking connectivity between the amygdala and the pregenual ACC to the regulation of affective processing (Etkin *et al.*, 2011b). Furthermore, previous studies have indicated that the ACC is involved in emotion regulation by having an inhibitory effect on amygdala function, which is consistent with the hypothesis that decreased cortical regulation of limbic activation may be present in depression (Phelps and LeDoux, 2005, Anand *et al.*, 2005). Taken together this suggests that the

decrease of RSFC between the right amygdala and the ACC could reflect or underlie diminished emotion regulation.

In the salience network we found a decreased positive connectivity between the bilateral dACC and right prefrontal regions in clinically depressed adolescents. We did not find an altered coupling between the dACC and the OFC, as was hypothesised. However, altered RSFC of the ACC with frontal cortical regions has been reported in adult depression (Sheline *et al.*, 2010). Several functions attributed to the right dorsolateral prefrontal cortex (dlPFC) and the ACC and their connections may be relevant for the interpretation of our results. For instance, within the scope of the salience network, one of the roles of the dACC is mediating the integration of information across events. An attention bias to negative emotions has been reported in clinical depression (Gotlib *et al.*, 2004), and research findings indicate that this bias is state-dependent, as it was found to be present in depressed adolescents but absent in remission (Maalouf *et al.*, 2012). This suggests that abnormal connectivity between these areas may be related to the increased negative self-attribution and biases in information processing typically observed in MDD patients (Grimm *et al.*, 2009). This hypothesis is further strengthened by the association we found between the depression scores on the RCADS and dACC connectivity. Adolescent depression has previously been associated with a bias towards negative emotional stimuli (Maalouf *et al.*, 2012). With respect to the role of the salience network of identifying personally relevant stimuli among the myriad inputs (Seeley *et al.*, 2007), this bias is plausible in depressed adolescents. Aberrant functioning of the salience network could play a role in a heightened sensitivity to negative stimuli in depressed adolescents. Finally, prior work has shown that dACC connectivity is related to affective processing (Adelstein *et al.*, 2011), and that the PFC also plays a role in affective working memory and the representation of positive and negative affect states (Grimm *et al.*, 2009). Thus, aberrant coupling between the ACC and dlPFC could also represent or underlie a defective evaluation of negative emotion. Taken together, for the salience network, abnormal connectivity between the dACC and prefrontal regions may be specifically related to disturbed assessment of affective stimuli.

In contrast to prior findings in adult depression, (Greicius *et al.*, 2007, Broyd

et al., 2009, Wang, 2012), we did not find differences in DMN connectivity. A study comparing RSFC between healthy children and young adults found that the size of functional connectivity (number of voxels) and RSFC strength differed between the two groups, independent of grey matter differences (Jolles *et al*., 2011). This also specifically applied to the DMN (Fair *et al*., 2008, Supekar *et al*., 2010). These results indicate an ongoing change of functional connectivity during adolescence. It could be argued that the inconsistency between findings of DMN RSFC abnormalities in adult depression and the lack of DMN RSFC abnormalities in the present study may be linked to such developmental differences. However, DMN connectivity abnormalities have also been reported by studies in depressed non-adult samples, for example by Gaffrey *et al*., who reported DMN connectivity in children with a history of preschool depression prior to the age of 6 (Gaffrey *et al*., 2012). Another study of depressed adolescents recently found altered RSFC of the subgenual ACC, a region linked to the DMN, and an associated increased rumination and higher depression levels in depressed subjects (Connolly *et al*., 2013). These discrepancies with our findings might be due to differences in study samples and methodology, like age ranges and inclusion of various comorbid conditions.

We believe our study has several strengths. We studied a substantial sample of clinically depressed, treatment-naïve adolescents, and investigated three networks putatively involved in affective disorders with a seed-based approach, allowing easier replicability (Cole *et al*., 2010). However, several limitations should also be noted. By using seed-based correlation instead of a data-driven approach, possible RSFC abnormalities in other resting-state networks might be overlooked. Also, the interpretation of abnormalities in RSFC in a cross-sectional observational design cannot be conclusive in terms of causality and should be done with caution. It is also of note that we only explored the functional connectivity of specific key regions of each network. Future studies should explore other seed regions or networks. Finally, anxiety co-occurred with depression in most of our clinically depressed adolescents, which could affect the specificity of the results. However, other studies investigating adolescent depression reported a similar comorbidity, and comorbidity with anxiety is considered to be characteristic for clinical depression in adolescence. Exclusion of anxiety comorbidity would therefore have resulted in an atypical sample (Zahn-Waxler *et al*., 2000, Cullen *et al*., 2009).

Conclusion

In summary, as expected, abnormalities were found in RSFC of the limbic and salience networks in clinically depressed adolescents. However, our results did not support the hypothesis that DMN RSFC is deviant. The aberrant RSFC of the amygdala network and dACC network may be related to altered emotion regulation and self-referential processing in depressed adolescents. Taken together, our results could provide new insights into disturbances of functional connectivity in depressed adolescents and suggest that models for affective disorders in adolescents may include abnormalities in the connectivity of the salience network. Clearly, future studies are needed to replicate the current findings and to further explore RSFC in adolescent depression.

Appendix and supplementary information

Appendix S1. Description of clinical measures

For all participants, several clinical measures were used for categorical and dimensional assessment of DSM-IV disorders. After clinical assessment of the patients by child and adolescent psychiatrists, categorical DSM-IV diagnoses were further assessed with the child and parent versions of the Anxiety Disorders Interview Schedule (ADIS) (Silverman and Albano, 1996). Additionally, the Children's Depression Inventory (CDI) (Kovacs, 1992b), the Revised Child Anxiety and Depression Scale (RCADS) (Chorpita *et al.*, 2000), the Youth Self Report (YSR) (Achenbach, 1991d) and its parent version the Child Behaviour Check List (CBCL) (Achenbach, 1991b) These instruments were also used to assess the healthy controls.

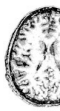
The ADIS is a structured diagnostic interview consistent with the DSM-IV criteria for depressive and anxiety disorders. The child and parents are interviewed separately and based on the outcomes of these interviews a final clinical severity score is given by the interviewer. The ADIS is shown to have good psychometric properties and was used in this study by trained clinicians and researchers.

The CDI is a self-report questionnaire with 27 items that correspond with DSM-IV dimensions of depressive disorders, and is scored on a 3-point Likert scale describing the severity of symptoms (0 = *absence* of symptomatology to 2 = *severe symptomatology*).

The RCADS is a self-report questionnaire with 47 items that

correspond with DSM-IV dimensions of depressive and anxiety disorders. The items are descriptive statements that are scored on a 4-point Likert scale (0 = *never* to 3 = *always*). The questionnaire covers six scales, corresponding to DSM-IV dimensions of anxiety and depressive disorders: Separation Anxiety Disorder (SAD), Generalized Anxiety Disorder (GAD), Social Phobia (SP), Major Depressive Disorder (MDD), Panic Disorder (PD), and Obsessive Compulsive Disorder (OCD). In the present study, only two scale scores were used: the total anxiety score (sum of the five scale scores about the anxiety disorders SAD, GAD, SP, PD and OCD) and a depression score (the MDD scale score). Because only three items were missing, expectation maximization as regression method was used to calculate the scale scores. The internal consistencies of these scale scores in both groups available (three were missing in the depressed group) are high (.93/.93 for the total anxiety score and .74/.86 for the depression score in respectively the patient and control groups).

The YSR covers 113 items concerning behavioural and emotional problems in the past 6 months, as reported by the adolescent. The internalising scale of the YSR contains 31 items in the form of descriptive statements that are scored on a 3-point Likert scale (0 = *not true* to 2 = *very or often true).*
The CBCL covers 118 items concerning behavioural and emotional problems in the past 6 months, as reported by parents or primary caregivers. The internalising scale of the CBCL contains 33 items in the form of descriptive statements that are scored on a 3-point Likert scale (0 = *not true* to 2 = *very or often true*).
All participants were tested with Dutch versions of the Wechsler Intelligence scales for children (Wechsler, 1991b) or adults (Wechsler, 1997b).



Supplementary tables S1 – S4

Supplementary Table 1. Group comparison for motion parameters

Statistic	Depressed adolescents	Controls
N	26	26
Mean (SD) AD	0.2100 (0.09011)	0.0657 (0.01550)
Minimum AD	0.07	0.06
Maximum AD	0.48	0.44
Mean (SD) RD	0.0735 (0.02560)	0.0657 (0.01550)
Minimum RD	0.04	0.05
Maximum RD	0.13	0.11
	Absolute displacement	Relative displacement
Mann-Whitney U	441.000	307.500
p	.059	.570

AD = absolute displacement in mm; RD = relative displacement in mm

Supplementary Table 2. Amygdala Resting-State Connectivity, group differences

Left amygdala RSFC[1]: DEP[2] > HC[3]

Cluster 1, 1785 voxels	Side	*Z*-value	*p*-value	MNI[4] Coordinates		
Region				*x*	*y*	*z*
Middle Frontal Gyrus	R	4.0816	0.0001	36	16	28
Precentral Gyrus	R	4.04997	0.0001	60	-4	34
Postcentral Gyrus	R	3.94122	0.0001	56	-20	38
Precentral Gyrus	R	3.86925	0.0001	38	0	30
Supramarginal Gyrus, BA[5] 3	R	3.71335	0.0002	62	-20	40
White Matter	R	3.55519	0.0004	42	-8	24

Right amygdala RSFC: HC[3] > DEP[2]

Cluster 2, 977 voxels	Side	*Z*-value	*p*-value	MNI[4] Coordinates		
Region				*x*	*y*	*z*
Frontal Pole	L	4.10506	0.0001	-8	60	38
Frontal Pole	L	4.09019	0.0001	-14	64	26
Anterior Cingulate Gyrus, BA[5] 32	R	3.81057	0.0001	14	40	12
Frontal Pole	L	3.53309	0.0004	-8	68	24
Paracingulate Gyrus, BA[5] 9	R	3.51748	0.0004	6	48	18
Frontal Pole, BA[5] 10	L	3.51675	0.0004	-20	58	14
Cluster 1, 639 voxels	Side	*Z*-value	*p*-value	MNI[4] Coordinates		
Angular Gyrus, BA[5] 39	L	3.65803	0.0003	-40	-58	32
Lateral Occipital Cortex, BA[5] 39	L	3.51779	0.0004	-52	-64	38
Lateral Occipital Cortex	L	3.35477	0.0008	-40	-64	38
Angular Gyrus	L	3.26345	0.0011	-48	-54	32
Lateral Occipital Cortex	L	3.14998	0.0016	-42	-72	34
Angular Gyrus	L	2.92358	0.0035	-56	-60	32

[1] RSFC = resting-state functional connectivity; [2] DEP = clinically depressed adolescents; [3] HC = healthy controls; [4] MNI = Montreal Neurologic Institute; [5] BA = Brodmann Area. Group differences are significant in all regions after correction for multiple testing over 3 network analyses (0.05/3 = p<0.017).

Supplementary Table 3. Bilateral dACC Resting-State Connectivity: HC[1] > DEP[2], group difference

Cluster 1, 609 voxels	Side	*Z*-value	*p*-value	MNI[3] Coordinates		
Region				*x*	*y*	*z*
Middle Frontal Gyrus	R	3.75842	0.0002	48	24	38
Middle Frontal Gyrus	R	3.67548	0.0002	50	32	22
Middle Frontal Gyrus, BA[4] 9	R	3.55386	0.0004	48	28	28
Frontal Pole	R	3.41668	0.0006	56	40	12
Precentral Gyrus	R	3.0845	0.0020	48	6	34
Middle Frontal Gyrus	R	2.70543	0.0068	54	12	42

[1] HC = healthy controls; [2]DEP = clinically depressed adolescents; [3] MNI = Montreal Neurologic Institute;
[4] BA = Brodmann Area.
Group differences are significant in all regions after correction for multiple testing over 3 network analyses (0.05/3 = p<0.017).

Supplementary Table 4. Associations between Clinical Scores and RSFC

	Depression scores			Anxiety scores		
Brain region	Unstandardized *b*	*t*	*p*	Unstandardized *b*	*t*	*p*
l amyg – r MFG	-1.211	-.585	.566	-.2033	-.327	.747
r amyg – r ACC	-1.481	-.805	.431	-3.855	-.699	.493
r amyg – l LOC	-1.351	-.968	.346	-.667	-.156	.878
bil dACC – r MFG	3.006	3.252	.004*	8.157	2.819	.011

RSFC = resting-state functional connectivity; l = left; r = right; amyg = amygdala; MFG = middle frontal gyrus; ACC = anterior cingulate cortex; LOC = lateral occipital cortex; bil = bilateral; dACC = dorsal ACC.
* = significant at Bonferroni corrected p < .006

Supplementary figure S1

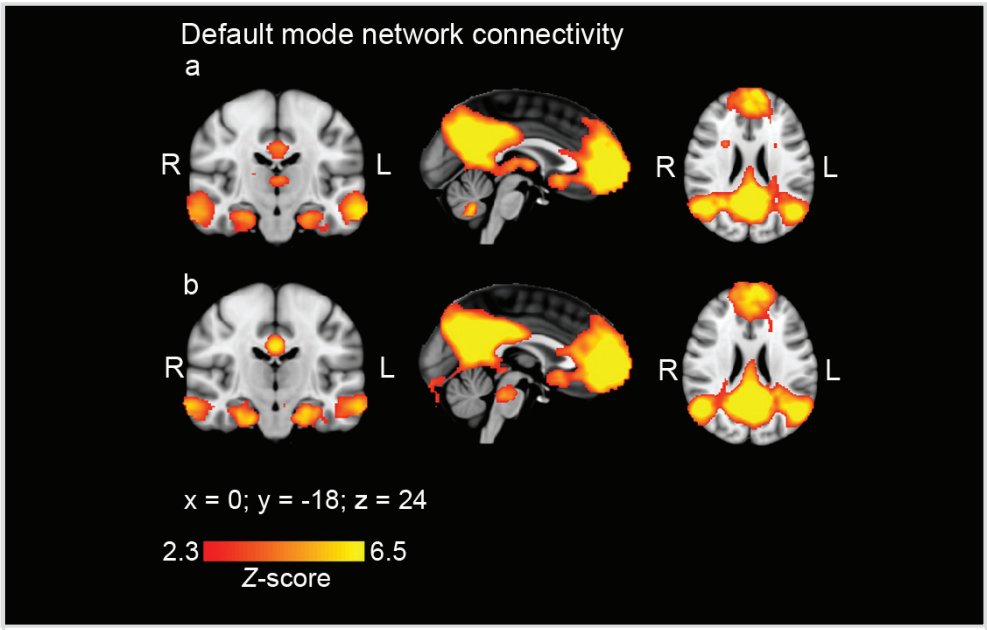


Figure S1. Default mode network connectivity
(a) main effect for the control group in default mode network connectivity; (b) main effect for the clinically depressed group in default mode network connectivity. Images are z¬-statistics, overlaid on the MNI-152 standard brain. The left hemisphere of the brain corresponds with the right side of the images. Yellow to red are z-values, ranging from 2.3 to 6.5. MNI coordinates displayed at the bottom of the figure correspond with the coordinates of the displayed slices.

Chapter 6
Reduced anterior cingulate grey matter volume in treatment-naïve clinically depressed adolescents

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Abstract

Adolescent depression is associated with increased risk for suicidality, social and educational impairment, for smoking, substance use, obesity, and depression in adulthood. It is of relevance to further our insight in the neurobiological mechanisms underlying this disorder in the developing brain, as this may be essential to optimize treatment and prevention of adolescent depression and its negative clinical trajectories. The equivocal findings of the limited number of studies on neural abnormalities in depressed youth, stress the need for further neurobiological investigation of adolescent depression. We therefore performed a voxel-based morphometry study of the hippocampus, amygdala, superior temporal gyrus, and anterior cingulate cortex (ACC) in 26 treatment-naïve, clinically depressed adolescents, and 26 pair-wise matched healthy controls. Additionally, an exploratory whole-brain analysis was performed. Clinically depressed adolescents showed a volume reduction of the bilateral dorsal ACC compared to healthy controls. However, no association was found between grey matter volume of the ACC and clinical severity scores for depression or anxiety. Our finding of a smaller ACC in clinically depressed adolescents is consistent with literature on depressed adults. Future research is needed to investigate if grey matter abnormalities precede or follow clinical depression in adolescents.

Introduction

Many psychiatric disorders have their onset during adolescence, including affective disorders such as depression (Kessler *et al.*, 2005a, Paus *et al.*, 2008, Hulvershorn *et al.*, 2011). Adolescent depression is a major risk factor for increased suicidality, social and educational impairments, and an increased risk for smoking, substance use, and obesity (Thapar *et al.*, 2012). Moreover, adolescent depression is associated with an increased risk for recurrence in adulthood (Clark *et al.*, 2012). Although effective treatments for this condition have been developed, a substantial number of children suffer from persistent depression or will have recurrences in adulthood (Curry *et al.*, 2011). Therefore, it is of relevance to further our insight in the neurobiological mechanisms underlying this disorder in the developing brain, as this may be essential to optimize treatment and prevention of adolescent depression and its negative clinical trajectories. For this reason, adolescent depression has recently become a focus in neuroimaging studies (Paus *et al.*, 2008, Hulvershorn *et al.*, 2011, Thapar *et al.*, 2012). Neuroimaging has already provided valuable insights into the anatomy and physiology of the developing brain of healthy youth as well as of those with neuropsychiatric illnesses (Paus *et al.*, 2008, Giedd and Rapoport, 2010, Crone and Ridderinkhof, 2011, Hulvershorn *et al.*, 2011).

Currently, only a limited number of studies on brain structure in paediatric affective disorders are available, focusing on bipolar disorder (Blumberg *et al.*, 2003, DelBello *et al.*, 2004, Chen *et al.*, 2004), post-traumatic stress disorder (PTSD) (De Bellis *et al.*, 2002a), anxiety disorders (De Bellis *et al.*, 2000, De Bellis *et al.*, 2002b, Milham *et al.*, 2005), and depression (Shad *et al.*, 2012, Ducharme *et al.*, 2013). In these studies, differences were found in amygdala, hippocampus, superior temporal gyrus (STG), and prefrontal cortex (PFC) volumes; all areas known to be part of the emotion generating and regulating neurocircuitry (Shin and Liberzon, 2010, Price and Drevets, 2012). More specifically, studies in adolescent depression reported altered grey matter of the PFC (Nolan *et al.*, 2002, Botteron *et al.*, 2002, Shad *et al.*, 2012), amygdala (MacMillan *et al.*, 2003, Rosso *et al.*, 2005, Caetano *et al.*, 2007), and hippocampus (MacMillan *et al.*, 2003, MacMaster and Kusumakar, 2004, Caetano *et al.*, 2007, MacMaster *et al.*, 2008). White matter differences were also reported, with altered structural connectivity between the right amygdala and the right subgenual anterior cingulate cortex (ACC) (Cullen *et al.*, 2010) and

in the lower frontal lobe white matter volume (Steingard *et al*., 2002).

One recent longitudinal study investigating cortical thickness in healthy adolescents showed an association between developmental rate of the ventromedial PFC and anxiety/depressive scores, with differences in cortical thinning rate between adolescents with low scores and those with high scores. This suggests that abnormalities in the development of the prefrontal cortex may be related to the vulnerability for affective pathology in adolescents (Ducharme *et al*., 2013). These studies highlight the relevance of investigating adolescent developmental changes and the potential impact they may have on mood disorders.

While one study did not report differences in hippocampal volume between adolescents and healthy control subjects (Rosso *et al*., 2005), most findings suggested a smaller hippocampal volume in depressed adolescents (MacMillan *et al*., 2003, MacMaster and Kusumakar, 2004, Caetano *et al*., 2007, MacMaster *et al*., 2008) and even in adolescents at risk for depression (Rao *et al*., 2010). Findings regarding frontal cortical areas are less univocal. A meta-analysis of adult patients consistently found volume reductions of prefrontal and frontal regions, in particular the ACC (Koolschijn *et al*., 2009). However, whereas larger left PFC volumes in depressed children were previously reported (Nolan *et al*., 2002), a recent study found reduced bilateral PFC volumes in depressed adolescents (Shad *et al*., 2012). Another study investigated the orbitofrontal cortex volumes using voxel-based morphometry (VBM) and did not find any differences between depressed and healthy children (Chen *et al*., 2008).

Reports on amygdala volume abnormalities in depressed and anxious youth are also inconsistent, with reports of volume decreases in depression (Rosso *et al*., 2005), bipolar disorder (Blumberg *et al*., 2003, DelBello *et al*., 2004) and anxiety (Milham *et al*., 2005), as well as volume increases in anxiety (De Bellis *et al*., 2000). Additionally, larger bilateral amydala:hippocampal volume ratios were found in depressed paediatric patients (MacMillan *et al*., 2003). However, another study failed to find any differences in amygdala volume between depressed young female adults, their non-depressed high-risk twins, and healthy controls (Munn *et al*., 2007).

Similar inconsistencies are found with respect to the STG. Whereas two studies reported a decreased STG volume in children and adolescents with bipolar disorder (Chen *et al*., 2004) and in depressed adolescents

(Shad *et al.*, 2012), another study found an increased STG volume in anxious children (De Bellis *et al.*, 2002b).

Co-occurrence with other psychiatric symptomatology, especially anxiety, is common in adolescent depression (Zahn-Waxler *et al.*, 2000, Costello *et al.*, 2003, Ghandour *et al.*, 2010, Simms *et al.*, 2012). Most neuroimaging studies in depressed youth have included depressive subjects with comorbid anxiety symptoms, but frequently also with attention deficit hyperactivity disorder (ADHD), oppositional defiant disorder (ODD) and conduct disorder (CD) (Nolan *et al.*, 2002, MacMaster and Kusumakar, 2004, Rosso *et al.*, 2005, Caetano *et al.*, 2007, Gabbay *et al.*, 2007, MacMaster *et al.*, 2008, Shad *et al.*, 2012). Co-occurrence of adolescent depression with anxiety has been associated with more severe internalising symptomatology and impaired functioning (Guberman and Manassis, 2011, Simms *et al.*, 2012). It is therefore of importance to take co-occurrence of anxiety symptoms into account.

The limited number of studies on neural abnormalities in depressed youth and the equivocal reports on the involvement of certain brain areas stress the need for further investigation in depressed adolescents. We therefore performed a VBM study in treatment-naïve adolescents with clinical depression and pair-wise matched healthy controls, assessing symptoms of depression and co-occurrent anxiety both categorically and dimensionally. Using VBM, we opted to examine grey matter volume in brain areas putatively involved in affective psychopathology, as well as to perform an explorative whole-brain analysis. Based on the available literature, we hypothesised that depressed adolescents would show reduced grey matter volume in the hippocampus and ACC. We also hypothesised altered grey matter volumes in the STG and the amygdala, although we had no a priori hypothesis about the directionality of the findings. Finally, given the frequent co-occurrence of anxiety symptoms, we planned to examine correlates of clinical severity scores for both depression and anxiety with structural abnormalities.

Material and methods

Participants

Fifty-two adolescents (26 patients, 26 controls) were selected as part of the EPISCA study (Emotional Pathways' Imaging Study in Clinical Adolescents). EPISCA is a longitudinal MRI study in which adolescents with clinical depression and matched healthy controls were followed over a six-month period (January 2010 till August 2012). The clinically depressed group underwent an MRI scanning protocol before the start of regular cognitive behavioural therapy (CBT), and three and six months after the start of CBT. The healthy controls were examined over similar periods. The current study reports on cross-sectional baseline data from both groups.

Inclusion criteria for the patient group were: having clinical depression as assessed by categorical and dimensional measures of DSM-IV depressive and anxiety disorders (see below under clinical measures), no current and prior use of antidepressants, and being referred for CBT at an outpatient care unit. Inclusion criteria for the control group were: no current or past DSM-IV classifications, no clinical scores on validated mood and behavioural questionnaires, no history of traumatic experiences, and no current psychotherapeutic and/or psychopharmacological intervention of any kind. Exclusion criteria for all participants were: primary DSM-IV clinical diagnosis of ADHD, ODD, CD, pervasive developmental disorders, post-traumatic stress disorder, Tourette's syndrome, obsessive-compulsive disorder, bipolar disorder, and psychotic disorders; current use of psychotropic medication; current substance abuse; history of neurological disorders or severe head injury; age <12 or >21 years; pregnancy; left-handedness; IQ score <80 as measured by the Wechsler Intelligence Scale for Children (WISC) (Wechsler, 1991a) or Adults (Wechsler, 1997a); and general MRI contraindications. Patients and controls were pair-wise matched by age, gender and IQ.

From the original total group of 57 adolescents (29 patients, 28 controls), two participants (two controls) were excluded due to poor data quality, two participants (one patient, one control) because of macroscopic anomalies found on their anatomical scan after inspection the structural images by a neuroradiologist, and one control was excluded due to pair-wise matching issues. Consequently, 52 participants were included in the final analysis: 26 clinically depressed treatment-naïve patients (mean

age 15.4 ± 1.5 years) and 26 healthy controls (mean age 14.7 ± 1.5 years). Participants were scanned within two weeks of initial screening, and all were new to MRI scanning procedures.
The study was approved by the Medical Ethics Committees of the Leiden University Medical Centre and written informed consent was obtained from the participants and their parents.

Clinical measures

For all participants, several clinical measures were used for dimensional and categorical assessment of DSM-IV disorders. For the clinically depressed adolescents, after receiving a diagnosis following the clinical assessment by child and adolescent psychiatrists, categorical DSM-IV diagnoses were further assessed with the Anxiety Disorders Interview Schedule (ADIS) for children and parents (Silverman, 1996). In addition, they had to have (sub)clinical scores on questionnaires assessing the severity of depressive, anxiety or internalising symptoms. We used standardized cut-off scores as provided by the manuals of the different questionnaires. The following questionnaires were used: the Children's Depression Inventory (CDI) (Kovacs, 1992a), the Revised Child Anxiety and Depression Scale (RCADS) (Chorpita *et al.*, 2000) the Youth Self Report (YSR) (Achenbach, 1991c) and its parent version the Child Behaviour Check List (CBCL) (Achenbach, 1991a). For the controls, the same instruments were applied. Controls were excluded when they fulfilled the criteria for a DSM-IV diagnosis or had (sub)clinical scores on clinical questionnaires.

The ADIS is a semi-structured diagnostic interview with child and parents separately to obtain DSM-IV-based diagnoses of anxiety and depressive disorders in children and adolescents of 7 to 18 years old. The CDI is a self-report questionnaire with 27 items that correspond with DSM-IV dimensions of depressive disorders, and is scored on a 3-point Likert scale describing the severity of symptoms (0 = *absence of symptomatology* to 2 = *severe symptomatology*). The RCADS is a self-report questionnaire with 47 items that correspond with DSM-IV dimensions of depressive and anxiety disorders. The items are descriptive statements that are scored on a 4-point Likert scale (0 = *never* to 3 = *always*). The questionnaire covers six scales, corresponding to DSM-IV dimensions of anxiety and depressive disorders: Separation Anxiety Disorder (SAD), Generalized Anxiety

Disorder (GAD), Social Phobia (SP), Major Depressive Disorder (MDD), Panic Disorder (PD), and Obsessive Compulsive Disorder (OCD). In the present study, only two scale scores were used: the total anxiety score (sum of the five scale scores about the anxiety disorders SAD, GAD, SP, PD and OCD) and a depression score (the MDD scale score). The YSR covers 113 items concerning behavioural and emotional problems in the past 6 months, as reported by the adolescent. The internalising and externalising scales of the YSR contain 31 and 32 items respectively, in the form of descriptive statements that are scored on a 3-point Likert scale (0 = not true to 2 = very or often true). The CBCL covers 118 items concerning behavioural and emotional problems in the past 6 months, as reported by parents or primary caregivers. The internalising and externalising scales of the CBCL contain 33 en 35 items respectively, in the form of descriptive statements that are scored on a 3-point Likert scale (0 = *not true* to 2 = *very or often true*).

All participants were tested with Dutch versions of the Wechsler Intelligence scales for children (Wechsler, 1991a) or adults (Wechsler, 1997a).

Image data acquisition

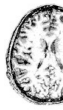
Images were acquired on a Philips 3T magnetic resonance imaging system (Philips Healthcare, Best, The Netherlands), equipped with a SENSE-8 head coil. Scanning took place at the Leiden University Medical Centre. Prior to scanning, all participants were introduced to the scanning situation by lying in a dummy scanner and hearing scanner sounds. For each subject, a sagittal 3-dimensional gradient-echo T1-weighted image was acquired (repetition time 9.8 ms; echo time 4.6 ms; flip angle 8°; 140 sagittal slices; no slice gap; field of view 256 × 256 mm; 1.17 × 1.17 × 1.2 mm voxels; duration 4:56 minutes) as part of a larger, fixed imaging protocol.

Statistical analysis

Demographic and clinical characteristics were analysed using SPSS 20.0 (SPSS Inc, Chicago, Illinois) using an independent-samples t-test with significance set at $p<0.05$. If data did not meet the assumptions required to perform parametric analysis and transformation did not solve this, the non-parametric Mann-Whitney U-test was performed.

For the MRI data, the structural data was analysed with FSL-VBM

(Douaud *et al.*, 2007) (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSLVBM), an optimized VBM protocol (Good *et al.*, 2001) carried out with FSL tools (Smith *et al.*, 2004). First, structural images were brain-extracted and grey matter-segmented before being registered to the MNI 152 standard space using non-linear registration (Andersson, 2007). The resulting grey matter partial volume images were averaged and flipped along the x-axis to create a left-right symmetric, study-specific grey matter template. Second, all native grey matter images were non-linearly registered to this study-specific template. The Jacobian of the warp field obtained in this registration reflects the voxel-wise relative volume change between the original and the study specific template (i.e., a Jacobian of 5 indicates that a volume in the original image has been shrunk by a factor of 5). In order to correct for local expansion or contraction, the registered partial volume images were then modulated by multiplying them with the Jacobian of the warp field. The modulated segmented images were then smoothed with an isotropic Gaussian kernel with a sigma of 3 mm. The Gaussian outputs a weighted average of each voxel's neighbourhood, with the average weighted more towards the value of the centrally located voxels. The application of this type of smoothing reduces the noise in the data substantially. The Harvard-Oxford Cortical and Subcortical Structural Atlases implemented in FSL were used to create masks for our regions of interest (ROIs): the hippocampus, the amygdala, ACC and STG. Probability range was set to 75-100% for all four structures. FSL was then used to create one mask encompassing the four structures, which was applied to the grey matter image from the study-specific template. Finally, groups were compared using a general linear model (GLM) including age as confound regressor. A voxel-wise GLM was applied using permutation-based (5000 permutations) non-parametric testing, correcting for multiple comparisons across space. First, volumes were compared in our regions of interest, using the created mask. Second, an exploratory whole-brain analysis was done; using the grey matter image from the study-specific template to investigate whether any not predicted differences existed between depressed and healthy adolescents. Third, a separate analysis was conducted to investigate the effect of age on grey matter. Threshold-Free Cluster Enhancement was used as a method for finding clusters in the data (Smith and Nichols, 2009) with thresholds for the ROI comparison as well as the whole-brain analysis set on $p<0.05$, corrected.



Additional analyses in SPSS were conducted in the patient group to examine voxel-wise correlations of clinical characteristics with grey matter volume in the structural effects found in the VBM analyses. We performed an analysis of variance to test whether there was a significant group × age interaction on grey matter volume.

Results

Sample characteristics

All participants were treatment-naïve for pharmacotherapy and psychotherapy. The patient group comprised 26 treatment-naïve adolescents with clinical depression, as diagnosed by a clinician and assessed by categorical and/or dimensional measures of depression and internalising symptomatology. Based on our own further assessment using the ADIS, 18 patients fulfilled criteria for a comorbid anxiety disorder and five also fulfilled the ADIS criteria for ADHD (two), CD (two) or ODD (one). It is of note that these five patients only had a clinical diagnosis of depressive and anxiety disorders as established by their clinician, but met the ADIS criteria for depression, anxiety, and an additional externalising disorder. As shown in Table 1, the patient and control groups both consisted of 23 females and 3 males. The two groups were comparable on age and full-scale IQ score. Patients scored significantly higher on the self-report questionnaires CDI, RCADS, and YSR, and on the parent-report questionnaire CBCL ($p<.001$).

VBM results

The VBM ROI analyses showed differences in grey matter volumes between depressed youth and healthy controls in an area within the ACC. The effect was present in Brodmann area 24/32 in the right hemisphere, extending to a lesser extent into the left hemisphere (Figure 1). On average, depressed adolescents showed a 14.4% volume reduction of grey matter in this area compared to the healthy controls. We found no group differences for the volumes in the ROIs for the amygdala, hippocampus and STG.

The exploratory whole-brain analysis did not reveal any grey matter volume differences between patients and controls. However, when the threshold was lowered to $p<.30$, an effect in the same part of the ACC was observed.

There was no significant group x age interaction effect on grey matter volume ($F = 1.049$, $p = .394$).

Table 1. Demographic and Clinical Characteristics of the Sample

Characteristic	Patients *N* = 26	Controls *N* = 26
Age (Mean ± SD)	15.4 ± 1.5	14.7 ± 1.5
Sex (*N* male/*N* female)	3/23	3/23
IQ (Mean ± SD)	104.2 ± 8.7	106.6 ± 7.8
CDI[a] (Mean ± SD)	18.6 ± 9.5**	4.6 ± 3.4**
RCADS—Depression[b] (Mean ± SD)	11.2 ± 5.7**	3.9 ± 3.0**
RCADS—Anxiety[b] (Mean ± SD)	32.7 ± 14.6**	14.8 ± 10.8**
YSR—Internalizing[b] (Mean ± SD)	24.2 ± 8.7**	8.3 ± 6.3**
YSR—Externalizing[b] (Mean ± SD)	12.5 ± 1.4*	6.6 ± 1.1*
CBCL—Internalizing[b] (Mean ± SD)	19.4 ± 7.3**	3.9 ± 3.6**
CBCL—Externalizing[b] (Mean ± SD)	10.7 ± 1.9*	3.5 ± 0.8*

Note: Because less than 20% of the items in CDI, RCADS, YSR and CBCL were missing, expectation maximization as regression method was used to calculate the scale scores.

IQ = Intelligence quotient

CDI = Children's Depression Inventory

RCADS = Revised Child Anxiety and Depression Scale

YSR = Youth Self Report

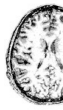
CBCL = Child Behaviour Check List

[a] One patient did not complete the questionnaire

[b] Three patients and their parents/primary caregivers did not complete the questionnaire

** Significant at $p<0.001$

* Significant at $p<0.005$



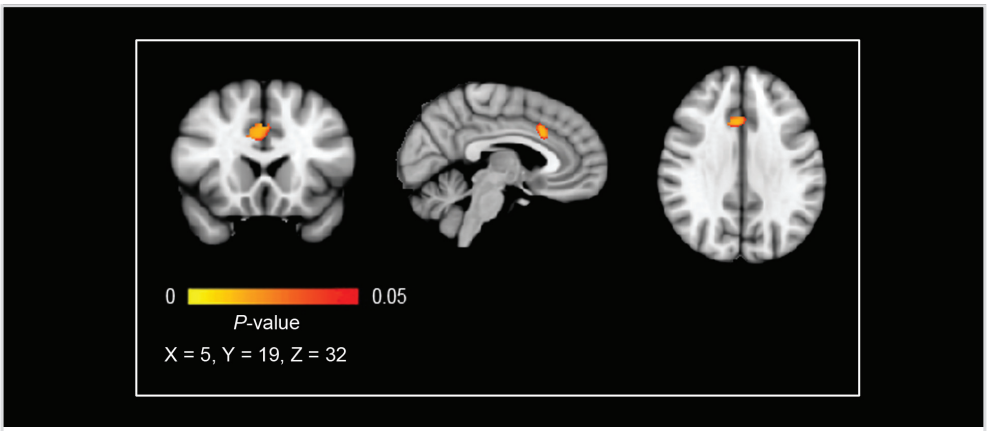


Figure 1.
Reduced ACC grey matter in depressed adolescents compared with healthy controls. Results are displayed at $p < .05$, corrected, 153 voxels, and 2 mm isotropic. The effect is presented on the MNI-152 1mm standard brain. The left hemisphere corresponds with the right side of the image.

Correlates with clinical severity scores

In order to examine whether the significant difference in grey matter volume was associated with symptom severity in the patient group, we performed a linear regression analysis, corrected for age across all subjects. To correct for multiple testing, significance was set to $p<.006$ after applying a Bonferroni correction. No correlations were found in the patient group between the volume reduction in the ACC, and severity of depression and anxiety scores as measured with the RCADS (r=-.248; p=.127 and r=-.275; p=.102 respectively), CDI (r=-.295; p=.086), YSR internalising (r=-.247; p=.128) and externalising (r=-.115; p=.301) scales, and CBCL internalising (r=-.078; p=.362) and externalising (r=-.122; p=.290) scales.

Discussion

We investigated differences in grey matter volumes in clinically depressed, treatment-naïve adolescents with and without comorbid anxiety. We hypothesised volume reductions in the ACC and hippocampus, and altered volumes of the STG and amygdala, using a region of interest analysis. We also performed an exploratory whole-brain VBM analysis. The grey matter of a region within the ACC was 14.4% smaller in the patient group compared

to healthy controls; no differences were found in the other a priori defined regions of interest, or in the whole-brain analysis.

To our knowledge, our study is the first to report a specific volume reduction of the ACC in a cohort of treatment-naïve, clinically depressed adolescents. A recent meta-analysis of grey matter volume in adult depression revealed that the most striking differences between depressed and healthy subjects were volume reductions of the ACC and of the hippocampus (Koolschijn *et al.*, 2009), which is partly in line with our findings in adolescents. The reduction in ACC grey matter volume in our study was found in the dorsal part. Results from functional MRI studies suggest that subregions of the ACC are implicated in different functions. For example, the dorsal ACC has been linked to higher cognitive processes (Bush *et al.*, 2000), focusing attention to relevant events, monitoring for response conflict, and cognitive control (Weissman *et al.*, 2005). Problems in controlling and inhibiting the processing of negative material in depression relates to dysfunction in higher-order cognitive control regions, including the ACC, dorsolateral PFC, and ventrolateral PFC (Foland-Ross and Gotlib, 2012). Abnormalities in grey matter densities of the dorsal ACC could be related to such problems. However, we did not assess these specific higher order cognitive processes in the current study. Therefore, conclusions cannot be made based on these results, and any reference toward an association between the ACC grey matter volume reduction and higher order cognitive processing remains speculative.

Whereas an increase in white matter density is seen during adolescence, a decrease in grey matter is observed (Paus, 2005, Lenroot and Giedd, 2006, Giedd and Rapoport, 2010, Blakemore, 2012). It has been proposed that the emergence of psychopathologies could be related to anomalies or exaggerations of typical maturation processes in interaction with psychosocial and biological environmental factors (Paus *et al.*, 2008). In the light of these suppositions, a reduction of grey matter volume in the ACC in clinically depressed adolescents could be interpreted as a result of abnormal neural maturation processes. The fact that the PFC shows the slowest reduction rate of all areas during maturation (Petanjek *et al.*, 2011) could potentially explain the discrepancy in findings between our study and other studies with a similar age range, and the finding by Nolan *et al.* (2002). The authors reported larger prefrontal areas in a group of depressed adolescents with a younger age (Nolan *et al.*, 2002), with both the larger volume at younger age and the reduction at later adolescence

reflecting a disturbed trajectory of maturation.

Our study did not confirm previous reports of reduced hippocampal volumes in depressed adolescents (MacMillan *et al*., 2003, MacMaster and Kusumakar, 2004, Caetano *et al*., 2007, MacMaster *et al*., 2008). However, a study by Rusch *et al*. (2001) investigating hippocampal volume in depressed young adults also failed to find differences between patients and controls, in contrast to other reports of hippocampal reduction in depressed adults. The authors noted that their sample was relatively young. One explanation for their null result was that atrophy of the hippocampus in depression is a chronic process and that measurable volumetric changes may not be noticeable until later in life (Rusch *et al*., 2001), which could also apply to our study. Furthermore, illness duration seems to be critical in the detection of hippocampal volume deficits in depressed patients (Campbell *et al*., 2004). We had no detailed assessment of illness duration in our sample, but patients were treatment-naïve, which might suggest a relatively short illness duration. Previous studies showing reduced hippocampal volumes in depressed adolescents used patient samples with mean illness durations of 27.4 months (Caetano *et al*., 2007), 27.7 months (MacMaster *et al*., 2008), and 2.89 years (MacMaster and Kusumakar, 2004).

Previous studies investigating brain volume in adolescent affective disorders have also reported altered amygdala (De Bellis *et al*., 2000, Blumberg *et al*., 2003, DelBello *et al*., 2004, Milham *et al*., 2005) and STG volumes (De Bellis *et al*., 2002b). Reports on amygdala abnormalities were inconsistent: decreases in left and total amygdala volume were found in adolescents with bipolar disorder or anxiety (Blumberg *et al*., 2003, DelBello *et al*., 2004, Milham *et al*., 2005) but also an increase of right and total amygdala volume in children with anxiety (De Bellis *et al*., 2000). Our study did not confirm any of those results, finding no amygdala volume differences between patients and healthy controls. The nature of the samples studied may have contributed to this discrepancy in findings, since other studies included patients with bipolar disorder or anxiety disorders with various other psychiatric comorbidities (e.g. ADHD, ODD, PTSD, depression, obsessive-compulsive disorder), which could have influenced the effect. Furthermore, studies investigating amygdala volume in MDD in adults also report inconsistent results. Increased amygdala volumes were reported in patients with a first depressive episode (Frodl *et al*., 2002, Frodl *et al*., 2003), whereas a meta-analysis did not reveal significant volumetric abnormalities in the amygdala and emphasized the inconsistencies in findings regarding the amygdala (Koolschijn *et al*., 2009).

We did not find abnormalities of grey matter volumes of the STG in our group of clinically depressed adolescents. The limited data on grey matter volume of the STG in adolescents with affective disorders is inconsistent, with one study reporting increased STG grey and white matter in paediatric generalized anxiety disorder patients (De Bellis *et al.*, 2002b), whereas another study investigated bipolar adolescents and found decreased left STG grey matter volume in patients (Chen *et al.*, 2004). Additionally, Shad (2012) investigated depressed adolescents and found decreased right STG grey matter volume in patients (Shad *et al.*, 2012). The inconsistent findings may be due to differences in study populations and methodologies.

Contrary to expectation, we did not find an association between grey matter volume of the effect found in the dorsal ACC and clinical severity scores within the patient group. Our study might have been underpowered to detect possible correlations between clinical data and grey matter volumes. However, an absence of correlates between structural brain abnormalities and clinical severity scores is not limited to our study, as it is reported in many studies of depression. This may be due to the more general and heterogeneous nature of clinical rating scales, typically assessing multiple aspects of depressive symptomatology.

Our sample consisted predominantly of females, which may limit comparison with previous studies reporting on study samples with more equal numbers of males and females. On the other hand, depression and anxiety are much more prevalent in girls than in boys (Costello *et al.*, 2003, Ghandour *et al.*, 2010, Thapar *et al.*, 2012). Our sample may therefore be more representative of depressed youth. Excluding the three boys from the analysis yielded the same effect in the ACC.

To our knowledge, the present study was the first to investigate grey matter volumes in a sample of clinically depressed adolescents and pair-wise matched healthy controls while assessing symptoms of depression and co-occurrent anxiety both categorically and dimensionally. Nevertheless, some limitations are important to note. The cross-sectional nature of this study does not allow for conclusions regarding causality. Hence, we cannot ascertain whether grey matter differences reported in this study preceded or followed the onset of a clinical depression. Also, since socioeconomic status (SES) was not assessed in a standardized

manner, we cannot rule out the possibility that grey matter differences are attributable to variations in SES. Additionally, no detailed information was available on illness duration, but the included group did not receive treatment for their clinical depression prior to this study, which might suggest a relatively short illness duration. We chose to include adolescents who were clinically depressed and allowed comorbid anxiety. Clearly, this limits the possibility to draw conclusions about grey matter abnormalities unique to depression. On the other hand, since depression and anxiety are so highly comorbid in adolescents, exclusion of anxiety would have resulted in an atypical and less ecologically valid sample (Zahn-Waxler *et al.*, 2000, Costello *et al.*, 2003, Cullen *et al.*, 2009, Ghandour *et al.*, 2010, Simms *et al.*, 2012).

In summary, our findings point to the involvement of the dorsal ACC in treatment-naïve, clinically depressed adolescents. Whether grey matter abnormalities in the ACC precede or result from affective disorders in adolescence, how they interact with (social) environmental and genetic factors and whether they are malleable by treatment is yet unknown, and further research is needed to shed more light on this issue (Lau, 2012).

Chapter 7
Discussion

This thesis aimed to address gaps in the current knowledge of the underlying neurobiology of adults and adolescents with depression and anxiety, using innovative neuroimaging modalities such as resting-state fMRI and voxel-based morphometry to investigate brain structure and functional connectivity. Resting-state fMRI was used to study the functional connectivity patterns unique to SAD without comorbidity, and also, for the first time, to PD without comorbidity. These adult patients were directly compared to pair-wise matched healthy control subjects. Additionally, the shared and unique characteristics of MDD and anxiety disorders were examined in a comparison between adult MDD patients without comorbidity, anxiety patients without comorbidity, and patients presenting with comorbid MDD and anxiety disorder(s), compared to healthy controls. Anxiety disorders included in this study were PD, SAD, and GAD, and any combination of the three.
To address gaps in the knowledge on developmental features of affective disorders, resting-state fMRI and structural MRI were employed in a study on clinically depressed adolescents.

The studies in this thesis were based on samples derived from two longitudinal studies: the Netherlands Study of Depression and Anxiety (NESDA) and the Emotional Pathways' Imaging Study in Clinical Adolescents (EPISCA).
In this chapter, the results of the studies in Chapters 2 – 6 will be summarised, and their implications will be discussed.

Summary of results

The unique resting-state characteristics of PD were examined in Chapter 2. We demonstrated altered resting-state functional connectivity (RSFC) in the limbic and salience networks in a sample of PD patients without any other psychiatric comorbidity. For the limbic network, patients showed increased RSFC between the right amygdala and the bilateral precuneus and lateral occipital cortex. This effect may be linked to typical panic symptoms such as derealisation and depersonalisation. However, as associations between the effect and clinical severity were lacking, these speculations could not be confirmed. For the salience network, PD patients had decreased RSFC between the left dACC and the bilateral frontal pole and superior/medial frontal gyrus compared to healthy controls. In contrast, PD patients showed an increased RSFC with the bilateral

precentral and postcentral gyrus, right supplementary motor cortex, and right ACC. Furthermore, an increased RSFC between the right dACC and the right superior parietal lobule, lateral occipital cortex, angular gyrus, and central opercular cortex in PD patients was found. These findings could be related to panic symptoms such as a heightened awareness of bodily sensations, or disturbances in self-awareness that could be reflected in symptoms such as the feeling that one is going crazy or loses control (Pannekoek *et al*., 2013b).

In Chapter 3, RSFC in medication-naïve SAD patients without psychiatric comorbidity was discussed. For the limbic network, SAD patients showed increased negative right amygdala RSFC with the left middle temporal gyrus, supramarginal gyrus, and lateral occipital cortex compared to healthy controls. These areas have been linked to facial perception, and an aberrant RSFC could be interpreted along the lines of an enhanced sensitivity for facial expressions in SAD patients. For the salience network, increased positive connectivity was reported between the bilateral dACC seeds and the left precuneus and lateral occipital cortex in SAD patients. This could underlie a disturbed self-awareness and biased information gathering in SAD patients (Pannekoek *et al*., 2013c).

Chapter 4 discussed the shared and unique RSFC characteristics of substantial groups of MDD patients without comorbidity, anxiety patients without comorbidity, and comorbid depressed and anxious patients, compared to healthy control subjects. Using the data-driven independent component analysis (in combination with dual regression) approach, differences were found between the comorbid group and healthy controls in the limbic network, where comorbid depressed and anxious patients showed increased connectivity in a cluster containing the bilateral precuneus, intracalcarine cortex, lingual gyrus, and posterior cingulate, as well as a cluster including the right precentral gyrus, inferior frontal gyrus, and middle frontal gyrus. It was suggested that the aberrant RSFC pattern found in the current study is specific to comorbid depression and anxiety, only becoming evident when patients present with both disorders and not in patients with a single diagnosis of either (Pannekoek *et al., submitted for publication*).

Our fourth RS study was conducted in a sample of treatment-naïve adolescents with a clinical diagnosis of depression and pair-wise matched healthy controls. Chapter 5 describes the RSFC differences we found

between these groups. For the limbic network, depressed adolescents showed increased RSFC between the left amygdala and the right middle frontal gyrus, inferior frontal gyrus, precentral gyrus, and postcentral gyrus. These findings could be related to a disturbed cognitive control over emotion processing in adolescent depression. There was also an attenuated RSFC in the clinical group between the right amygdala and the left frontal pole, right ACC, paracingulate gyrus, and superior frontal gyrus, as well as with the left angular gyrus, lateral occipital cortex, and supramarginal gyrus. Altered connectivity between the amygdala and the ACC is typical for depression, and could be associated with abnormalities in emotion regulation. For the salience network, decreased RSFC was found in the clinical group between the dACC and the right middle frontal gyrus, frontal pole, and inferior frontal gyrus. Altered connectivity between the dACC and prefrontal areas may be associated with a bias towards negative emotional stimuli and disrupted assessment of affective stimuli (Pannekoek *et al*., 2014a).

In addition to RSFC in the previous chapter, grey matter volume in clinically depressed adolescents was studied in Chapter 6. A region-of-interest VBM revealed a smaller ACC volume by 14.4% in the patient group compared to pair-wise matched healthy controls. This is consistent with literature on adult depression. The ACC has been linked to higher cognitive functions, and problems with inhibiting the processing of negative material are common symptoms of depression. Abnormalities in grey matter volume of the ACC could be related to such problems. The emergence of psychopathologies in adolescence has been related to anomalies in brain maturation in combination with psychosocial, biological environmental factors. Our results may be interpreted as a result of abnormal maturational processes (Pannekoek *et al*., 2014b).

In sum, these findings point to the involvement of a wide array of brain regions in depression as well as in anxiety disorders that is not limited to a specific age range. The next sections will discuss our findings in light of neurobiological models and findings of contemporary studies of depression and anxiety.

Panic disorder

The most influential and extensive neuroanatomical model on PD was initiated by Gorman (1989; 2000). In his revised version of the original

neuroanatomical hypothesis, it was proposed that panic originates from an abnormally sensitive fear network including the insula, PFC, thalamus, amygdala, and projections from the amygdala to the brainstem and hypothalamus (Gorman *et al.*, 2000). Gorman's model was presented as a hypothesis, inviting further studies to confirm its assumptions.

In our RS study of PD patients without comorbidity, we focused on three RS networks that had been implicated in anxiety disorders before, but not in PD. With Gorman's neuroanatomical hypothesis in mind, the limbic network, probed with seeds in the bilateral amygdala, was particularly of interest. For this network, an increased negative RSFC was found with the bilateral precuneus and lateral occipital cortex. Whereas the amygdala plays a central role in Gorman's theory, the precuneus and lateral occipital cortex are not considered. With a strong focus on subcortical and limbic structures and the mPFC, the hypothesis does not include any posterior regions. The interpretation of our results that we offered was that the reported RSFC abnormalities could be associated with typical panic symptoms such as disturbances of self-processing and consciousness occurring during panic attacks. These functions are linked to the precuneus, which plays a role in self-reflection and self-processing activities like mental imagery and episodic/autobiographical memory retrieval. A heightened RSFC between the precuneus and the amygdala/limbic network, which plays a key role in emotional and fear processes, could be related to symptoms such as losing control, depsonalisation, and derealisation.

Similar to the precuneus and lateral occipital cortex, the regions found to have altered RSFC with the salience network are also new to models on PD. The left and right dACC showed altered RSFC with frontal and occipito-parietal areas that are involved in the processing of somatosensory information, attentional control, and self-awareness. With the salience network serving as an assessor of relevance of internal and external stimuli, an exaggerated significance attributed to such stimuli is much in line with the symptomatology of PD. Although Gorman notes in his revised model that misinterpretation of (benign) bodily signals is a hallmark of PD, and a potential deficit in the processing of sensory information is recognised as a contributor to the onset of panic attacks, brain areas involved in the appraisal of somatosensory input (e.g. precentral and postcentral gyrus) are not incorporated in the model.

The involvement of a more extensive neurocircuitry than what was described in the neuroanatomical model is also emphasised in other contemporary brain imaging studies. The past years have witnessed a substantial increase in the use of neuroimaging studies to further investigate the role of brain regions and circuitry that may be involved in the pathophysiology of PD (Pannekoek *et al.*, 2013a). De Carvalho and colleagues (2010) reviewed fMRI studies in PD, and included a discussion of treatment-related research (de Carvalho *et al.*, 2010). Functional studies show that brain structures such as the PFC, ACC, hippocampus, amygdala, and brainstem regions may play a major role in panic circuitry. The authors further stated that in particular the amygdala, PFC and hippocampus seem to be important for cognitive behavioural therapy (CBT) in PD, which serves to change dysfunctional thoughts regarding bodily sensations. On a neural level, reduced PFC activity could reflect an impaired top-down control of the fear response. Abnormal hippocampal activity may be related to an exaggerated appreciation of potentially threatening stimuli (de Carvalho *et al.*, 2010). PET studies point to the metabolic changes brought about by CBT in cortical areas such as the inferior frontal gyrus (IFG) (Prasko *et al.*, 2004) and the mPFC and hippocampus (Sakai *et al.*, 2006). Consistent with these findings, a recent fMRI study showed that CBT normalised a pre-treatment increase in IFG activation in PD patients compared to healthy controls, demonstrating an effect of CBT on neural mechanisms in PD (Kircher *et al.*, 2013). These studies provide further evidence for the involvement of cortical brain areas in PD that are not part of the neuroanatomical hypothesis.

Dresler and colleagues (2013) conducted a comprehensive literature review of PD studies, and discussed the findings in light of Gorman's model (Dresler *et al.*, 2013). Based on the existing literature and the ongoing gaining of insights by studies applying modern neuroimaging techniques, some amendments to the neuroanatomical model were suggested. The emphasis on the amygdala might be tempered, since findings on its involvement in PD are not univocal. Simultaneously, the role of several cortical areas such as the insula and ACC seems to be underestimated in the hypothesis. It is of note that the specific involvement of these hypothesised brain areas remains to be confirmed, as there is no conclusive evidence on the increase or decrease of regional volume and function (Dresler *et al.*, 2013).

In summary, our results are in line with other contemporary imaging studies on PD with respect to the neuroanatomical hypothesis from Gorman, suggesting the involvement of additional cortical areas in the pathogenesis of PD. It is evident that much more research is needed before a model can be proposed that is based on conclusive findings. Imaging genetics studies, experimental and longitudinal designs, and neurochemical approaches in future research could prove highly relevant for a better understanding of the neurobiology underlying PD.

Social anxiety disorder

Our resting-state study in SAD appeared to be a timely contribution to the research on the neurobiology of this disorder. Simultaneously with our publication, several other resting-state studies in SAD also came out. This increase in publications seems to parallel the accumulating interest in the neurobiology of SAD, one of the most prevalent psychiatric disorders.

We found altered RSFC between the right amygdala and the left middle temporal gyrus, supramarginal gyrus, and lateral occipital cortex in SAD patients. We also found increased RSFC between the bilateral dACC and the left precuneus and lateral occipital cortex. Our results are largely consistent with resting-state reports that appeared simultaneously with our study, of altered amygdala and ACC functional connectivity in SAD patients. One group found decreased RSFC in the primary somatosensory, motor, and visual networks in SAD patients. This group also reported increased RSFC in a medial prefrontal cortex network, and bidirectional abnormalities in a range of other networks (Liao *et al.*, 2010a). The same group showed an association between clinical severity and decrease of functional connectivity in the frontal and occipital lobe (Ding *et al.*, 2011). A third study by this group showed altered effective connectivity in SAD patients between the amygdala and the inferior temporal gyrus and visual cortices (Liao *et al.*, 2010b). Finally, the authors used regional homogeneity analysis to investigate RSFC in SAD patients, and reported decreased coherence in the angular gyrus and mPFC within the DMN in individuals with SAD (Qiu *et al.*, 2011).

Contemporary studies recognise the role of multiple brain regions in emotion, which is reflected in the use of neuroimaging modalities that highlight the involvement of neural networks rather than singular brain regions in SAD. Where resting-state fMRI studies address functional

connectivity of the brain, diffusion tensor imaging (DTI) is used to examine the structural integrity of white matter and to map white matter tracts. The uncinate fasciculus and superior longitudinal fasciculus have particularly shown involvement in the four DTI studies that exist in SAD. The uncinate fasciculus is a white matter tract that links the amygdala with the orbitofrontal cortex, and the superior longitudinal fasciculus connects occipital, parietal and frontal regions (Ayling *et al*., 2012, Fouche *et al*., 2013). Decreased fractional anisotropy was reported in the right uncinate fasciculus in SAD patients (Phan *et al*., 2009). Another study found decreased fractional anisotropy and volume in the left uncinate fasciculus and the left superior longitudinal fasciculus, and this decrease in fractional anisotropy was associated with higher trait anxiety in SAD patients (Baur *et al*., 2011). The same authors proceeded to find significantly reduced volume and fractional anisotropy at a trend level of the left uncinate fasciculus in SAD patients. Additionally, a decreased global mean fractional anisotropy was found in SAD patients (Baur *et al*., 2013). An increase in fractional anisotropy and volume of the genu of the corpus callosum was also reported (Liao *et al*., 2011). These studies suggest that distributed networks are involved in the mediation of anxiety in SAD (Fouche *et al*., 2013), and emphasise the importance of the connectivity between the amygdala and frontal cortical regions.

It is of note that despite this increased interest in the neurobiology of SAD, the entire imaging literature on SAD remains relatively small (Fouche *et al*., 2013). There is still no neuroanatomical model on SAD, like there is on PD. However, Rapee & Heimberg (1997) proposed their cognitive-behavioural model of social phobia. The model comprises 5 components, which build upon the fundamental notion that people with SAD assume that others are overly critical of them, and judge them negatively. They also attribute a great importance to being evaluated positively by others (Rapee and Heimberg, 1997).
The first component entails forming a mental representation of how the audience (i.e. any one person or group of people present) presumably perceives the individual's external appearance and behaviour. The second component is attentional resource allocation, meaning that the individual will rapidly turn his or her attention to the detection of threat. However, at the same time, the individual must monitor the mental representation

of the self and in particular those aspects of the individual's external appearance or behaviour that can elicit negative evaluation. The third component is formed by a prediction of the expected performance standard set by the audience, which is based on the characteristics of the situation and the audience (i.e. is it a formal or informal situation, does the audience consist of people that are more important or less important than the individual). This, in turn, will determine the perceived likelihood of negative evaluation. The fourth component is anxiety. This anxiety follows from the predicted negative evaluation and has physiological, cognitive and behavioural aspects. These aspects influence the individual's mental representation of his or her own external appearance or behaviour as seen by the audience, which renews the cycle starting with component one (Rapee and Heimberg, 1997).

Representation of the self and the external world (i.e. the audience) is evidently the central focus of this model. Although not explicitly stated by Rapee and Heimberg (1997), it is very plausible that creating a mental representation of how one is perceived by an audience relies heavily on information derived from facial expressions from the audience. In support of this, findings of functional imaging studies in SAD have been fairly consistent in reporting that individuals with SAD demonstrate increased amygdala and frontal-striatal cortices activity when exposed to negative or threatening facial expressions (Fouche *et al.*, 2013). Additionally, heightened self-referential processing has also been shown (Blair *et al.*, 2011b), and representation of the self is an important aspect of Rapee and Heimberg's model. However, it should be noted that these are interpretations that have not been incorporated in a neuroanatomical model on SAD.

Taken together, in line with other current literature on functional and structural connectivity studies, our results point to the involvement of widespread brain regions in SAD, including limbic, temporal, frontal, parietal and occipital areas. The recent growth in number of neuroimaging studies (particularly connectivity) may be an indicator that a neuroanatomical model of SAD may be developed in the near future.

Generalised anxiety disorder

Whereas the unique contributions of PD and SAD were discussed in Chapters 2 and 3 respectively, GAD was not separately addressed in this

thesis. The NESDA sample, from which the participants were drawn for Chapters 2, 3, and 4, did not include participants with GAD alone, preventing us from addressing this explicitly in our research. We have therefore not been able to contribute to the literature on GAD alone.

It is of note that GAD is distinctly understudied, despite its frequent comorbidity with depression and other anxiety disorders (Kessler *et al.*, 2005b). In addition, where other anxiety disorders and depression have been contrasted to one another, this has only been done once for GAD, making it difficult to outline the similarities and differences with related disorders (Hilbert *et al.*, 2014). However, based on the small body of available literature on the neurobiology, Hilbert and colleagues (2014) propose a tentative neurobiological model of GAD in their very recent review. The authors suggest that in GAD patients, apprehensive expectation leads to amygdala hyperactivation, which in turn leads to elevated cortisol levels. Due to disturbed emotion regulation by the vlPFC and ACC, cognitive top-down control in the form of worrying as a coping mechanism is ineffective, leading to uncontrollable worries as well as chronic cortisol secretion. As a result, serotonin uptake reaches a maximum and causes the affective changes in GAD patients. Additionally, an increased cortisol level decreases functional connectivity between the amygdala and the PFC, also affecting emotion regulation and increasing anxiety (Hilbert *et al.*, 2014). Whereas the authors feel that their neurobiological model matches contemporary models and theories in other modalities (e.g. psychology), they acknowledge that further work is needed to substantiate and expand this model. However, this newly proposed neurobiological model of GAD could be a fitting and relevant framework that can be used as a reference for future studies.

Depression

Helen Mayberg proposed a pioneering neurobiological model of depression with a focus on disruption of a widely distributed and functionally interactive network of cortico-limbic pathways (Mayberg, 1997). In her model, three compartments (dorsal, ventral, and rostral) are held responsible for maintaining an intact mental state, and dysfunction of either element as well as dysregulated collaboration between them is hypothesised to underlie depressive symptomatology. The inhibition of hyperactive ventral areas and restored activity of hypoactive dorsal regions

is proposed to result in remission (Mayberg, 1997). By offering the model as an adaptable framework, Mayberg invited researchers from various fields to integrate their findings. Indeed, depression has attracted much attention over the past decades with a growing amount of neuroimaging research. Subsequent studies contributed to the development of a model of emotion perception by Phillips and colleagues, with an interpretation for depression pointing to the involvement of a ventral and a dorsal system (Phillips *et al.*, 2003a, Phillips *et al.*, 2003b). For a normal emotional perception, the ventral system is responsible for identifying the emotional significance of environmental stimuli and for the production of affective states. This system is proposed to be disturbed in depression. Similarly, the dorsal system is involved in higher cognitive processes such as planning and selective attention, and contributes to a more effortful regulation of the affective state. Phillips *et al.* advocate that these processes are biased by emotional input in depression (Phillips *et al.*, 2003a, Phillips *et al.*, 2003b).

Providing support for Mayberg's and Phillips' theories, many studies have investigated the roles of separate brain regions in anatomical and functional studies, with findings consistently highlighting abnormalities of the hypothalamus, pituitary, hippocampus, amygdala, and prefrontal regions as the ACC, dorsolateral and orbitofrontal cortex (Hulvershorn *et al.*, 2011). Particularly the role of anterior cingulate regions has received a great deal of attention. A meta-analysis by Pizzagalli (2011) revealed that pre-treatment elevated rostral – also known as pregenual – ACC resting cerebral blood flow is a reliable predictor of better treatment response in depression (Pizzagalli, 2011). This was originally reported by Mayberg herself in a PET study in hospitalised depressed subjects, where hypometabolism of the pregenual ACC marked non-responders to treatment (Mayberg *et al.*, 1997). Subsequent studies confirmed this finding and also described the link between better treatment response and hyperactivity in subgenual ACC regions (Mayberg, 2003) as well as hypoactivity in dACC regions (Davidson *et al.*, 2002, Pizzagalli, 2011).
Aberrancies of the anterior cingulate also emulate from structural studies, with reports of reduced grey matter volume in depressed subjects compared to healthy controls (Koolschijn *et al.*, 2009). In contrast, there are also reports of no dACC volume differences between groups (Pizzagalli, 2011). However, our finding of reduced dACC grey matter volume in clinically

depressed adolescents (Pannekoek *et al.*, 2014b) confirms findings from the majority of structural studies in depressed subjects. This further substantiates the neurobiological models emphasising a role of the ACC in depression. It is still unknown whether abnormalities of this brain region precede or follow the onset of MDD. However, our findings stem from a study in treatment-naïve depressed adolescents that were included straight after first referral to a clinician. This suggests that our participants were in an early stage of the disorder, which could indicate that the smaller dACC grey matter volume was present before disease onset. Nevertheless, we stress that future studies should confirm this speculation. Our resting-state study in the same adolescent sample also showed abnormalities of the ACC in depressed subjects. Confirming previous literature as well as Mayberg's model and Phillips' theory on emotion perception (Mayberg, 1997, Phillips *et al.*, 2003b, Etkin *et al.*, 2011a), we reported decreased resting-state functional connectivity between the amygdala and the pregenual ACC (Pannekoek *et al.*, 2014a). This provides further support for disturbances in the ventral system in which such deviations are proposed to underlie depressive symptomatology by both models (Mayberg, 1997, Phillips *et al.*, 2003b). Additionally, we reported decreased dACC connectivity with prefrontal regions, indicating that the dorsal system is also affected in depression. Mayberg depicts connections between dorsal cortical regions and prefrontal regions, suggesting that remission will occur when hypoactivity in the dorsal areas is restored (Mayberg, 1997).

All in all, the results of our studies are in favour of two important neurobiological models on depression (Mayberg, 1997) and emotion perception (Phillips *et al.*, 2003a, Phillips *et al.*, 2003b). It is plausible that frontocingulate dysfunction contributes to key cognitive and affective abnormalities in depression, such as rumination, a tendency to engage in and enhance negative information, and emotion dysregulation (Pizzagalli, 2011).

Comorbid depression and anxiety

Investigation of comorbid depression and anxiety can have important treatment implications. With the topic of the shared or separate etiology of depression and anxiety disorders still hotly debated (Ressler and Mayberg, 2007), the available literature is not yet able to provide a definitive answer.

The results from Chapter 4 of this thesis, reporting altered RSFC in a limbic network in comorbid depressed and anxious adults compared to healthy controls, but no differences in depression or anxiety alone, suggested that the effect is specific to comorbid depression and anxiety, only becoming evident when patients present with both disorders and not in patients with a single diagnosis of either. No correlations with symptom severity were found. Whereas comorbidity is associated with higher severity (Kessler *et al.*, 2012), tailored treatments for anxiety disorders can prove as effective for individuals with and without comorbid depressive and anxiety disorders, and significantly reduce symptoms (Allen *et al.*, 2010).

Developmental aspects

Rapid and dramatic developmental changes, for example in hormonal and neural systems, are characteristic for the pubertal years. Adolescence is therefore regarded as a particularly sensitive period for vulnerability for and the development of psychopathology. Indeed, many psychiatric illnesses, including mood and anxiety disorders, most commonly have their origin during adolescence and are likely to be associated with abnormalities of typical maturational processes. The onset of affective disorders in youngsters is not only related to a greater disease severity, but anxiety symptoms frequently precede depression in adolescence (Paus *et al.*, 2008). In turn, adolescent onset of depression is a predictor for other mental health disorders in adulthood, such as anxiety disorders, substance-related disorders and suicidal behaviour (Thapar *et al.*, 2012). Because of these far-reaching and even lifelong implications and the paucity of early interventions and treatments to address these implications, more insight into the underlying normal and abnormal developmental trajectories is warranted (Paus *et al.*, 2008). In Chapters 5 and 6 we demonstrated functional and structural abnormalities in clinically depressed adolescents that were included in the study immediately after referral by their clinician, suggesting that these aberrancies may have been present prior to the development of psychopathology. However, causality could not be assessed in the current study design. Our results are nevertheless a valuable contribution to the research field of adolescent affective disorders. Neuroimaging studies on the typically developing brain are essential to construct a template that can serve as a reference for the abnormally developing brain. In addition, integrating multiple imaging modalities

with other factors (e.g. genetic, psychosocial and environmental) in a longitudinal design will provide a more comprehensive view on the various contributors to the development of affective disorders (Paus *et al*., 2008).

Clinical implications

The current diagnostic system for mental disorders is based on a categorical approach that relies on the presence of absence of certain symptoms (Brown and Barlow, 2005, Krishnan and Nestler, 2008). A DSM diagnosis of depression requires the presence of a certain number of symptoms that significantly impair functioning for a specific duration. As a result, two patients with the same diagnosis can actually only have one symptom in common (Krishnan and Nestler, 2008). On the other hand, the symptoms can overlap between different disorders (such as depression and anxiety) that are thought to have a different etiology (Krishnan and Nestler, 2008). The ongoing debate about the advantages and disadvantages of a dimensional diagnostic approach instead of or in addition to a categorical approach is beyond the scope of this thesis. However, these variations within one disorder and overlap of symptoms with other disorders signify how challenging it is to offer a straightforward interpretation of study results (Krishnan and Nestler, 2008).

Considerations and limitations

NESDA

The NESDA neuroimaging study is embedded in the large longitudinal framework of the general NESDA study. This unique design offers the opportunity to monitor a large, well-characterised sample presenting with an extensive variety of symptoms over a long period: multiple assessment waves have been done since its commencement in 2004 and the study is still ongoing. Despite the advantages of such a design, some considerations are important to note.

Patients for the NESDA sample were recruited through general practitioners, primary care practices, and outpatient clinics of mental health organisations. It is therefore possible that the most severely affected patients are not included. On a related note, it is probable that patients with the severest symptomatology are not motivated to partake in the extensive initial assessment, with a consecutive two-hour MRI scanning session and interview for the neuroimaging study.

Some issues concerning the imaging component of NESDA should also be considered. The resting-state scan was acquired at the end of the imaging protocol: after completion of three task-related functional MRI runs and the acquisition of an anatomical scan (scan sequence: Tower of London, word encoding, T1-weighted scan, word recognition, perception of facial expression, resting-state). It is therefore possible that the task-related functional MRI session preceding the resting-state fMRI run have influenced RSFC. Positioning the resting-state fMRI run at the beginning of a scanning protocol could help prevent these so-called spillover effects, which is something that would be recommended for future studies. Also, the current design did not include a DTI run. With the current increased emphasis on connectivity in (neuro)psychiatric imaging studies, including DTI in the NESDA neuroimaging protocol would have resulted in a considerable amount of cross-sectional and longitudinal data, and thus provided a valuable contribution to contemporary mental health research. In line with this, it would also be of interest to investigate whether abnormalities in functional connectivity are accompanied by abnormalities in structural connectivity, and whether they are associated or even predictive.

EPISCA

The EPISCA study captures a representative cohort of adolescents with internalising problems by including youngsters with depressive and anxious symptomatology, as well as a group of adolescents with a history of sexual trauma (the latter is not discussed in this thesis). Because the participants were included before starting any type of treatment and were scanned twice more with periods of three months in between, this longitudinal study design is suited for the assessment of treatment effects. The resting-state run was the first run of the scanning protocol, thus preventing the potential influence of task-induced activity on the RSFC.

It is of note that the EPISCA sample is relatively small, and larger groups would be desirable in future studies. Another consideration for future research would be to explicitly define illness duration. The adolescents were recruited after referral by a clinician and before treatment onset, suggesting that they were in an early stage of the disease. Nevertheless, it would be beneficial to assess the exact duration of the complaints. Also, the male/female distribution was very unequal: only

three males were included versus 23 females in the two EPISCA studies in this thesis. Gender comparisons were therefore impossible. However, as females are more likely to develop depression (Kessler *et al.*, 2005b), the EPISCA sample is representative of the general population. Finally, the resting-state fMRI run had a duration of 6 minutes. Whereas it was standard in studies to use 5–7 minutes of resting-state data, it has been shown that reliability of RSFC improves significantly with an increase in scan duration as well as in the number of volumes (Birn *et al.*, 2013).

Resting-state

A big advantage of resting-state fMRI is that it offers an unbiased approach to investigate functional connectivity of the brain. This is of especially great importance in clinical groups, where persons suffering from, for example, neurodegenerative or (neuro)psychiatric disorders are less capable or even incapable of dealing with the cognitive demand that functional tasks often entail. However, interpretation of RSFC results is not necessarily straightforward. Functional connections between brain regions are correlations between timeseries of particular nodes (or regions of interest), and do not imply causality or directionality (i.e. is the connectivity between two nodes direct, or is it mediated by a third). These correlations are therefore also more susceptible to being influenced by confounders, such as noise and artefacts (Smith, 2012).

In addition to head motion, cardiac and respiratory fluctuations are examples of physiological noise that can have an effect on RSFC, and ways to eliminate them are constantly improved(Birn, 2012). Physiological activity was not monitored during scanning in the studies discussed in this thesis. However, heart rate variability was investigated in the entire NESDA sample, and showed that heart rate variability was lower in depressed subjects (Licht *et al.*, 2008) as well as in anxious subjects (Licht *et al.*, 2009) compared to healthy controls. It is important to note that in both these studies the lower heart rate variability in the patient groups appeared to be driven by antidepressant use (Licht *et al.*, 2008, Licht *et al.*, 2009). In our studies, using subsamples of the NESDA neuroimaging study, we only included medication-free and largely even medication-naïve subjects. Also, it has been shown that independent component analysis (applied in Chapter 4) can detect signal sources associated with confounding physiological activity and that it can distinguish these from signals of

interest (Beckmann *et al.*, 2005). We therefore think that it is unlikely that the differences in Chapter 4 were introduced by these physiological signals. Additionally, in our studies using a seed-based region-of-interest approach (Chapters 2, 3 and 5 (Pannekoek *et al.*, 2013b, Pannekoek *et al.*, 2013c, Pannekoek *et al.*, 2014a)), the global signal was regressed out to reduce artefacts as a result of physiological signal sources, in accordance with previous studies indicating that such a procedure is a successful tool for this purpose (Birn *et al.*, 2006, Fox and Raichle, 2007, Birn, 2012).

A topic of some controversy concerning resting-state fMRI is the existence of negative functional connectivity. This refers to negative correlations between the timeseries of two brain regions, and is currently still poorly understood. It has been argued that these correlations are introduced by global signal regression during data preprocessing (Murphy *et al.*, 2009, Cole *et al.*, 2010, Chen *et al.*, 2011), suggesting that this method may have some disadvantages. However, global signal regression is also a powerful and important tool to remove motion artefacts in resting-state fMRI (Power *et al.*, 2014, Yan *et al.*, 2013) and as discussed above, as well as physiological noise. In addition, it has been shown that the negative connectivity exists with or without correction for global signal (Chang and Glover, 2009). At present, as reports have been equivocal, the precise meaning of negative connectivity remains open to debate (Cole *et al.*, 2010).

It is clear that like other MRI modalities, resting-state fMRI has its advantages as well as limitations. As one of the newest imaging approaches, it is in constant development and the research techniques are continuously improved. Experts in the field of functional connectivity point out the links that are already made between changes in connectivity and diseases, and predict an even further growth in its clinical applications. They are confident that in the future, functional connectivity has the potential to become a powerful instrument for investigating disease mechanisms, particularly when combined with other imaging modalities including structural MRI and diffusion-based connectivity, but also non-MRI modalities such as magnetoencephalography and electroencephalography (Smith, 2012).

Future directions

Structural and functional magnetic resonance imaging have provided important insights into the involvement of candidate brain regions. It is unlikely that simple increases and decreases in brain activity alone are sufficient to explain the complex diversity of symptoms that define depression and anxiety (Krishnan and Nestler, 2008). Combined efforts using various research modalities (for example, MRI, (epi)genetics, neuroendocrinology, and environmental studies) could significantly improve knowledge about the neurobiology and pathophysiology of depression and anxiety disorders. Performing such complementing multi-modal studies, and thus acknowledging and embracing the heterogenetic nature of these disorders, would be a promising next phase of neurobiological research on affective disorders. This multivariate approach is a critical next step in the eventual development of diagnostic guidelines to distinguish patient subgroups, map comorbidity and the extent of its influence on disease course and treatment outcome, construct optimal intervention strategies, predict the disease course and identify biomarkers for vulnerability.

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Nederlandse samenvatting

Introductie

Depressie en angststoornissen vallen onder de meest voorkomende psychiatrische stoornissen. Bovendien gaan ze vaak met elkaar gepaard. Zowel depressie als angst wordt geassocieerd met een verhoogd risico op suïcide, persoonlijk lijden en een verminderd functioneren op sociaal vlak en op werk. Dit leidt gezamenlijk tot een aanzienlijke beperking in het dagelijks leven, wat grote economische lasten tot gevolg heeft. Vaak begint een depressie of een angststoornis tijdens de adolescentie. Het ziektebeeld en de daarmee gepaard gaande beperkingen zijn des te ernstiger bij een vroegere uiting van de stoornis.

Veel patiënten met angststoornissen kampen ook met symptomen van een depressie en andersom. Als iemand beide stoornissen heeft – dan wordt van zogeheten comorbide depressie en angst gesproken – heeft dit een grotere ernst van de symptomen tot gevolg, is er minder respons op behandeling en heeft de patiënt een slechtere prognose dan wanneer iemand slechts één stoornis heeft. Comorbiditeit is zelfs een voorspeller voor suïcide. Drie angststoornissen die het meest voorkomen in combinatie met depressie, ofwel 'major depressive disorder' (MDD), zijn sociale angststoornis (SAS), gegeneraliseerde angststoornis (GAS) en paniekstoornis (PS).

Neuroimaging bij depressie en angst

Juist omdat depressie en angst zo regelmatig samen voorkomen, is de vraag gerezen of ze een gezamenlijke oorzaak hebben of echt aparte stoornissen zijn. Dit laatste komt overeen met het huidige classificatiesysteem. Om een beter beeld te krijgen van de neurobiologie die ten grondslag ligt aan depressie en angst, kan beeldvormend onderzoek van de hersenen (neuroimaging) worden ingezet. Met structurele 'magnetic resonance imaging' (MRI) kunnen anatomische eigenschappen van het brein in kaart worden gebracht. Met functionele MRI (fMRI) wordt de activiteit van en connectiviteit tussen hersengebieden gemeten. Dit kan bijvoorbeeld door de deelnemer een taakje te laten uitvoeren in de scanner, zoals het kijken naar plaatjes of het indrukken van een knop. Op die manier kan men zien welke hersengebieden er op dat moment actief zijn. Een ander voorbeeld van fMRI is zogeheten resting-state fMRI, waarbij degene in de scanner helemaal geen taakje hoeft te doen, maar slechts zo stil mogelijk moet

liggen zonder in slaap te vallen. Hierbij wordt verondersteld dat wanneer diverse hersengebieden hetzelfde activatiepatroon laten zien, deze bij hetzelfde proces betrokken zijn en dus functioneel met elkaar verbonden zijn; dan wordt gesproken van functionele connectiviteit.

Resultaten van zowel structurele als functionele neuroimaging studies hebben aangetoond dat de structuur en functie van bepaalde hersengebieden verschillen bij mensen met angst en depressie, wanneer zij worden vergeleken met de hersenen van gezonde mensen. Bij depressie worden de anterieure cingulate cortex (ACC), hippocampus, amygdala, thalamus, het cerebellum, de posterieure cingulate cortex (PCC), en de temporale en pariëtale gebieden in de cortex stelselmatig in verband gebracht met de ziekte. Als we kijken naar studies naar angststoornissen, wordt bij PS een rol toebedeeld aan de hippocampus, amygdala en de hersenstam, maar tevens worden de ACC en de PCC genoemd. Daarnaast worden andere corticale gebieden als de insula en de prefrontale cortex (PFC) gerapporteerd. Bij SAS komen de amygdala, frontale corticale gebieden en de precuneus veel naar voren. Vergeleken met andere angststoornissen zijn er bij GAS bijzonder weinig studies uitgevoerd. Het geringe aantal studies laat echter zien dat de amygdala en de PFC een rol spelen bij deze angststoornis.

Neurobiologische modellen van depressie en angst

In de afgelopen decennia is de interesse voor de neurobiologie die ten grondslag ligt aan depressie en angst enorm gegroeid, wat heeft geleid tot de ontwikkeling van enkele theorieën.

In 1997 verscheen het neurobiologische model over depressie van Helen Mayberg, waarin zij oppert dat een reeks hersengebieden en met name verstoorde verbindingen en interacties tussen cortico-limbische hersengebieden een rol spelen bij depressie. Aangezien dergelijke interacties noodzakelijk zijn voor een normale regulatie van stemming en daarmee gepaard gaande processen, veronderstelt Mayberg dat dysfunctionele interacties de veroorzaker zijn van depressieve symptomen. De drie basiselementen uit haar theorie worden gevormd door een dorsaal, ventraal en rostraal component.

Vergelijkbaar met deze theorie ontwikkelden Mary Phillips en haar collega's hun functionele neuroanatomische model van emotie-perceptie in 2003. Het model van Phillips bouwt voort op het idee dat twee

systemen, een ventraal en dorsaal systeem, verantwoordelijk zijn voor emotie-perceptie en tevens voor het tot stand brengen en reguleren van een emotionele staat. Het model brengt duidelijke patronen van structurele en functionele afwijkingen in deze systemen in verband met specifieke symptomen van psychiatrische stoornissen, waaronder MDD.

Jack Gorman ontwikkelde in 1989 een zeer invloedrijke hypothese over paniekstoornis, welke hij in 2001 in herziende vorm opnieuw uitbracht. Gorman suggereert dat paniek voortvloeit uit een abnormaal gevoelig angstnetwerk dat de amygdala, thalamus, PFC en insula omvat, alsmede de projecties van de amygdala naar de hersenstam en hypothalamus.

Tot op heden zijn er nog geen neurobiologische modellen verschenen over SAS en GAS. Dit onderstreept de noodzaak voor meer onderzoek naar en beter begrip van deze angststoornissen.

De bestaande modellen van depressie en angststoornissen gaan uit van mechanismen die afhankelijk zijn van een adequate communicatie tussen bepaalde hersengebieden, maar waarin afwijkingen zitten. Sinds het verschijnen van deze theorieën, heeft de neuroimaging een grote ontwikkeling ondergaan en zijn de onderzoekstechnieken verbeterd en aangescherpt.

Doel van dit proefschrift

Het doel van de studies in dit proefschrift was om de onderliggende neurobiologie van depressie en angst bij volwassenen en jongeren te onderzoeken met recent ontwikkelde neuroimaging technieken, en daar waar nodig fundamentele en invloedrijke modellen aan te vullen. Volwassen deelnemers kwamen uit de Nederlandse Studie naar Depressie en Angst (NESDA). Jongere deelnemers waren afkomstig van de Emotional Pathways' Imaging Study in Clinical Adolescents (EPISCA).

Ondanks het belang van connectiviteitsstudies voor een beter begrip van depressie en angst, is er nog erg weinig bekend over de netwerkinteracties tussen hersengebieden bij deze stoornissen. Het doel van dit proefschrift was te onderzoeken of nieuwe imaging-technieken zoals resting-state fMRI resultaten zouden bevestigen uit eerdere studies waarbij gebruik werd gemaakt van andere imaging-modaliteiten, of dat er neurale trajecten zouden worden gevonden die betrokken zijn bij depressie en angst die nog niet eerder werden gerelateerd aan deze ziektebeelden.

De unieke resting-state eigenschappen van paniekstoornis (PS)

werden onderzocht in Hoofdstuk 2. Wij toonden aan dat resting-state functionele connectiviteit (RSFC) in het limbisch en salience netwerk afweek in een groep PS patiënten van 11 personen, die geen enkele andere psychiatrische comorbiditeit hadden, vergeleken met 11 gezonde controles. Voor het limbisch netwerk lieten PS patiënten een verhoogde RSFC zien tussen de rechter amygdala en de bilaterale precuneus en laterale occipitale cortex. Dit effect zou gelieerd kunnen zijn aan typische panieksymptomen zoals derealisatie en depersonalisatie, waarmee het gevoel wordt bedoeld dat iemand het contact met de realiteit of zichzelf kwijt is. Deze speculaties konden echter niet worden bevestigd, aangezien wij geen associatie hebben aangetoond tussen deze verhoogde RSFC en de ernst van de symptomen. Voor het salience netwerk lieten PS patiënten een verminderde RSFC zien tussen de linker dorsale ACC (dACC) en de bilaterale polus frontalis vergeleken met gezonde controles. Tevens werd een verhoogde RSFC gevonden bij PS patiënten met de bilaterale centrale precentrale en postcentrale gyrus, de rechter supplementaire motorische cortex en de rechter ACC. Bovendien werd een verhoogde RSFC gevonden bij PS patiënten tussen de rechter dACC en de rechter superieure pariëtale lobule, laterale occipitale cortex, angulaire gyrus en de centrale operculaire cortex gevonden. Deze resultaten zouden gerelateerd kunnen zijn aan panieksymptomen zoals een verhoogde gewaarwording van lichamelijke sensaties, of problemen met het zelfbewustzijn. Dergelijke panieksymptomen zijn bijvoorbeeld het gevoel dat iemand gek wordt of de controle verliest.

De RSFC van 12 medicatie-naïeve SAS patiënten zonder psychiatrische comorbiditeit, vergeleken met 12 gezonde controles, werd onderzocht in Hoofdstuk 3. Voor het limbisch netwerk lieten SAS patiënten een verhoogde negatieve RSFC zien van de amygdala met de linker middel temporale gyrus, supramarginale gyrus en de laterale occipitale cortex, vergeleken met gezonde controles. Deze gebieden zijn betrokken bij het waarnemen van gezichten, en een afwijkende RSFC ervan bij SAS patiënten zou kunnen worden geïnterpreteerd als een verhoogde gevoeligheid voor gezichtsuitdrukkingen van anderen. Voor het salience netwerk werd een verhoogde positieve connectiviteit getoond in SAS patiënten tussen de bilaterale dACC en de linker precuneus en laterale occipitale cortex. Dit zou te maken kunnen hebben met een verstoord zelfbewustzijn en een tendentieuze manier van informatieverzameling bij SAS patiënten.

Hoofdstuk 4 behandelde de gedeelde en unieke RSFC kenmerken bij groepen patiënten m*et al*leen MDD, patiënten m*et al*leen angst, en patiënten met comorbide depressie en angst, vergeleken met gezonde controles. Door gebruik te maken van de data-gedreven zogeheten 'independent component analysis' methode in combinatie met 'dual regression', werden RSFC verschillen gevonden tussen de comorbide groep en gezonde controles. Deze verschillen werden gevonden in het limbisch netwerk, waar comorbide patiënten een verhoogde connectiviteit vertoonden in de bilaterale precuneus, intracalcarine cortex, linguale gyrus en de PCC, alsmede met de rechter precentrale gyrus, inferieure frontale gyrus en de middel frontale gyrus. Wij suggereerden dat deze afwijkende RSFC specifiek is voor een comorbide presentatie van angst en depressie en dus alleen tot uiting komt als patiënten beide stoornissen hebben, en niet met slechts één diagnose.

Onze vierde resting-state studie werd gedaan in een groep adolescenten met een klinische diagnose van depressie die nog geen enkele vorm van behandeling hadden ondergaan, en gekoppelde gezonde controles. In Hoofdstuk 5 werden de RSFC verschillen beschreven die wij tussen deze twee groepen vonden. Voor het limbisch netwerk toonden depressieve adolescenten een verhoogde RSFC tussen de linker amygdala en de rechter middel frontale gyrus, inferieure frontale gyrus, precentrale gyrus en de postcentrale gyrus. Deze bevindingen zouden gerelateerd kunnen zijn aan een verstoorde cognitieve controle van emotieverwerking bij depressie in adolescenten. Wij vonden tevens een verminderde RSFC in de depressieve groep tussen de rechter amygdala en de linker polus frontalis, de rechter ACC, paracingulate gyrus en de superieure frontale gyrus, alsmede de linker angulaire gyrus, laterale occipitale cortex en de supramarginale gyrus. Abnormale connectiviteit tussen de amygdala en de ACC is kenmerkend voor depressie en zou geassocieerd kunnen zijn met afwijkingen in emotieregulatie. Voor het salience netwerk werd een verminderde RSFC gevonden in de depressieve groep tussen de bilaterale dACC en de rechter middel frontale gyrus, polus frontalis en de inferieure frontale gyrus. Een veranderde connectiviteit tussen de dACC en prefrontale hersengebieden zou geassocieerd kunnen zijn met een tendens naar negatieve emotionele stimuli, en een verstoorde beoordeling van affectieve stimuli.

Naast de RSFC studie uit Hoofdstuk 5, werd in Hoofdstuk 6

bij dezelfde groep klinisch depressieve adolescenten ook het grijze stof volume onderzocht. Een zogeheten 'region-of-interest' VBM ('voxel-based morphometry') toonde aan dat het volume van de ACC bij de depressieve groep 14,4% kleiner was dan bij gekoppelde gezonde controles. Dit komt overeen met bevindingen in de literatuur over depressie bij volwassenen. De ACC wordt in verband gebracht met hogere cognitieve functies, en inhibitieproblemen bij de verwerking van negatieve materie zijn veelvoorkomende symptomen bij depressie. Afwijkingen in het grijze stof volume van de ACC zouden aan zulke problemen gerelateerd kunnen zijn. Het ontstaan van psychiatrische stoornissen tijdens de adolescentie wordt geassocieerd met afwijkingen in de ontwikkeling van het brein, in combinatie met psychosociale, biologische en omgevingsfactoren. Onze resultaten zouden kunnen worden geïnterpreteerd als een resultaat van een verstoorde ontwikkeling.

Paniekstoornis

In de revisie van zijn neuroanatomische hypothese van PS stelt Gorman dat paniek voortkomt uit een hypergevoelig angstnetwerk, waarin de insula, PFC, thalamus, amygdala en de projecties van de amygdala naar de hersenstam en hypothalamus zitten. Wij toonden binnen een limbisch netwerk afwijkingen aan tussen enerzijds de amygdala en anderzijds de precuneus en laterale occipitale cortex. De amygdala wordt al genoemd in het model van Gorman. De precuneus en de laterale occipitale cortex, beide posterieure gebieden, komen echter niet aan bod; de hypothese bevat helemaal geen posterieure hersengebieden.

Eveneens nieuw zijn de verschillen tussen mensen met PS en gezonde personen die we vonden in het salience netwerk. De linker en rechter dACC toonden afwijkende RSFC met frontale en occipito-pariëtale gebieden, die betrokken zijn bij processen als het verwerken van somatosensorische informatie, aandachtscontrole en zelfbewustzijn. Het salience netwerk speelt een rol bij het toekennen van relevantie aan interne en externe prikkels. Het toeschrijven van een te grote betekenis aan dergelijke stimuli past in het kader van symptomen die typisch zijn voor PS.

Uit de resultaten van onze studie bleek dat er een breder netwerk aan hersengebieden betrokken is bij PS dan wat tot nu toe in modellen beschreven staat. Dit komt overeen met de bevindingen van andere

hedendaagse neuroimaging onderzoeken, op basis waarvan recentelijk ook enige aanvullingen op de hypothese van Gorman werden voorgesteld. De nadruk die nu op de amygdala wordt gelegd in het model zou wat afgezwakt mogen worden, aangezien ni*et al*le studies even sterk wijzen op de betrokkenheid van dit gebied. Tegelijkertijd lijkt de rol van corticale gebieden zoals de insula en de ACC juist te weinig onderstreept. Het is echter duidelijk dat nog veel meer onderzoek moet worden gedaan voordat een nieuw model kan worden opgesteld dat gebaseerd is op eenduidige bevindingen.

Sociale angststoornis

Omstreeks dezelfde tijd waarin onze publicatie over SAS verscheen waren andere, internationale onderzoeksgroepen eveneens bezig met resting-state fMRI onderzoek bij SAS. Dit reflecteert de behoefte naar meer aandacht voor deze stoornis. Onze resultaten komen grotendeels overeen met dergelijke studies, waarin eveneens een afwijkende functionele connectiviteit van de amygdala en ACC – en tevens andere gebieden in de cortex – bij SAS patiënten werd gerapporteerd. Met name de connectiviteit tussen de amygdala en frontale corticale gebieden is belangrijk en dit komt ook naar voren in structurele connectiviteitsstudies. Hedendaags onderzoek benadrukt dan ook de rol van meerdere hersengebieden bij emotie, door gebruik te maken van neuroimaging technieken die betrokkenheid van neurale netwerken in plaats van een enkel hersengebied aan het licht brengen.

Uit neuroimaging onderzoek komt helder naar voren dat hersengebieden die betrokken zijn bij de perceptie en verwerking van gezichten een heel grote rol spelen bij SAS. Ook cognitieve theorieën over SAS zijn gebaseerd op de gevoeligheid van patiënten voor andermans goedkeuring en angst voor een negatief oordeel. Wij denken dat het beeld dat iemand heeft van hoe anderen over hem of haar denken voor een groot deel gebaseerd is op hun gezichtsuitdrukkingen.

Hoewel er in recente jaren beduidend meer neuroimaging onderzoek naar SAS is gedaan, heeft dit tot op heden nog niet geleid tot de ontwikkeling van een neuroanatomisch model van SAS. Wellicht is deze groei echter wel een voorbode voor de totstandkoming van een dergelijk model in de nabije toekomst.

Generaliseerde angststoornis

In de hoofdstukken 2 en 3 werden de unieke resting-state eigenschappen van respectievelijk PS en SAS onderzocht. De Nederlandse Studie naar Depressie en Angst (NESDA) had geen deelnemers die alleen de diagnose GAS hadden, waardoor wij niet de mogelijkheid hadden om dit ziektebeeld in dit proefschrift op dezelfde manier te beschrijven zoals PS en SAS.

Depressie

In de afgelopen decennia is er steeds meer aandacht gekomen voor depressie, met daarmee ook een groeiend aantal structurele en functionele neuroimaging studies. De theorieën van zowel Mayberg als Phillips werden veelal ondersteund door dergelijk onderzoek, waarin de rol van diverse op zichzelf staande hersengebieden werd onderzocht. Afwijkingen van de hypothalamus, hypofyse, hippocampus en amygdala werden consistent gerapporteerd, alsmede prefrontale gebieden zoals de dorsolaterale en orbitofrontale cortex. Bovendien was er vooral veel aandacht voor de ACC. Onze bevinding van een kleiner grijze stof volume in dit gebied bij adolescenten met een klinische depressie bevestigt dan ook de resultaten van de meeste structurele MRI studies, waarin eveneens kleinere ACC volumes werden beschreven.

Ook onze resting-state fMRI studie in dezelfde groep depressieve jongeren toonde afwijkingen van de ACC aan. Onze bevinding van een verminderde resting-state functionele connectiviteit tussen de amygdala en de pregenuale ACC komt overeen met bestaande literatuur en met het model van Mayberg en de theorie van Phillips over emotieperceptie. Beide hypothesen veronderstellen dat dergelijke verstoringen in het ventrale systeem de basis te vormen van depressieve klachten, wat met onze resultaten nog meer draagvlak krijgt. Het is aannemelijk dat het dysfunctioneren van deze frontocingulaire gebieden bijdraagt aan belangrijke cognitieve en affectieve afwijkingen bij depressie, waarbij moet worden gedacht aan verstoorde emotieregulatie en aan rumineren: de neiging om bezig te zijn met en het uitvergroten van negatieve informatie.

Comorbide depressie en angst

Er is nog altijd een hevige discussie gaande omtrent een gezamenlijke of aparte ontstaansgeschiedenis van depressie en angststoornissen. De bestaande literatuur slaagt er nog niet in om hier een eenduidige oplossing

voor te bieden. Hoofdstuk 4 van dit proefschrift beschrijft dat RSFC van een limbisch netwerk afwijkt in patiënten die comorbide depressie en angst hebben vergeleken met gezonde personen, maar niet vergeleken met patiënten die ofwel alleen depressie, ofwel alleen angst hebben. Op basis van onze bevindingen lijkt dit effect dus uniek te zijn voor de meervoudige diagnose van zowel angst als depressie, terwijl het niet tot uiting komt op het moment bij personen die één diagnose van of angst, of depressie hebben.

Overwegingen

De longitudinale opzet van NESDA en het feit dat de studie aan drie verschillende centra wordt uitgevoerd, heeft ervoor gezorgd dat er een grote en goed omschreven groep deelnemers uit het hele land en met een breed scala aan symptomen kon worden geïncludeerd en over een lange periode kon worden gevolgd. Ondanks deze belangrijke sterke punten zijn er ook bepaalde kanttekeningen waar rekening mee moet worden gehouden. De patiënten in de NESDA studie werden geworven via huisartspraktijken en eerstelijns GGZ-instellingen, waardoor het mogelijk is dat patiënten met zeer ernstige problematiek niet zijn geïncludeerd. Het is eveneens mogelijk dat patiënten met de meest ernstige klachten het uitgebreide onderzoek, waarin meerdere metingen werden gedaan en vragenlijsten werden afgenomen met nog een extra twee uur durende MRI sessie en interview voor de neuroimaging studie, als te belastend zagen en daardoor niet gemotiveerd waren om mee te doen. Een eventuele beperking van het imaging protocol zou de positie van de resting-state scan kunnen zijn, welke aan het eind van het protocol werd gemaakt. Het is niet uitgesloten dat de diverse voorafgaande taak-gerelateerde functionele scans van invloed waren op de resting-state scan.

Bij de EPISCA studie was de resting-state scan aan het begin van het protocol geplaatst, om dergelijke invloeden te voorkomen. Een kanttekening die bij dit onderzoek moet worden geplaatst is de relatief beperkte grootte van de groepen.

Een groot voordeel van resting-state fMRI is dat het onderzoekers in staat stelt om naar de functionele connectiviteit van het brein te kijken zonder enige manipulatie door middel van taakjes of stimuli. Met name klinische groepen van mensen die bijvoorbeeld aan een neuropsychiatrische of neurodegeneratieve stoornis zoals dementie lijden, zijn veelal verminderd

of helemaal niet in staat om de cognitieve belasting van taken in de scanner aan te kunnen. Eén van de moeilijkheden van resting-state fMRI is het interpreteren van de resultaten. Functionele connecties tussen hersengebieden zijn correlaties en daaruit valt geen richting of oorzakelijk verband af te leiden. Deze correlaties zijn ook gevoeliger voor andere invloeden van bijvoorbeeld fysiologische ruis door ademhaling en hartslagvariabiliteit. De techniek om dergelijke invloeden zoveel mogelijk te beperken of zelfs helemaal uit te sluiten blijft echter steeds ontwikkelen en verbeteren.

Tot slot

Structurele en functionele MRI hebben een belangrijke bijdrage geleverd aan een beter inzicht in de rol die bepaalde hersengebieden spelen bij depressie en angst. Het is onwaarschijnlijk dat alleen het stijgen en dalen van hersenactiviteit afdoende is om de complexe diversiteit van angst- en depressiesymptomen te verklaren. Het combineren van verschillende onderzoeksmethoden (zoals MRI, (epi)genetica, neuroendocrinologie en omgevingsstudies) kunnen de neurobiologische en pathofysiologische kennis over depressie en angst beduidend vergroten. Het uitvoeren van dergelijke complementerende multimodale studies zou een veelbelovende volgende fase zijn in neurobiologisch onderzoek bij affectieve stoornissen.

English summary

Introduction

Depressive and anxiety disorders are amongst the most prevalent of all psychiatric disorders, and also co-occur frequently. Separately, they are associated with an increased risk for suicide, subjective suffering, impaired social and work functioning, and the resulting disability is in turn responsible for a high economic burden. Depression and anxiety disorders often have an onset during adolescence, and an early onset is associated with more disease severity and disability.

Patients with anxiety disorders often report symptoms of depression, and vice versa. Having comorbid depression and anxiety is related to a higher symptom severity, lower response to treatment, and poorer prognosis than in patients with only one disorder, and it is even a predictor of suicide. In adults, three of the most frequently co-occurring anxiety disorders with major depressive disorder (MDD) are social phobia or social anxiety disorder (SAD), generalised anxiety disorder (GAD), and panic disorder (PD).

Neuroimaging of depression and anxiety

Exactly because these disorders co-occur so frequently, there is an ongoing debate about whether they share the same aetiology or are, concurring with the current classification system, distinct disorders. Neuroimaging offers a non-invasive way to obtain a better insight into what underlies depression and anxiety. Anatomical characteristics of the brain can be mapped with structural magnetic resonance imaging (MRI), whereas functional MRI (fMRI) measures activity of and connectivity between brain regions. One way of applying fMRI is by asking the individual in the scanner to perform a certain task, such as pressing a button or looking at pictures, in order to see what brain areas are activated during that task. Another possibility lies with so-called resting-state fMRI, where the person in the scanner does not have to do any kind of task, but simply must lie as still as possible without falling asleep. It is understood that when various brain areas show the same pattern of activity during this resting-state, they are engaged in the same process and thus defined as functionally connected to one another.

Results from structural and functional neuroimaging studies have shown that certain brain areas show differences in structure and function in individuals with depression and anxiety when compared to healthy

individuals. In depression, brain regions that are consistently reported include the anterior cingulate cortex (ACC), hippocampus, amygdala, thalamus, cerebellum, posterior cingulate cortex (PCC), and temporal and parietal cortical areas. When looking at anxiety disorders, in PD, studies point to the involvement of the hippocampus, amygdala and the brain stem, but also highlight roles for the ACC and PCC as well as other cortical areas such as the insula and the prefrontal cortex (PFC). In SAD, frequent mention is made of the amygdala and frontal cortical areas, as well as the precuneus. GAD appears to be understudied in comparison to other anxiety disorders, but the few studies that are available show involvement of the amygdala and the PFC.

Neurobiological models on depression and anxiety

Over the past decades, there has been an accumulating interest in the underlying neurobiology of depression and anxiety, which has led to the development of several theories.

Helen Mayberg created a neurobiological model on depression in 1997, in which she suggested that an array of brain regions and, more specifically, maladaptive functional interactions of cortico-limbic brain regions are involved in depression. Since such interactions are critical for the normal regulation of mood and associated processes, dysfunctional interactions are proposed to underlie depressive symptomatology. The three elements that form the basis of her model include a dorsal, ventral and rostral component.

Similarly, Mary Phillips and colleagues proposed their functional neuroanatomical model of emotion perception in 2003. Building upon the notion that two systems, a ventral and dorsal system, are responsible for emotion perception, as well as the production and regulation an affective state, their model relates distinct patterns of structural and functional abnormalities in these systems to specific symptoms of psychiatric disorders including MDD.

In his influential original neuroanatomical hypothesis on PD from 1989 and its revision in 2001, Jack Gorman proposed that panic originates from an abnormally sensitive fear network including the amygdala, thalamus, PFC, and insula, as well as amygdalar projections to the brainstem and hypothalamus.

It should be noted that no neurobiological models on SAD and

GAD have been proposed. This underlines the need for further studies and a better understanding of these anxiety disorders. The existing neurobiological models of depression and anxiety have been built around dysfunctional mechanisms that rely upon the proper functioning of and communication between certain brain areas. Since the development of those models, neuroimaging techniques have been much improved and advanced.

Aim of this thesis

The studies presented in this thesis aimed to address gaps in the current limited knowledge of the neurobiology underlying depression and anxiety in adults and adolescents, by employing novel imaging approaches. Adult participants were derived from the Netherlands Study of Depression and Anxiety (NESDA), and adolescent participants from the Emotional Pathways' Imaging Study in Clinical Adolescents (EPISCA). Very little is known about the network interactions between brain regions in depression and anxiety, despite the importance of such connectivity for an understanding of these disorders. The objective of this thesis was to investigate whether novel imaging approaches such as resting-state fMRI would confirm results from previous studies using other neuroimaging modalities, or uncover neural pathways implicated in depression and anxiety that have not been linked to these disorders before.

The unique resting-state characteristics of PD were examined in Chapter 2. We demonstrated altered resting-state functional connectivity (RSFC) in the limbic and salience networks in a sample of 11 PD patients without any other psychiatric comorbidity, compared to 11 healthy control subjects. For the limbic network, patients showed increased RSFC between the right amygdala and the bilateral precuneus and lateral occipital cortex. This effect may be linked to typical panic symptoms such as derealisation and depersonalisation, which refer to sensations of being detached from reality or oneself. However, as we did not find a relationship between this increase in RSFC and the severity of symptoms, these speculations could not be confirmed. For the salience network, PD patients had decreased RSFC between the left dorsal ACC (dACC), and the bilateral frontal pole and superior/medial frontal gyrus compared to healthy controls. In contrast, PD patients showed an increased RSFC with the bilateral precentral and

postcentral gyrus, right supplementary motor cortex, and right ACC. Furthermore, an increased RSFC between the right dACC and the right superior parietal lobule, lateral occipital cortex, angular gyrus, and central opercular cortex in PD patients was found. These findings could be related to panic symptoms such as a heightened awareness of bodily sensations, or disturbances in self-awareness that could be reflected in symptoms such as the feeling that one is going crazy or loses control.

We investigated RSFC in medication-naïve SAD patients without psychiatric comorbidity in Chapter 3. For the limbic network, SAD patients showed increased negative right amygdala RSFC with the left middle temporal gyrus, supramarginal gyrus, and lateral occipital cortex compared to healthy controls. These areas have been linked to the perception of faces, and an abnormal RSFC could be interpreted as though SAD patients have an enhanced sensitivity for facial expressions of other people. For the salience network, increased positive connectivity was reported between the bilateral dACC seeds and the left precuneus and lateral occipital cortex in SAD patients. This could underlie a disturbed self-awareness and biased information gathering in SAD patients.

Chapter 4 discussed the shared and unique RSFC characteristics of substantial groups of patients with only MDD, patients with only anxiety, and patients with comorbid depression and anxiety, compared to healthy control subjects. Using the data-driven independent component analysis (in combination with dual regression) approach, differences were found between the comorbid group and healthy controls in the limbic network, where comorbid depressed and anxious patients showed increased connectivity in a cluster containing the bilateral precuneus, intracalcarine cortex, lingual gyrus, and posterior cingulate, as well as a cluster including the right precentral gyrus, inferior frontal gyrus, and middle frontal gyrus. It was suggested that the deviant RSFC pattern found in the current study is specific to comorbid depression and anxiety, only becoming evident when patients have both disorders and not in patients with a single diagnosis of either.

Our next RS study was conducted in a sample of treatment-naïve adolescents with a clinical diagnosis of depression and matched healthy controls. Chapter 5 describes the RSFC differences we found between these groups. For the limbic network, depressed adolescents showed increased RSFC between the left amygdala and the right middle frontal gyrus, inferior

frontal gyrus, precentral gyrus, and postcentral gyrus. These findings could be related to a disturbed cognitive control over emotion processing in adolescent depression. There was also a reduced RSFC in the clinical group between the right amygdala and the left frontal pole, right ACC, paracingulate gyrus, and superior frontal gyrus, as well as with the left angular gyrus, lateral occipital cortex, and supramarginal gyrus. Altered connectivity between the amygdala and the ACC is typical for depression, and could be associated with abnormalities in emotion regulation. For the salience network, decreased RSFC was found in the clinical group between the bilateral dACC and the right middle frontal gyrus, frontal pole, and inferior frontal gyrus. Altered connectivity between the dACC and prefrontal areas may be associated with a bias towards negative emotional stimuli and disrupted assessment of affective stimuli.

In addition to RSFC study that was discussed in the previous chapter, grey matter volume in clinically depressed adolescents was studied in Chapter 6. A region-of-interest VBM (voxel-based morphometry) revealed a smaller ACC volume by 14.4% in the patient group compared to matched healthy controls. This is consistent with literature on adult depression. The ACC has been linked to higher cognitive functions, and problems with inhibiting the processing of negative material are common symptoms of depression. Abnormalities in grey matter volume of the ACC could be related to such problems. The emergence of psychiatric illnesses in adolescence has been linked to abnormalities in brain maturation in combination with psychosocial, biological environmental factors. Our results may be interpreted as a result of abnormal maturational processes.

Panic disorder

According to Gorman's revised version of his original neuroanatomical hypothesis of PD, panic originates from an abnormally sensitive fear network including the insula, PFC, thalamus, amygdala, and projections from the amygdala to the brainstem and hypothalamus. Within the context of a limbic network, we reported abnormalities between the amygdala and the precuneus and lateral occipital cortex. Whereas the amygdala plays a central role in Gorman's theory, the precuneus and lateral occipital cortex are not considered; the hypothesis does not include any posterior regions.

The differences we reported for the salience network are also new to models on PD. The left and right dACC showed altered RSFC with

frontal and occipito-parietal areas that are involved in the processing of somatosensory information, attentional control, and self-awareness. With the salience network serving as an assessor of relevance of internal and external stimuli, an exaggerated significance attributed to such stimuli is much in line with the typical symptoms of PD.

Our finding that a more widespread set of brain regions is involved in PD than what is currently described in models, is consistent with other contemporary neuroimaging studies. Based on the currently available body of literature on PD, potential amendments of Gorman's hypothesis were suggested. The emphasis on the amygdala might be tempered, since findings on its involvement in PD are not univocal. Simultaneously, the role of several cortical areas such as the insula and ACC seems to be underestimated in the hypothesis. However, it is evident that much more research is needed before a model can be proposed that is based on conclusive findings.

Social anxiety disorder

The need for further studies in SAD is widely recognised, and was reflected in the number of resting-state fMRI studies that appeared around the time of our own publication. Our results are largely consistent with such studies also reporting altered amygdala and ACC functional connectivity in SAD patients, as well as other cortical regions. The particular importance of the connectivity between the amygdala and frontal cortical regions is not just emphasised in functional but also in structural connectivity studies. Contemporary studies recognise the role of multiple brain regions in emotion, which is highlighted in the use of neuroimaging modalities that point to the involvement of neural networks rather than singular brain regions in SAD.

It is evident that brain areas involved in facial perception and facial processing are heavily involved in SAD. The sensitivity for others' approval and fear of negative judgement is reflected in cognitive theories on SAD, and we speculate that the idea of how one is perceived by other people relies to a great extent on information derived from their facial expressions. Unfortunately the recent growth in number of neuroimaging research, particularly connectivity studies, has not yet resulted in a neuroanatomical model of SAD. However, it may be an indicator that such a model may be

developed in the near future.

Generalised anxiety disorder

Whereas the unique contributions of PD and SAD were discussed in Chapters 2 and 3 respectively, GAD was not separately addressed in this thesis. The Netherlands Study of Depression and Anxiety (NESDA) sample, from which the participants were drawn for Chapters 2, 3, and 4, did not include participants with GAD alone, preventing us from addressing this explicitly in our research. We have therefore not been able to contribute to the literature on GAD alone.

Depression

Depression has attracted much attention over the past decades with a growing amount of neuroimaging research. Providing support for Mayberg's and Phillips' theories, many studies have investigated the roles of separate brain regions in anatomical and functional studies, with findings consistently highlighting abnormalities of the hypothalamus, pituitary, hippocampus, amygdala, and prefrontal regions as the ACC, dorsolateral and orbitofrontal cortex. Particularly the ACC has received much attention, and our finding of reduced ACC grey matter volume in clinically depressed adolescents confirms findings from the vast majority of structural studies in depressed subjects reporting ACC volume reductions.

Our resting-state study in the same adolescent sample also showed abnormalities of the ACC in depressed subjects. Confirming previous literature as well as Mayberg's model and Phillips' theory on emotion perception, we reported decreased resting-state functional connectivity between the amygdala and the pregenual ACC. This provides further support for disturbances in the ventral system in which such deviations are proposed to underlie depressive symptomatology by both models. It is plausible that frontocingulate dysfunction contributes to key cognitive and affective abnormalities in depression, such as emotion dysregulation and rumination: a tendency to engage in and enhance negative information.

Comorbid depression and anxiety

With the topic of the shared or separate etiology of depression and anxiety disorders still hotly debated, the available literature is not yet able to provide a definitive answer. The results from Chapter 4 of this thesis, reporting

altered RSFC in a limbic network in comorbid depressed and anxious adults compared to healthy controls, but no differences in depression or anxiety alone, suggested that the effect is specific to comorbid depression and anxiety, only becoming evident when patients present with both disorders and not in patients with a single diagnosis of either.

Considerations

The longitudinal and multi-centre design of NESDA enables the monitoring of a large, well-characterised sample presenting with an extensive variety of symptoms over a long period. Despite these obvious advantages, there are also some limitations. Patients for the NESDA sample were recruited through general practitioners, primary care practices, and outpatient clinics of mental health organisations. It is therefore possible that the most severely affected patients were not included. It is also likely that patients with the severest symptomatology were not motivated to partake in the extensive assessments, with an additional two-hour MRI scanning session and interview for the neuroimaging study. A consideration regarding the scanning sequence lies with the position of the resting-state fMRI run at the end of the protocol. It is possible that the preceding task-related functional MRI sessions have influenced the resting-state scan.

This was not an issue in the EPISCA study, with the resting-state scan positioned at the beginning of the scanning protocol. However, one consideration that should be made regarding EPISCA is the relatively small sample size.

A big advantage of resting-state fMRI is that it offers an unbiased approach to investigate functional connectivity of the brain. This is of especially great importance in clinical groups, where persons suffering from, for example, neurodegenerative or (neuro)psychiatric disorders are less capable or even incapable of dealing with the cognitive demand that functional tasks often require. One of the difficulties with resting-state fMRI is that in contrast to task fMRI, it is more complicated to interpret, because functional connections between brain regions are correlations, and do not imply causality or directionality. These correlations are also more susceptible to being influenced by confounders, such as physiological noise caused by breathing and heart rate variability. Fortunately, technical advances

are continuously improving the ways to minimise or even eliminate the influence of such artefacts.

Concluding remarks

Structural and functional magnetic resonance imaging have provided important insights into the involvement of candidate brain regions in depression and anxiety. It is unlikely that simple increases and decreases in brain activity alone are sufficient to explain the complex diversity of symptoms that define depression and anxiety. Combined efforts using various research modalities (for example: MRI, (epi)genetics, neuroendocrinology, and environmental studies) could significantly improve knowledge about the neurobiology and pathophysiology of depression and anxiety disorders. Performing such complementing multimodal studies would be a promising next phase of neurobiological research on affective disorders.

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Curriculum Vitae

Justine Pannekoek werd geboren op 21 juni 1983 te Rotterdam. Zij kreeg naast de officiële naam Justine tevens de roepnaam Nienke en is altijd zo genoemd. In 1995 ging zij naar het Stedelijk Gymnasium Leiden waar zij in 2001 haar diploma behaalde. Hierna volgde zij de opleiding Psychologie aan de Universiteit Leiden met als specialisatie de master Klinische Neuropsychologie, waarvoor zij in het Leids Universitair Medisch Centrum onderzoek deed naar cognitieve achteruitgang en prikkelbaarheid bij patiënten met de ziekte van Huntington. Na het behalen van haar bul in 2007 verlegde zij de focus van geestelijk welbevinden naar lichamelijke gezondheid en was twee jaar werkzaam in de sportbranche. In december 2009 begon Nienke als promovenda aan de afdeling psychiatrie van het Leids Universitair Medisch Centrum. Zij onderzocht hersenafwijkingen bij volwassenen en jongeren met depressie en angststoornissen met behulp van magnetic resonance imaging technieken. Tijdens haar onderzoek bezocht zij tweemaal- een maand in 2013 en een half jaar in 2014- de groep van Professor Dan Stein aan de University of Cape Town in het kader van een grootschalig internationaal neuroimaging project naar sociale angststoornis. Per februari 2015 is Nienke werkzaam als postdoctoraal onderzoeker aan Imperial College London, waar zij de invloed van spijsverteringshormonen op verslavingsproblematiek onderzoekt.

Justine Pannekoek was born on 21 June 1983 in Rotterdam, The Netherlands. She was also given the middle name Nienke, which is her preferred name. After completing the highest level of secondary education in 2001, she studied Psychology at Leiden University and completed her Master's in Clinical Neuropsychology in 2007. She temporarily shifted her focus from mental wellbeing to physical health and worked in sports for two years. Nienke started her PhD at the department of psychiatry of the Leiden University Medical Centre in December 2009. During her PhD, she had the opportunity to visit the University of Cape Town twice, one month in 2013 and six months in 2014, to work on an international multi-centre neuroimaging project on social anxiety disorder. Nienke works as a postdoctoral researcher at Imperial College London from February 2015, where she investigates the role of appetitive gut hormones on addiction.