Untangling the adolescent internalizing brain: investigations on brain networks in youth with anxious and depressive problems

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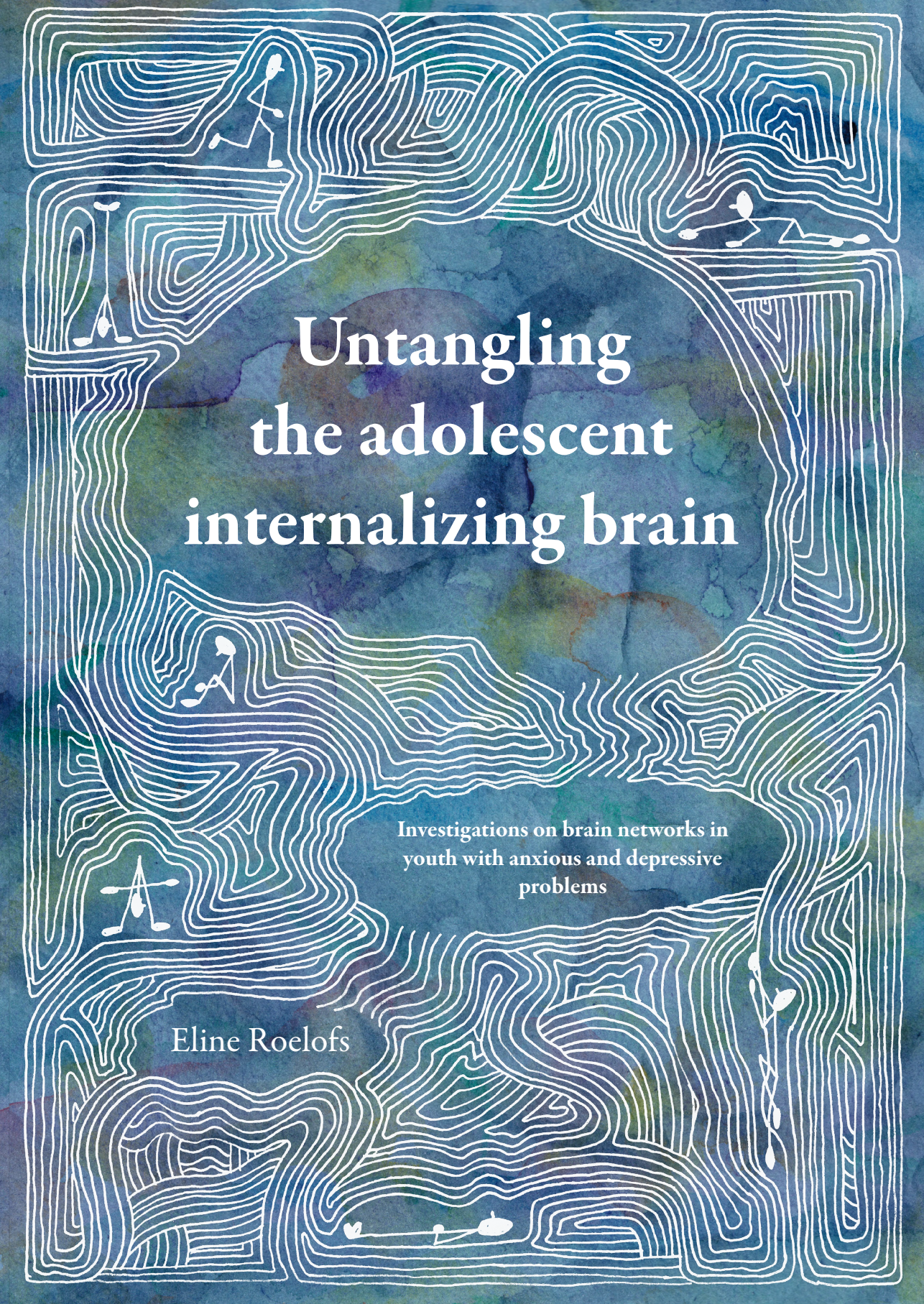
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ISBN: **978-94-6537-114-6**
Cover design: Merel Touw | www.mevrouwtouw.nl
Lay-out: Maya Schutte | www.mayaschutte.com
Printing: Ridderprint | www.ridderprint.nl

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Investigations on brain networks in youth with anxious and depressive problems

Proefschrift

ter verkrijging van

de graad van doctor aan de Universiteit Leiden,

op gezag van rector magnificus prof. dr. S. de Rijcke,

volgens besluit van het college voor promoties

te verdedigen op woensdag 11 maart 2026

klokke 11.30 uur

door

Eline F. Roelofs

geboren te 's-Hertogenbosch

in 1992

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General introduction

Introduction

Internalizing disorders

Internalizing disorders are psychological conditions characterized by inward-directed symptoms, including anxiety, low mood and social withdrawal [1]. These symptoms become clinically significant when they cause distress or impair daily functioning. The Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5), provides criteria for diagnosis. Throughout this thesis, the term *internalizing disorders* refers explicitly to anxiety disorders and depressive disorders as defined by the DSM-5 [2].

Both anxiety and depressive disorders constitute umbrella terms encompassing multiple distinct conditions. Specifically, in this thesis, depressive disorders include major depressive disorder and persistent depressive disorder. Anxiety disorders encompass separation anxiety, specific phobia, social anxiety disorder, panic disorder, agoraphobia and generalized anxiety disorder. While brief descriptions of these conditions are provided in Box 1.1, readers are directed to the DSM-5 for comprehensive diagnostic criteria [2].

Box 1.1 Internalizing disorders

***Depressive disorders** are primarily characterized by persistent low mood and anhedonia. **Major depressive disorder** requires the presence of depressed mood or loss of interest for at least two weeks, accompanied by at least four additional symptoms: significant weight changes, sleep disturbances, psychomotor changes, fatigue, feelings of worthlessness or guilt, cognitive difficulties (concentration, decision-making) and recurrent thoughts of death or suicidal ideation. **Persistent depressive disorder** (formerly dysthymia) involves chronic low mood, present more days than not for at least two years in adults (one year in adolescents) with at least two associated symptoms from the major depressive disorder criteria, excluding suicidal ideation.*

***Anxiety disorders** share the common feature of intense, disproportionate fear that causes significant distress or avoidance behaviors, typically persisting for six months or longer. Each disorder represents fear of specific stimuli or situations. First, **separation anxiety** is defined by an excessive fear or anxiety to be separated from attachment figures, inappropriate to the developmental stage. Specific phobia encompasses an extreme fear of a specific object or situation, such as heights, animals or seeing blood. Patients with **social anxiety disorder** intensely fear that they are scrutinized and evaluated by others. **Panic disorder** involves recurrent panic attacks coupled with persistent concern about future attacks or their consequences. **Agoraphobia** manifests as fear of situations where escape might be difficult, including public transportation, open or enclosed spaces, crowds or being outside alone. Lastly, patients with **generalized anxiety disorder** experience excessive fear and worry about several life domains, such as work performance or interpersonal relationships.*

Most internalizing disorders have a first onset in late childhood or early adolescence and are highly prevalent in young people. Annual prevalence of internalizing disorders in young people under the age of 20 years ranges from 2.5% to 8.5% in the Netherlands. In a wider scope, the annual prevalence ranges from 5.8% to 6.2% in high income countries, including those in Western Europe and North-America [3]. In general, adolescent patients with an internalizing disorder rarely experience only depressive or anxiety symptoms. Rather, there is a substantial symptom overlap within patients, leading to one or more comorbid DSM-5 diagnoses [4]. Etiological models suggest that interactions between

environmental, genetic, biological, developmental and temperamental factors could lead to internalizing disorders [5-7]. Previous studies have reported shared risk factors for depression and anxiety disorders [4, 8]. These findings suggest that internalizing symptoms might be seen more as a spectrum, rather than manifestations of single, unique disorders.

This spectrumlike comorbidity pattern is particularly concerning given its association with more severe clinical presentations and poorer short- and long-term outcomes. Depressed adolescents diagnosed with a comorbid anxiety disorder often display more severe symptoms, increased somatic concerns, poorer response to treatment and increased risk of suicidal behaviors when compared to depressed adolescent patients without comorbid psychopathology [4]. Furthermore, internalizing disorders in adolescence are linked to delayed or changed development in social, academic or personal fields [4]. Lower social performance has been reported in adolescents with anxiety disorders, and they experience more negative interpersonal relationships, loneliness and bullying [9]. Moreover, increased school avoidance and poorer academic performance has been found in adolescents with anxiety disorders and adolescents with depressive disorders [10, 11]. In addition, internalizing disorders in adolescence might have lifelong consequences, as longitudinal studies have reported negative associations between internalizing disorders in adolescence and functioning in adulthood. For example, adolescent anxiety and depression is linked to poorer adult physical and mental health. They are also associated with increased risky and criminal behavior, lower educational and financial attainment, including increased high school dropout, loss of jobs and impoverishment and lower quality of social relationships [10, 12-14].

Given these significant consequences, understanding the developmental context of adolescent internalizing disorders is crucial to increase our understanding of the underlying mechanisms and to improve therapeutic approaches. Therapy usually consists of psychotherapy such as cognitive behavioral therapy (CBT) with additional pharmacological therapy if needed [15, 16]. In clinical practice, treatment of adolescent internalizing disorders is highly personalized, as cognitive and emotional maturity can vary substantially among adolescents of the same age [16, 17]. In addition, most adolescents still live at home and aim to complete their education, which means their family and school situations have to be taken into account. This underscores the need for developmentally informed, context-sensitive treatment and research on adolescents with internalizing disorders.

Neurobiological development in adolescence

As described above, internalizing disorders in young people are prevalent and can have a detrimental influence on several domains in adolescent development and functioning, both during adolescence and in later life. Numerous complex interactions between several factors, among others neurobiological factors, contribute to the etiology of these disorders. To understand which neurobiological factors might contribute to adolescent internalizing disorders, I first need to address neurobiological development in healthy adolescents. In general, adolescence is a period of large changes, marked by physical, psychological and social development [18-20]. Adolescence is traditionally defined as a transitional period between 10 – 19 years old [21], although some argue that a definition of 10 – 24 years old resembles adolescent

growth more closely [22]. For the purpose of this thesis and in line with previous work of our research group, adolescence is defined as the age period between 10 and 21 years old [23].

Adolescence brings positive changes, including enhanced executive functioning that enables better control of thoughts and actions. Adolescents can also more effectively link their behaviors to long-term goals. On the other hand, adolescents tend to become more self-conscious, sensitive to opinions and evaluation of peers and prone to risk-taking behaviors [24-26]. Due to all these changes, adolescence can be seen as a period of adjustment and vulnerability [27]. Normal adolescent development requires coordinating emotions and behavior within social and academic environments. Psychopathology may emerge when adolescents struggle to balance their evolving emotional responses and behaviors while simultaneously meeting social expectations (from peers) and performance expectations (in educational settings) [27, 28].

On a neural level, studies on development of the adolescent brain have shown that widespread structural and functional changes are rapidly taking place [29-31]. Importantly, these neurobiological changes are nonlinear and wide individual variation in patterns of brain development have been found [32]. Moreover, intricate structural and functional connections within and between brain regions are present, although most research has been conducted on individual regions. For more information on neuroimaging techniques used to study these neurobiological changes, I refer the reader to Box 1.2. Regarding changes in brain function, investigations of the brain at rest have reported linear and non-linear changes in functional connectivity within and between resting-state networks. For example, increased functional connectivity was found in early adolescence within networks involved in cognitive control, while later adolescence was associated with decreased functional connectivity in networks involved in association and attention, possibly reflecting increased functional specialization [30]. When considering structural changes in white matter tracts throughout adolescence, nonlinear development of white matter of the brain has been reported. The general consensus is that the organization of white matter microstructure is fine-tuned throughout adolescence and young adulthood, taking into account that the timing and speed differs for each white matter tract [29]. Moreover, differences in functional and structural brain maturation across regions are thought to contribute to imbalances in brain function. These imbalances are linked to several typical adolescent behaviors, such as heightened emotionality, increased risk taking, independence seeking and increased immediate reward sensitivity [24, 31].

Box 1.2 Neuroimaging techniques

Brain connectivity refers to how brain regions communicate with each other and how information is transmitted between them. Several methods are available to examine this, using variations of Magnetic Resonance Imaging (MRI). In this thesis, I investigate structural connectivity and functional connectivity. ***Structural connectivity*** *is used to describe white matter tracts within the brain, connecting gray matter brain areas, while* ***functional connectivity*** *is used to describe brain function of the brain based on correlations between activational patterns.*

White matter microstructure

White matter forms long-range anatomical connections between gray matter regions throughout the brain. These connections are organized into distinct tracts that continue developing through adolescence and into adulthood. Some tracts continue developing well into the third decade of life. White matter can be investigated using ***Diffusion Tensor Imaging (DTI)****, which uses tensors to model the direction and amount of diffusivity of water molecules across the brain. Water molecules inside white matter tracts are more organized than those in gray matter and preferentially diffuse along the same direction as the actual white matter fibers, thus providing insight into the organization of white matter microstructure. One of the most commonly used characteristics of white matter microstructure is fractional anisotropy (FA) [33]. FA quantifies the directional preference of water diffusion, where higher FA values indicate that water molecules preferentially move in one direction rather than randomly, suggesting more organized white matter microstructure [33, 34]. This parameter thus provides valuable information about the white matter microstructure and can be investigated in several ways. One of the most widely used methods to analyze potential differences in white matter microstructure is tract-based spatial statistics (TBSS) [35], which we apply in several studies in this thesis (****Chapters 2, 4 and 5****). TBSS is a voxelwise method to investigate changes of white matter microstructure within and between participants or groups [35].*

Resting-state functional connectivity

Brain connectivity can also be investigated through functional neuroimaging approaches that measure neural activity patterns. The blood-oxygen level dependent (BOLD) signal provides an indirect measure of neuronal activity by detecting changes in local oxygenated blood flow. This signal increases slowly over several seconds following neural activation. ***Resting-state functional MRI (rs-fMRI)*** *captures spontaneous BOLD signal fluctuations in awake participants who are not performing any specific task, thereby enabling the study of functional intrinsic brain network organization. Functional connectivity is defined as the temporal correlation between BOLD signals from different brain regions. Brain regions that exhibit similar patterns of signal fluctuation over time are considered functionally connected. A resting-state network thus comprises a collection of brain regions that demonstrate correlated BOLD signal dynamics during rest, reflecting resting-state functional connectivity (RSFC). Multiple networks have been consistently identified and characterized across studies in healthy participants. Building on this foundation, researchers have applied functional connectivity approaches to examine how brain network alterations may contribute to the development and maintenance of internalizing disorders. We will examine RSFC in* ***Chapter 3****.*

Of particular importance in adolescent neurodevelopment is the characteristic imbalance between brain regions involved in impulse control on the one hand and emotionality on the other hand, as revealed by several studies on development of brain function and structure [24, 31]. This imbalance reflects different maturation timing across brain regions and connections between these regions, most importantly regions in the corticolimbic network (Figure 1). This network consists of limbic structures, such as the amygdala, and prefrontal regions, such as the medial, dorsolateral and ventrolateral prefrontal cortex (PFC) [36]. Together, these regions orchestrate emotion processing and regulation. While limbic regions are primarily involved in affective (emotional) processing and mature earlier in adolescence, prefrontal areas contribute to cognitive control and develop later [36]. Generally, successful emotion

regulation is thought to take place when prefrontal regions take control over limbic structures [37]. The neural imbalance in brain maturation that occurs in adolescence tends to be in favor of limbic structures over prefrontal regions. Thus, this developmental mismatch may contribute to heightened adolescent emotionality and risk-taking behavior.

In sum, these paragraphs highlight that changes occur in functional and structural connectivity of brain networks in all adolescents. Notably, adolescence is also a period when internalizing disorders often begin to emerge. This raises fascinating questions about what might be happening in the brains of adolescents affected by such disorders. It becomes increasingly insightful to explore structural and functional connectivity as neuroscience shifts toward a connectivity-based approach, focusing on the interactions between brain regions rather than examining them in isolation [38].

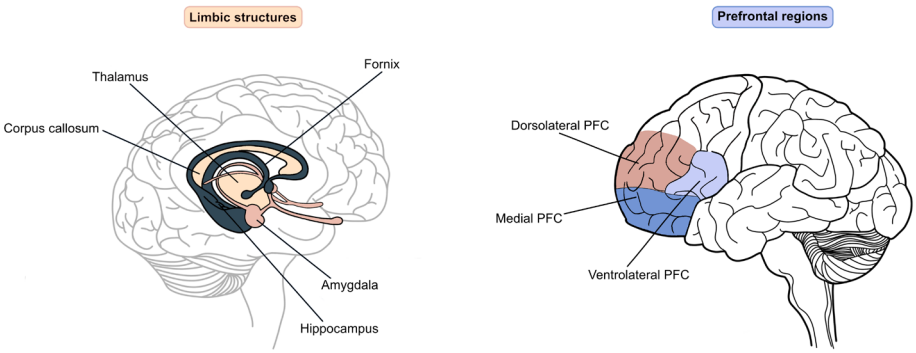

Figure 1 The corticolimbic network.

The corticolimbic network in internalizing disorders

While not the exclusive focus of this thesis, it is important to acknowledge the prominent role of the corticolimbic network in internalizing disorders, as it represents one of the most extensively studied brain networks in this context.

As described above, the corticolimbic network is involved in emotion processing and regulation. It comprises limbic structures and prefrontal regions (Figure 1). Impaired emotion regulation is considered a significant factor in internalizing psychopathology and research indicates that individuals with anxiety and depression often exhibit alterations within regions of the corticolimbic network [6, 39]. Specifically, limbic structures, which are primarily responsible for emotion processing, appear to exert excessive influence over prefrontal regions in depressed and anxious patients [40, 41]. It is therefore generally thought that alterations in corticolimbic connectivity might contribute to internalizing psychopathology.

Within this network, a particular region should be highlighted: the amygdala, an almond-shaped brain region situated deep within the temporal lobe, is a vital part of the limbic system and the corticolimbic

network [42, 43]. Functionally, it is principally involved in emotion processing, emotional memory formation, learning, motivation, social cognition, and stress responses [44]. Alterations in both the structure and function of the amygdala have been extensively reported in patients with internalizing disorders (for example, see meta-analyses by Zugman *et al.* [45], Tang *et al.* [46], Hamilton *et al.* [47]).

Having established the general importance of brain connectivity in internalizing disorders, in particular the corticolimbic network and the amygdala, we turn our attention to how these alterations manifest in adolescents. The following sections will delve into existing research on resting-state functional connectivity (RSFC) and white matter microstructure observed in adolescents with internalizing disorders. I will highlight key findings and identify gaps in the current literature, specifically concerning comorbid presentations.

Several studies have investigated RSFC in adolescent depression and anxiety disorders, mostly focusing on RSFC of the amygdala to several regions of the brain. Meta-analyses of cross-sectional studies report changes between regions of the corticolimbic network in adolescent samples, such as hypoconnectivity of the amygdala to frontal regions [45, 46], although these findings could not be replicated in a another meta-analysis [48]. Thus, we need to further investigate amgydala connectivity to address this gap.

Other brain networks implicated in internalizing disorders

Recent models also suggest involvement of other resting-state networks in patients with internalizing disorders [49]. Cross-sectional alterations have been found within and between several networks, including the affective (corticolimbic), default mode (DMN), salience and central executive network [46, 48, 50] (Figure 1, Figure 2A). In short, the affective network is involved in emotion processing and regulation [51]; the DMN in ruminative, negative self-referential processes [52]; the salience network in external stimulus detection and processing of emotionally salient information [53]; and the central executive network is involved in emotion regulation and goal-directed response initiation [54]. However, the specific role of these resting-state networks in adolescents with internalizing disorders is yet to be further elucidated.

When considering structural connectivity, previous cross-sectional studies in depressed adolescents reported lower white matter microstructure, measured by fractional anisotropy (FA), in several regions. These regions include the corpus callosum, cingulum, inferior fronto-occipital fasciculi (IFOF) and uncinate fasciculus (UF) (Figure 1, Figure 2B) [55-58]. Regarding adolescents with anxiety disorders, one study investigated white matter microstructure in adolescents with generalized anxiety disorder (GAD) and reported lower FA in, among others, the IFOF, UF and corona radiata [59]. The UF, corpus callosum, IFOF and cingulum are thought to be involved in regulation and communication within and between regions of the corticolimbic network. However, the role of the corona radiata in internalizing disorders is still unclear [60, 61]. Interestingly, these results seem to show overlapping patterns compared with patterns seen in adult depression and anxiety, although studies in the adolescent population are still sparse [62, 63]. However, no studies to date have examined white matter microstructure in adolescents

diagnosed with depressive disorders and comorbid anxiety disorders. Moreover, research focusing exclusively on adolescent depression or anxiety is still limited. Thus, we need to investigate differences in white matter microstructure in adolescents with anxiety and comorbid depression compared to healthy peers to narrow this knowledge gap.

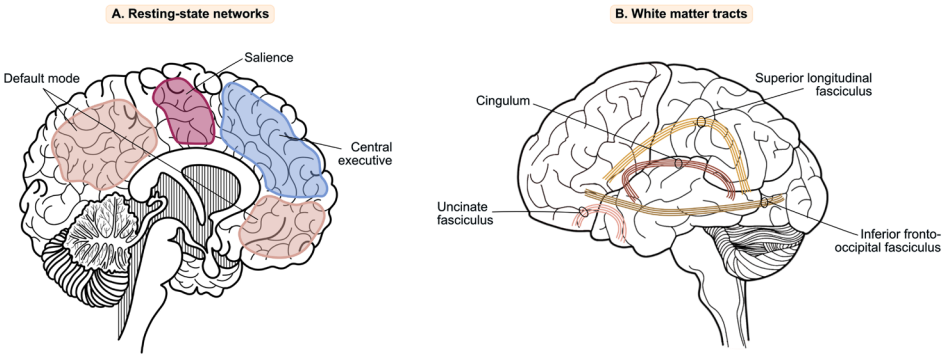

Figure 2 Functional and structural connectivity in adolescent internalizing disorders.

Understanding the neurobiological factors underlying these disorders is essential for improving diagnosis, prevention, and treatment approaches to lower the significant impairments that internalizing disorders can have on adolescent development across social, academic, and emotional domains. Despite this clinical importance, few studies have examined how brain changes unfold over time in adolescents with internalizing disorders. Longitudinal designs allow for the assessment of temporal dynamics in brain development and psychopathology. Furthermore, they can help unentangle the effects of treatment from the natural course of illness, reveal critical neurobiological mechanisms and identify potential targets for early intervention. Previous studies on longitudinal changes in RSFC in adolescent depression have revealed alterations over time in functional connectivity of regions involved in emotion processing, mainly in the amygdala [5, 64-69]. Moreover, associations with changes in symptom severity were reported [65, 69]. Studies on longitudinal changes in RSFC in adolescents with anxiety disorders have not yet been conducted until now. Furthermore, studies investigating longitudinal alterations in white matter microstructure in adolescents with internalizing disorders are lacking. Therefore, to address this gap we need to investigate longitudinal changes in functional and structural connectivity in adolescents with internalizing disorders.

Aims and outline

The etiology of adolescent internalizing disorders involves a complex interplay of multifactorial interactions across biological, psychological, and environmental domains. In this thesis, I aim to shed light on the role of functional and structural connectivity to expand our knowledge of the brain networks underlying anxiety and depression in adolescents.

This thesis is organized into two main parts:

- **Part 1: Changes in brain networks over time in internalizing disorders** – This section focuses on longitudinal alterations in structural and functional connectivity in adolescents with internalizing disorders.

- **Part 2: Structural connectivity in social anxiety disorder** – This section explores changes in structural connectivity in social anxiety disorder (SAD), examining vulnerability factors to develop SAD and structural alterations in the largest cohort to date.

Part 1: Changes in brain networks over time in internalizing disorders

In the sections above, I have discussed neurobiological changes in adolescence and the gaps in current literature. It becomes clear that longitudinal studies on functional and structural connectivity in adolescents with internalizing disorders are lacking, despite their clinical importance. Measuring adolescents multiple times over a longer timeframe is challenging, as research participation has to compete with busy schedules, filled with educational, social and other tasks. In addition, multiple stakeholders, such as parents or guardians and school, have to be taken into account [70].

In the first part of this thesis, we bridge this gap by using a unique longitudinal dataset of adolescents with internalizing disorders and healthy peers. We aim to identify alterations in functional and structural connectivity that contribute to internalizing disorders in adolescence, providing insights that could eventually lead to improved intervention and prevention strategies. In **Chapter 2**, we use this longitudinal dataset to compare development of white matter microstructure between adolescents with internalizing disorders and their healthy peers. In **Chapter 3**, we will investigate longitudinal changes in RSFC to explore differences between adolescents with internalizing disorders and healthy peers.

Part 2: Structural connectivity in social anxiety disorder

In the second part of this thesis, we zoom in on structural connectivity in SAD. SAD is one of the most prevalent psychiatric disorders with a lifetime prevalence rate between 4–13 % and has a typical onset in childhood or early adolescence [71, 72]. In addition, SAD often has a high psychiatric comorbidity, mostly with other internalizing disorders, and a chronic course [7, 73]. As described in box 1.1 of this introduction, patients with SAD are characterized by a persistent and intense fear of situations involving potential exposure and scrutiny from (unfamiliar) people [74]. Hence, it is not surprising that quality of life and everyday functioning are clearly impaired by SAD [75, 76]. Moreover, adolescents with SAD

and their parents reported increasing difficulties in overall, social and academic functioning as they grew older. In addition, other work revealed different cognitive functioning in adolescents with SAD compared to healthy controls [77, 78].

Neurobiological models of SAD suggest both hyperactivity in regions involved in fear processing (amygdala, insula, anterior cingulate cortex) and impaired emotion regulation. This might be due to disrupted communication between regulatory (ventral PFC) and evaluative (dorsal PFC) regions [7, 79, 80]. Several studies have investigated white matter microstructure in adults with SAD, as reviewed by Parsaei and colleagues [81]. Among others, decreased FA in patients with SAD has been found in regions involved in emotion regulation and processing like the UF. These findings underscore the evidence for altered brain connectivity in SAD. However, studies are limited and results are inconsistent. In addition, most of these studies had low sample sizes (ranging from n = 36 to n = 88 for the total sample) [82-86]. To address this gap, we need studies with a large sample size which use pre-defined methods that can be replicated.

SAD often runs in families and the overall vulnerability to developing SAD is thought to be based on complex interactions between genetic (dis)advantages and liabilities, epigenetic factors and environmental factors [7, 87, 88]. Moreover, a recent genome wide association study (GWAS) analysis confirmed a heritable basis of SAD [89]. To examine the genetic vulnerability to SAD more closely, an endophenotype approach could be used. Endophenotypes are defined as biological or psychological markers of a disorder. They are thought to be in the causal chain between genetic contributions to a disorder and diagnosable symptoms of psychopathology [90, 91] and include, for example, neurobiological changes in brain structure and function. Several neurobiological candidate endophenotypes for SAD have been examined in a study involving families genetically enriched for SAD. In this study, cortical and subcortical grey matter characteristics, increased and prolonged amygdala activation and increased brain activity whilst processing unintentional social norm violations have been revealed as promising SAD endophenotypes [92-96]. However, whether changes in structural connectivity are linked to the genetic vulnerability for SAD is until now unexplored.

Considering the above, SAD serves as an excellent model among internalizing disorders in adolescence for applying innovative research methods to explore neurobiological factors from novel perspectives. Therefore, the general aim of the second part of this thesis is to deepen our understanding of white matter microstructure in SAD by using two innovative study designs. First, we will examine whether alterations in white matter microstructure are candidate endophenotypes of SAD in a family study in **Chapter 4**. Next, in **Chapter 5**, we will explore differences in white matter microstructure between patients with SAD and healthy control participants in a mega-analysis of the largest dataset on white matter microstructure in SAD to date.

In **Chapter 6,** I will summarize the results of the studies included in this thesis, offer general reflections and propose directions for future research.

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Part 1

2

Exploring the course of adolescent anxiety and depression: associations with white matter tract microstructure

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Eur Arch Psychiatry Clin Neurosci 2022. Vol 272 Issue 5 Pages 849-858

Abstract

Objective: Cross-sectional Diffusion Tensor Imaging (DTI) studies have reported alterations in white matter (WM) microstructure in adolescents with internalizing psychopathology. Yet, longitudinal studies investigating the course of WM microstructure are lacking. This study explored WM alterations and its relation to clinical symptoms over time in adolescents with internalizing disorders.

Methods: DTI scans were acquired at baseline and after three months in 22 adolescents with clinical depression and comorbid anxiety (INT), and 21 healthy peers (HC) (age: 12 – 18). Tract-based spatial statistics was used for three voxelwise analyses: i) changes in WM microstructure between and within the INT and HC group; ii) associations between changes in symptom severity and changes in WM microstructure within youths with INT; iii) associations between baseline WM parameters with changes in symptom severity within youths with INT.

Results: Data did not reveal changes in WM microstructure between or within groups over three months' time nor associations between changes in WM microstructure and changes in self-reported symptoms (analyses corrected for age, gender and puberty stage). Lower baseline levels of fractional anisotropy (FA) in the right posterior corona radiata (PCR) and right cingulum were associated with a higher decrease of depressive symptoms within the INT group. Post-hoc analysis of additional WM parameters in the significant FA clusters showed that higher levels of baseline mean diffusivity and radial diffusivity in the PCR were associated with a lower decrease in depressive symptoms.

Conclusion: Baseline WM microstructure characteristics were associated with a higher decrease in depressive symptoms over time. These findings increase our understanding of neurobiological mechanisms underlying the course of internalizing disorders in adolescents.

Introduction

Depression is a prevalent mental illness in adolescence and comorbidity with one or more anxiety disorders is high [1, 2]. A substantial proportion of the adolescents diagnosed with depression display more severe symptoms, poorer response to treatment and increased risk of suicidal behaviors. This complex psychopathology is often linked to comorbidity with anxiety disorders [2]. Sadly, recent studies imply that comorbid anxiety disorders are rather the rule than the exception in clinically depressed adolescents as the majority of the adolescent clinical population with depression has one or more comorbid anxiety disorders [2]. Models suggest that complicated interactions between environment, genes, neurobiological characteristics and the timing of developmental stages contribute to the onset and course of internalizing (anxiety and depressive) disorders [3, 4]. Longitudinal studies may shed light on the neurobiological mechanisms involved in the development and course of these impairing disorders and could thus be used to elucidate these interactions. Previous longitudinal studies in at-risk or subthreshold populations have suggested a complex interaction between the course of anxious and depressive symptoms and white matter (WM) neurodevelopmental plasticity, thus underlining the need for longitudinal studies in clinical cohorts [4]. Yet, despite the clear relevance for developing prevention and intervention strategies, only limited longitudinal research into possible neurobiological targets has been conducted in adolescents with internalizing psychopathology (i.e. with clinical anxiety and/or depression).

There are, however, several cross-sectional studies in clinical adolescent anxiety and depression revealing structural and functional abnormalities in neural networks that subserve affective processing, such as abnormalities in the corticolimbic circuitry [3]. Main components of this network include the amygdala, insula, hippocampus, anterior cingulate cortex (ACC) and prefrontal cortex (PFC), which regulate the experience, expression and evaluation of emotions [3]. Alterations in emotion-processing networks have been reported in studies in adolescents with anxiety or depression such as functional and structural changes in amygdalar and ACC networks. For example, impaired amygdala habituation and decreased ACC volume have been reported previously [5, 6]. To our knowledge, few DTI studies in adolescents with clinical depression have been conducted. These studies have reported lower fractional anisotropy (FA), a general indicator of WM tract microstructure, in adolescents with depression compared to healthy peers in WM tracts such as the corpus callosum, cingulum, inferior fronto-occipital fasciculi (IFOF) and uncinate fasciculus (UF) [7-10]. In addition, only one DTI study has been conducted in adolescents diagnosed with an anxiety disorder. Liao *et al.* [11] reported lower FA in adolescents with Generalized Anxiety Disorder (GAD) compared to healthy peers in among others the IFOF, UF and corona radiata. We refer the reader to Supplemental Tables S1a and S1b for an overview of previous literature. The UF, corpus callosum, IFOF and cingulum are thought to be involved in regulation and communication within and between regions of the corticolimbic network [12, 13]. Interestingly, these results seem to show overlapping patterns compared to adult depression and anxiety, although studies in the adolescent population are still few [5, 6]. Considering the above, alterations in functional and

structural regions involved in the corticolimbic network are likely to play a role in adolescent anxiety and depression and could be targets for further investigation.

In addition to these cross-sectional studies, a few longitudinal, non-DTI studies have been conducted in adolescents with internalizing psychopathology. Resting-state functional connectivity (RSFC) studies in adolescent depression have shown alterations over time in RSFC within and between regions involved in emotion processing, such as the amygdala and insula, and cognitive controlling regions such as the ACC and prefrontal cortex. Importantly, baseline and longitudinal changes in RSFC were associated with changes in depression severity [14-16]. Furthermore, structural and functional alterations in the insula and structural changes in lateral and medial prefrontal regions have been associated with future onset and illness course in adolescents at-risk for depression or diagnosed with subthreshold depression [4]. Therefore, baseline characteristics are likely to be associated with the development and course of the disorder. However, longitudinal DTI studies in clinical adolescents with depression and comorbid anxiety are lacking.

The *Emotional Pathways' Imaging Study in Clinical Adolescents* (EPISCA) study is a longitudinal naturalistic study designed to investigate neurobiological mechanisms related to emotion processing and regulation in a clinical cohort of adolescents with stress-related psychopathology. Measurements were taken at baseline, three months and six months. The study consisted of three groups: adolescents with internalizing (depressive and anxiety) disorders, adolescents with trauma disorders and a healthy control group. Results of the neuroimaging measurements at baseline have been previously reported [9, 17-20]. The present work explored WM microstructure in adolescents with depression and anxiety and a control group of healthy peers over a three-month period, in order to gain more insight in longitudinal changes in WM microstructure involved in a clinically representative population of adolescents with internalizing psychopathologies.

In addition, we focused on the within-subject relationship between (baseline) WM microstructure and clinical symptoms over time within the internalizing group. Our main parameter of interest was FA. We used other DTI parameters, being axial diffusivity (AD), mean diffusivity (MD) and radial diffusivity (RD), to gain more insight into characteristics of underlying WM microstructure involved in adolescent anxiety and depression. DTI uses tensors to model diffusivity of water molecules across the brain. These tensors consist of three main eigenvalues (λ_1, λ_2 and λ_3) which in turn can be used to calculate the four most commonly used characteristics of WM microstructure: fractional anisotropy (FA), axial diffusivity (AD), radial diffusivity (RD), and mean diffusivity (MD) [21]. FA provides a relative difference between the largest eigenvalue as compared to the others, reflecting the tendency of water molecules to diffuse in one direction as opposed to all others and could therefore be described as a general indicator for WM microstructure (e.g. myelin thickness, membrane integrity) [21, 22]. AD is defined as the first eigenvalue (l_1) and reflects water diffusion along the principal direction of the fiber, displaying fiber bundle coherence and axonal integrity [23]. RD is defined as the average of the second and third eigenvalue (λ_2 and λ_3) and reflects water diffusion perpendicular to the principal direction of the fiber, thus being more

indicative of the level of myelinization [24]. MD is defined as the average of the three eigenvalues and hence reflects average water diffusion in all directions within a fiber, thus putatively reflective of a degree of myelination [25]. In general, decreased FA is coupled with decreased AD and / or increased RD and MD and vice versa [21, 26].

Based on previous cross-sectional studies of white matter in anxious or depressed adolescents compared to healthy controls and longitudinal studies in at-risk groups [4, 8-11, 27], we expected different FA changes over time in the clinical group compared to healthy peers in WM tracts previously implied in emotion processing, such as the cingulum, UF and corpus callosum. Directionality of these changes will be investigated exploratory as results of previous literature are few and heterogenous.

Methods

Participants

Participants took part in the *Emotional Pathways' Imaging Study in Clinical Adolescents* (EPISCA) study, and were examined three times in a six-month interval between January 2010 to August 2012 [28]. In the present study, only data from two groups of adolescents between 12 and 18 years old were included from the first (baseline) and second visit (three months after baseline), as loss-to-follow-up was too great after six months (loss-to-follow-up after three months: 16%, loss-to-follow-up after six months: 24%).

The first group consisted of adolescents with clinical depression and comorbid anxiety disorders (n = 30; internalizing disorders, hereafter referred to as 'INT'). These participants were recruited in outpatient departments of two child-and-adolescent psychiatric clinics and included in the study before start of care-as-usual (see Supplemental Table S2 for more information about received treatments). They (i) were diagnosed with clinical depression and at least one clinical anxiety disorder as assessed by categorical measures of DSM-IV depressive or anxiety disorders, (ii) were being referred for cognitive behavioral therapy at an out-patient care unit, and (iii) recent change in antidepressant treatment.

The second group consisted of healthy control peers (n = 32, HC), who were recruited through local advertisements. They had (i) no current or past DSM-IV diagnoses of Axis I and/or Axis II disorders, (ii) no clinical scores on validated mood and behavioral questionnaires, (iii) no history of traumatic experiences, and (iv) no current psychotherapeutic and/or psychopharmacological intervention of any kind.

Exclusion criteria for both groups were: (i) a primary DSM-IV diagnosis of attention deficit hyperactivity disorder, oppositional defiant disorder, conduct disorder, pervasive developmental disorders, posttraumatic stress disorder, Tourette's syndrome, obsessive–compulsive disorder, bipolar disorder, and psychotic disorders, (ii) current use of psychotropic medication, (iii) current substance abuse, (iv) a history of neurological disorders or severe head injury, (v) age < 12 or > 21 years, (vi) pregnancy, (vii) left-handedness, (viii) IQ score < 80, as measured by either the Wechsler Intelligence Scale for Children [29]

(WISC) or the Wechsler Adult Intelligence Scale [30] (WAIS), and (ix) general MRI contraindications (e.g. metal implants, claustrophobia).

At the first visit, participants underwent clinical assessment by a child and adolescent psychiatrist. Afterwards, the Anxiety Disorders Interview Schedule [31] (ADIS) was performed to obtain DSM-IV-based classifications of anxiety and depressive disorders. To assess severity of anxious and depressive symptoms, additional self-report questionnaires were completed at each visit, including the Children's Depression Inventory [32] (CDI) and the Revised Child Anxiety and Depression Scale [33] (RCADS). More specifically, total scores on the CDI were used to assess depressive symptoms and total t-scores on the anxiety subscale for the RCADS to assess anxiety symptoms. Clinical scores were defined as ≥16 and ≥70, respectively. Pubertal stage was assessed using the self-report Pubertal Development Scale [34] (PDS), according to the following categories: 1) prepubertal, 2) early pubertal, 3) midpubertal, 4) late pubertal, and 5) postpubertal. HC-participants were excluded when criteria for a (history of) DSM-IV diagnosis or (sub)clinical scores on clinical questionnaires were met.

Ethics

The EPISCA study was approved by the medical ethics committee of Leiden University Medical Center. All participants provided informed consent according to the Declaration of Helsinki; both participants and parents signed the informed consent form. All anatomical scans were reviewed by a radiologist.

Analysis of demographic data and symptom severity

To examine differences in demographic data and within-subject changes of symptoms in the INT group, (paired) t-tests or two-sided Fisher exact tests were used for continuous and dichotomous data respectively. For non-normally distributed data, as defined by a significant Shapiro-Wilk test, the Mann-Whitney U test or Wilcoxon signed-rank test were used. Incidental missing values on the self-report questionnaires were replaced using expectation maximization (see Online Resource Methods). The Bonferroni method was used to correct p-values for multiple comparisons of the symptom-related questionnaires (4 tests, being comparisons on each timepoint of scores on the RCADS and CDI between the INT and HC group, corrected p-value = 0.0125).

MRI data acquisition

DTI data were collected using a Philips 3.0 T Achieva MRI scanner (Philips Medical Systems, The Netherlands) with an eight-channel sensitivity encoding (SENSE) head coil. A single-shot echo–planar imaging (EPI) sequence was used with the following scan parameters: single band, repetition time = 11 000 ms, echo time = 56 ms, flip angle = 90°, b factor = 1000 s/mm^2, voxel dimensions = 2.3 mm isotropic, number of slices = 73, no slice gap. DTI data were acquired along 32 directions, together with a baseline image without diffusion weighting (b = 0). Total scan time was approximately 7 minutes. In addition, a sagittal 3-dimensional gradient-echo T1-weighted image was acquired for registration purposes with the following scan parameters: TR = 9.8 ms; TE = 4.6 ms; flip angle = 8°; 192 x 152 matrix; FOV = 224 x 177 x 168 mm, 140 sagittal slices; no slice gap; 1.16 x 1.16 x 1.20 mm voxels. Prior

to scanning, all participants were introduced to the scanning situation by lying in a dummy scanner and hearing scanner sounds. All participants were scanned within 2 weeks of initial screening and were new to MRI-scanning procedures.

Processing of DTI data

Image pre-processing and analyses were performed using the Oxford Centre for Functional Magnetic Resonance Imaging of the Brain (FMRIB) Software Library (FSL), version 6.0.3 [35]. The Brain Extraction Tool (BET) was used to remove non-brain tissue from the non-diffusion images. Image distortion and motion artefacts induced by eddy currents, inter-volume and intra-volume head motions were corrected and outliers were replaced with Gaussian Process predictions [36]. Image quality was statistically evaluated afterwards [37]. Individual FA images and primary (l_1), secondary (l_2) and tertiary (l_3) eigenvalues were created by fitting a tensor model to the raw diffusion data using FMRIB's Diffusion Toolbox (FDT).

Standard protocols designed to facilitate harmonized image analysis across multiple sites (http://enigma.ini.usc.edu/protocols/dti-protocols/) were used to visually and statistically evaluate individual vector and raw FA images. These protocols are designed for mega- and meta-analyses within working groups of the ENIGMA consortium (Thompson et al., 2014; Thompson et al., 2020). All DTI data in the present study were collected in one MRI scanner.

Afterwards, a study specific template was created and registered to MNI standard space (see Online Resource Methods). Individual maps of diffusivity measures were calculated using the eigenvalues and aligned onto the template, defining axial diffusivity (AD) as l_1, radial diffusivity (RD) as $l_{23} = (l_2 + l_3) / 2$ and mean diffusivity (MD) as $l_{123} = (l_1 + l_2 + l_3) / 3$. Then, using TBSS, all participants' FA and non-FA images were projected onto the template [38].

Subsequently, quality control was performed twofold: we visually inspected the registered images for misalignment onto the skeleton and calculated individual projection distances of the extracted skeletons onto the template to detect outliers. Outliers were defined as individual projection distance to the template exceeding the threshold of 3.8 mm, which could represent bad alignment to the template [39]. All images were well aligned, and no outliers were detected.

Tracts of interest (TOI)

Based on previous literature, three tracts of interest (TOI) were combined in one binary mask, being the UF (bilateral), cingulum (bilateral) and corpus callosum (genu, body and splenium) using the Johns Hopkins University (JHU) ICBM-DTI-81 white-matter labels atlas [40] provided by FSL. Subsequently, this mask was combined with the mean FA skeleton to include only voxels comprised in both the tract and the skeleton. This confined the statistical analysis to voxels from the center of the tract, thereby minimizing anatomic inter-subject variability, deviations in registration and partial volume effects [41].

Statistical analysis of DTI data

First, we examined voxelwise changes in FA over time between patients and controls by conducting a multi-level block permutation analysis to allow permutation inference with repeated measures data, using 5000 permutations and threshold-free cluster enhancement (TFCE) [42, 43]. Three contrasts were investigated: i) change over time within the INT group (T2 > T1 and T1 > T2), ii) change over time within the HC group (T2 > T1 and T1 > T2), and iii) a group x time interaction using an F-test. A Bonferroni correction of 0.05 / 2 = 0.025 was applied to correct the first two contrasts assessing changes within group over time.

Second, we examined voxelwise associations between longitudinal changes in FA and changes in anxiety and depressive symptoms using FSL's randomise with 5000 permutations and TFCE [43]. Changes in symptom severity were calculated for each patient by subtracting baseline scores on the self-report questionnaires from scores after three months (ΔCDI, ΔRCADS), where a negative Δ-score implied a decrease in self-reported symptoms. Similarly, the change in FA (ΔFA) was calculated by subtracting individual voxelwise values of FA on baseline from voxelwise FA values after three months. Then, associations between ΔFA and i) ΔCDI and ii) ΔRCADS were investigated in separate models. For each analysis, gender, age and puberty stage at baseline (centered) were modelled as covariates of no interest and variance smoothing of sigma = 2.1 mm and family-wise error (FWE) correction of $p < 0.05$ were applied.

Third, we investigated within-subject in the INT group whether baseline FA showed an association with changes in anxious (ΔRCADS) and depressive symptoms (ΔCDI) as described above. Again, for each analysis, gender, age and puberty stage at baseline (centered) were modelled as covariates of no interest and variance smoothing of sigma = 2.1 mm and FWE correction of $p < 0.05$ were applied.

In all analyses, we first investigated FA, as this is a general indicator of WM microstructure. Additional parameters (AD, MD and RD) were examined in significant clusters only. Analyses were conducted within the a-priori defined TOIs and in exploratory whole brain analyses to investigate changes outside the TOI. The JHU ICBM-DTI-81 white-matter labels atlas and JHU white-matter tractography atlas were used to locate significant findings [40]. Participants were included in the respective association analyses if the questionnaire (RCADS or CDI) was filled out on baseline and after three months.

Results

Sample

Two participants (n = 1 INT, n = 1 HC) were excluded due to an anomaly on the structural T1-scan and one HC participant was excluded as criteria for clinical psychopathology were met. Six participants (n = 3 INT, n = 3 HC) were excluded due to technical issues and an additional six participants did not follow up after the first visit (n = 4 INT, n = 2 HC). Thus, the final sample for initial analysis consisted of 47 participants (n = 22 INT, n = 25 HC).

After pre-processing, data from n = 43 participants (n = 22 INT, n = 21 HC) were available for further analysis, as for one HC participant BET was unable to adequately extract non-brain tissue images and an additional three HC participants had to be excluded due to excessive head motion (defined as relative head motion with respect to the previous volume > 2.5 mm).

Demographics

Sample characteristics are summarized in Table 1. Participants in the INT group reported significantly higher levels of internalizing symptoms (self-reported anxiety and depressive symptoms) and puberty stage compared to their healthy peers at both time points. They did not, however, differ with respect to gender distribution, age, IQ or days between visits. Within the INT group, participants reported a significant decrease in depressive symptoms ($T = 50.00$, $z = -2.05$, $p = 0.04$; Wilcoxon signed-rank test) but not in anxiety symptoms after three months ($t(20) = 1.33$, $p = 0.20$; paired t-test).

Table 1 Demographic characteristics of participants with and without clinical depression and comorbid anxiety.

	INT (n = 22)	HC (n = 21)	Statistical analysis
Primary diagnoses[a] (n)			
MDD + GAD	14		
MDD + SAD	14		
MDD + specific phobia	9		
MDD + panic disorder	5		
MDD + separation anxiety	9		
Demographics			
Age in years (mean ± SD; range)	15.93 ± 1.45 (13.23–17.99)	15.09 ± 1.80; (12.33-17.80)	t(41) = 1.68, p = 0.10
Male / Female (n)	2 / 20	4 / 17	p = 0.41, OR = 1.08, 95% CI = 0.24-4.76
IQ (mean ± SD)	105.18 ± 8.17	106.67 ± 8.36	t(36) = 0.59, p = 0.56
Puberty stage (median ± SD)	4.00 ± 0.73	4.00 ± 0.73	U = 253.00, z = 2.32, p = 0.03
Days between visits (median ± SD)	100.00 ± 10.72	98.00 ± 12.48	U = 271.00, z = .98, p = 0.33
Ethnicity (% white)	90.00	96.88	p = 0.61, OR = 3.44, 95% CI = 0.34-35.09
Self-report measures (median ± SD)			
CDI on baseline	20.00 ± 8.91	4.00 ± 3.48	U = 419.50, z = 4.59, p < 0.001***
CDI after three months[b]	11.00 ± 9.12	2.00 ± 3.84	U = 371.00, z = 4.22, p < 0.001***
RCADS on baseline	33.00 ± 15.01	9.00 ± 10.63	U = 374.50, z = 3.90, p < 0.001***
RCADS after three months[c]	25.00 ± 15.32	10.00 ± 8.66	U = 359.50, z = 3.90, p < 0.001***
Secondary (externalizing) diagnoses (n)			
ADHD; inattention	1		
ADHD; hyperactive	1		
Oppositional Defiant Disorder	1		
Behavioral Disorder	2		

MDD: Major Depressive Disorder; GAD: Generalized Anxiety Disorder; SAD: Social Anxiety Disorder; ADHD: Attention Deficit Hyperactivity Disorder; CDI: Children's Depression Inventory; RCADS: Revised Child Anxiety and Depression Scale; OR: unadjusted Odds Ratio; CI: Confidence Interval; SD: Standard Deviation. [a] Diagnoses are not mutually exclusive. [b] Two patients did not complete the questionnaire. [c] One patient did not complete the questionnaire. *** Significant at Bonferroni corrected p-value of 0.0125.

DTI analyses

Significant results are summarized in Table 2 and illustrated in Figure 1 and Online Resource Figures S1 and S2. Permutation analyses did not reveal significant differences in FA over time within the INT or HC group or a significant time x group interaction. In addition, whole brain and TOI analyses did not show significant associations between ΔFA and ΔCDI or between ΔFA and ΔRCADS within the INT group.

Whole brain voxelwise analyses within the INT group revealed that baseline FA in the right posterior corona radiata (PCR; TFCE and FWE corrected p = 0.03) and a small peripheral cluster in the cingulum (TFCE and FWE corrected p = 0.04) were positively associated with ΔCDI (Table 2). Additional permutation analyses in the PCR further revealed that baseline RD (TFCE and FWE corrected p < 0.001) and MD (TFCE and FWE corrected p = 0.01) were significantly negatively associated with ΔCDI. AD was not significantly associated with ΔCDI. In other words: lower baseline FA and higher baseline MD and RD were associated with a higher decrease in self-reported depressive symptoms, as defined by a negative ΔCDI score. Data did not reveal significant associations between ΔCDI and additional WM parameters in the cingulum or significant associations between baseline FA and ΔRCADS.

Interestingly, participants who reported ≥5 points lower on the CDI after three months compared to baseline had significantly lower FA on baseline in the PCR (ΔCDI scores ≤ -5; n = 9, mean ΔCDI ± SD = -10.55 ± 4.91; mean FA ± SD = 0.52 ± 0.06) compared to those who did not change more than ≤ -5 or even had positive ΔCDI scores (n = 11, mean ΔCDI ± SD = 1.64 ± 4.64; mean FA ± SD = 0.57 ± 0.04; t(14) = 2.42, p = 0.03; illustrated in Figure 1B and Online Resource Figure S2).

Table 2 Significant associations between baseline FA and longitudinal changes in depressive symptoms within adolescents with depression and comorbid anxiety.

Cluster index	Voxels (mm^3)	p	Peak MNI coordinates			Location
			x	*y*	*z*	
2	924	0.03	20	-39	39	Right corona radiata (posterior)
1	122	0.04	16	31	32	Right cingulum (cingulate gyrus)

Threshold-free cluster enhancement (TFCE) and family-wise error (FWE) corrected at p-values < 0.05.

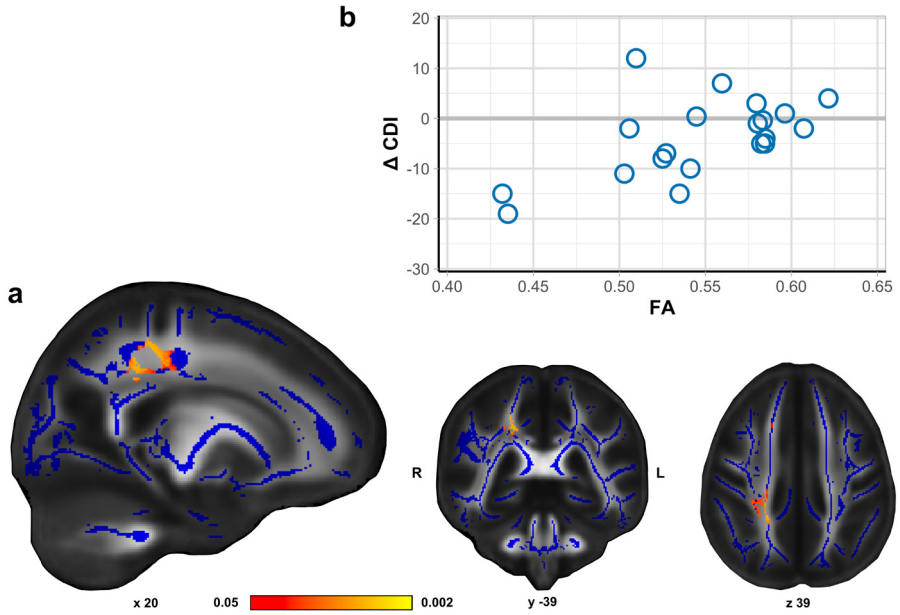

Figure 1 Significant associations between baseline FA and longitudinal changes in depressive symptoms within adolescents with depression and comorbid anxiety. A) Significant cluster from voxelwise whole brain analyses of fractional anisotropy (FA). Sagittal, coronal and axial sections of the WM skeleton (blue), with a subregion of the posterior corona radiata (PCR) showing significant associations of baseline fractional anisotropy (FA) with longitudinal changes in depressive symptoms within adolescents with depression and comorbid anxiety ($p < 0.05$, threshold-free cluster enhancement (TFCE) and family-wise error (FWE) corrected (yellow/orange)). The color bar indicates p-values. B) Association between baseline fractional anisotropy in the PCR and longitudinal changes in depressive symptoms within adolescents with depression and comorbid anxiety. CDI: children's depression inventory; FA: fractional anisotropy.

Discussion

In the present naturalistic study, we investigated the longitudinal course of parameters of WM microstructure in a group of adolescents with clinical depression and anxiety (INT) and compared these to a group of healthy peers (HC), using voxelwise TOI-based and exploratory whole-brain analyses. Furthermore, we examined within-subject associations between clinical symptoms and WM parameters in the INT group: we explored associations between changes in WM parameters and changes in clinical symptoms, and we investigated whether baseline WM parameters were associated with subsequent changes in clinical symptoms over a three-month period. As far as we are aware, this is the first longitudinal DTI study investigating WM microstructure in a naturalistic cohort of adolescents with clinical depression and anxiety, thus providing a first step in exploring WM microstructure in the course of these impairing mental illnesses.

Data revealed no significant changes in FA within and between the INT group and HC group over a three-month period; neither did we find evidence for significant correlations between changes in FA and changes in self-reported internalizing symptoms in the INT group. Voxelwise whole brain analyses

revealed that lower baseline values of FA in the right posterior corona radiata (PCR) were associated with a higher decrease in depressive symptoms after three months. Additional analyses showed that higher baseline values of MD and RD in the PCR were associated with a higher decrease in depressive symptoms. Furthermore, lower baseline values of FA in the right cingulum were associated with a higher decrease in depressive symptoms after three months, but no associations between depressive symptoms and other WM parameters in this cluster were found.

The corona radiata is part of the limbic-thalamo-cortical circuitry and has been implicated in several psychiatric disorders such as major depressive disorder and schizophrenia [44, 45]. It consists of several white matter tracts, such as the superior corona radiata (SCR) and posterior corona radiata (PCR). Lower FA in the SCR has been associated with an increased risk of psychopathology in a sample of healthy adolescents with a familial history of internalizing and/or externalizing psychopathology [46]. The PCR consists of ascending fibers connecting the thalamus with the cerebral cortex, and descending fibers connecting the frontoparietal cortex with subcortical nuclei and the spinal cord, including areas functionally involved in the default mode network, which regulates attention during cognitive performance, and in top-down control of emotional experience and mood [47, 48]. In general, low FA coupled with high MD and RD and unchanged AD could be indicative of demyelination, low axonal packaging or less spatial coherency of fiber alignment [21, 49]. Thus, our results might point at less spatial coherency in the PCR, as bundles of the corona radiata follow a geometrically complex path with a high degree of intra-voxel changes in fiber orientation before reaching the corpus callosum [48, 50]. However, the role and function of the PCR in the onset and development of adolescent depression and anxiety is yet to be further unraveled.

The cingulum, the other track implicated in this study, is functionally involved in among others regulation of emotional states. Anatomically, the cingulum bundle runs in the cingulate gyrus from anterior to posterior sections of the brain and is thought to mediate communication between the cingulate gyrus and regions involved in the limbic system, as well as within regions of the cingulate gyrus [12, 47]. Previous research has implicated lower FA in the cingulum in adolescent depression compared to healthy controls [10, 27]. In addition, decelerated maturation of myelin and lower age-related FA increase in the cingulum has been associated with anxiety symptoms in healthy adolescents [51, 52]. These results could imply a role of the cingulum in impaired regulation of emotional states, contributing to onset and persistence of internalizing disorders. However, as we discovered associations between lower baseline FA and changes in depressive symptoms without associations with other WM parameters in a small cluster (size: 122 voxels), directionality of underlying WM microstructure and thus interpretation of this finding is limited [21, 49].

Data did not reveal any significant associations between changes in FA and changes in self-reported symptoms. Since WM development is associated with learning new skills, this finding was unexpected. For example, studies in mice reported on FA increases after learning a new skill and steeper FA development curves were associated with higher performance [53, 54]. In agreement with this, knock-out mice showed

impairment in acquiring new skills when no myelination was possible [55]. We hypothesize that three months was too short to detect any significant changes; alternatively, our sample might have been too small to achieve sufficient power to detect significant associations due to type 2 errors.

There are some more limitations to consider. First, only a few boys participated in this study although the gender distribution was not significantly different between the clinical and healthy control group. However, as girls are more likely to develop internalizing psychopathology, and the female gender has been associated with an increased risk on comorbid anxiety and depression, we feel our study is representative of the clinical population [1]. Second, sample size and study duration were modest. Yet, previous longitudinal studies in adolescents with internalizing disorders have assessed longitudinal effects of cognitive behavioral therapy (CBT) over three months and reported functional brain changes in amygdalar – prefrontal connections, which in some studies were associated with improvement of clinical symptoms [15, 56-58]. In addition, the present design does not allow to differentiate between symptoms of clinical anxiety and depression. Furthermore, several studies concerning white matter development in healthy adolescents have reported continuing white matter microstructure developmental changes during adolescence well into young adulthood [59-61]. Taken together, we expected three months to be sufficient to detect any changes. Our modest sample size could have induced type 2 errors. Indeed, perhaps our study had too little power to detect a significant effect. Furthermore, treatment was not standardized, but all participants were treatment-naïve at baseline, thus enabling us to examine the course of symptoms in this clinically representative population. Lastly, we did not account for social-economic status or illness duration.

To better understand the course and neurodevelopment of WM in adolescent anxiety and depression, we recommend future studies to use a longitudinal study design with larger groups of patients and healthy controls and a standardized treatment in an elongated study period, as standardization of treatment would aid the interpretation of intervention effects on the course of underlying microstructure of WM tracts.

In conclusion, this study revealed no significant changes in WM characteristics within and between adolescents with internalizing disorders and healthy peers over a three-month period; neither were significant correlations between changes in FA and changes in self-reported internalizing symptoms in adolescents with internalizing disorders found. However, analysis revealed associations between parameters of WM microstructure and changes in self-reported depressive symptoms in adolescents with clinical depression and comorbid anxiety. We found an association between a higher decrease in depressive symptoms over a period of three months, and characteristics of baseline WM microstructure in the posterior corona radiata (lower FA, higher RD and MD). These results further entangle the contribution of underlying neurobiological factors in the course of adolescent depression and comorbid anxiety disorders.

Supplemental methods

Statistical analysis: expectation maximization

A limited number of items were missing in the CDI questionnaire (10 items in total, 6 items for 6 participants at session 1 and 4 items for 1 participant at session 2) and in the RCADS (4 items in total, 3 items for 3 participants at session 1 and 1 item for 1 participant at session 2). Expectation maximization was allowed as Little's MCAR tests showed that data was completely missing at random for both questionnaires at both sessions.

DTI registration

DTI-TK uses tensor-based registration and has proven additional value to TBSS [62, 63]. In the present study, a within-subject template was created for each subject by computing an initial average template from the two time points. The template was iteratively refined using the following procedure: the within-subject tensor images were registered to the template and a refined template was computed as an average of the registered tensor images for the next iteration. The process was repeated until the change between templates from consecutive iterations became sufficiently small, first with affine and then with non-linear registrations. Once a suitable non-linear alignment has been established, a study-specific (group-wise) template was created from all of the within-subject templates using the described iterative method. Afterwards, this improved study-specific template was resampled to isotropic $1mm^3$ resolution and each original within-subject tensor image was registered to the study-specific template using non-linear alignments. Finally, the FA maps and refined scalar diffusivity maps (e.g. the eigenvalues and eigenvectors) were obtained for each participant using singular-value decomposition. To warp all DTI images to standard space, the FA map of the final study-specific tensor template was linearly registered to the ENIGMA DTI-MNI-space [64].

Supplemental results

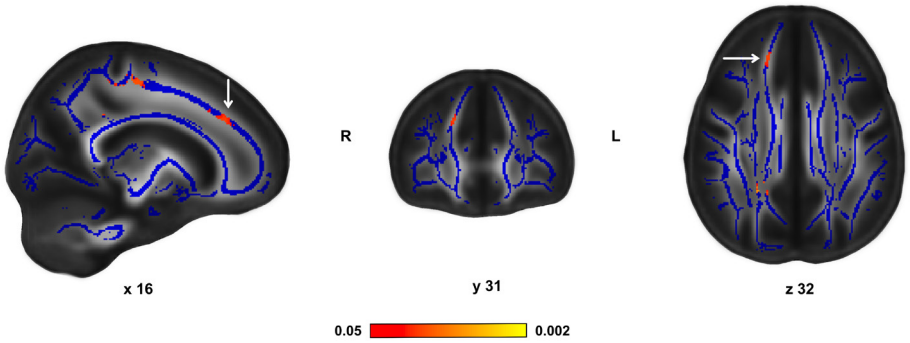

Figure S1 Significant associations between baseline FA in the cingulum and longitudinal changes in depressive symptoms within adolescents with depression and comorbid anxiety. Significant cluster from voxelwise whole brain analyses of fractional anisotropy (FA). Sagittal, coronal and axial sections of the WM skeleton (blue), with a subregion of the cingulum (indicated by white arrow) showing significant associations of fractional anisotropy (FA) with longitudinal changes in depressive symptoms within adolescents with depression and comorbid anxiety ($p < 0.05$, threshold-free cluster enhancement (TFCE) and family-wise error (FWE) corrected (yellow/orange)). The color bar indicates p-values. The cluster not indicated by arrow depicts other the significant cluster in the posterior corona radiata shown in Figure 1 in main article.

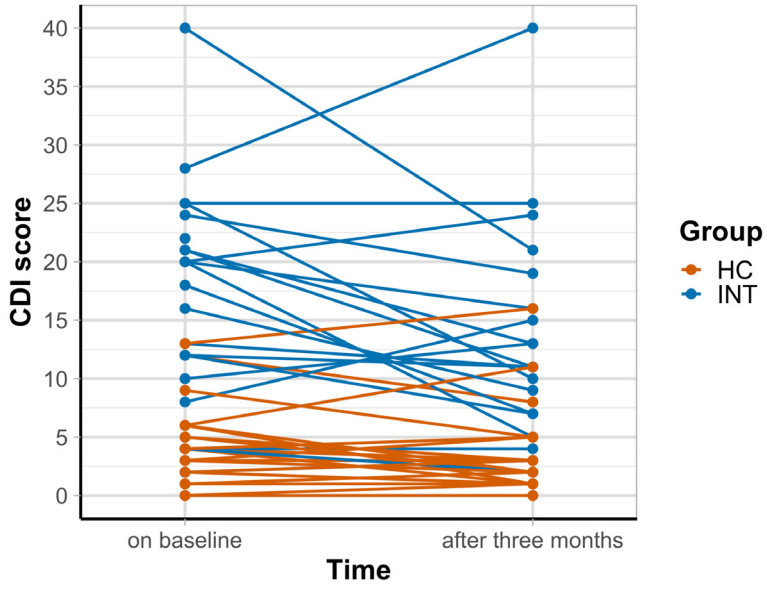

Figure S2 Association between baseline fractional anisotropy in the PCR and longitudinal changes in depressive symptoms within adolescents with depression and comorbid anxiety. CDI: children's depression inventory; FA: fractional anisotropy.

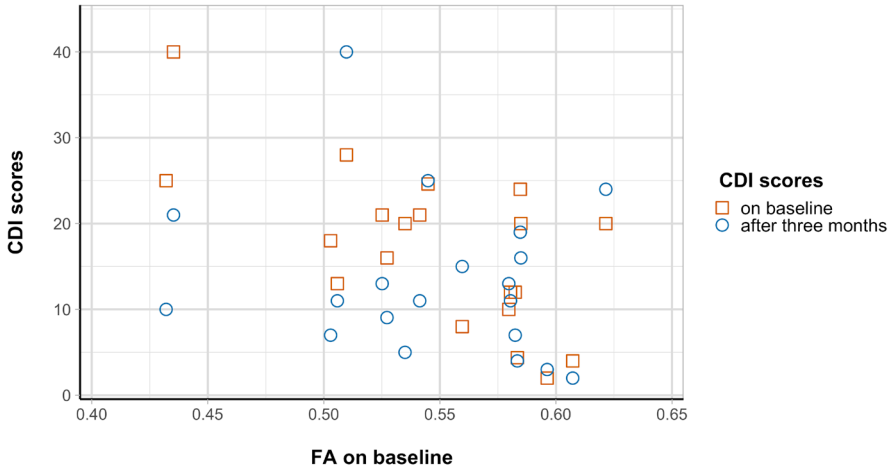

Figure S3 Longitudinal changes in depressive symptoms within adolescents with depression and comorbid anxiety and healthy control participants. CDI: children's depression inventory; HC: healthy control participants; INT: adolescents with depression and comorbid anxiety.

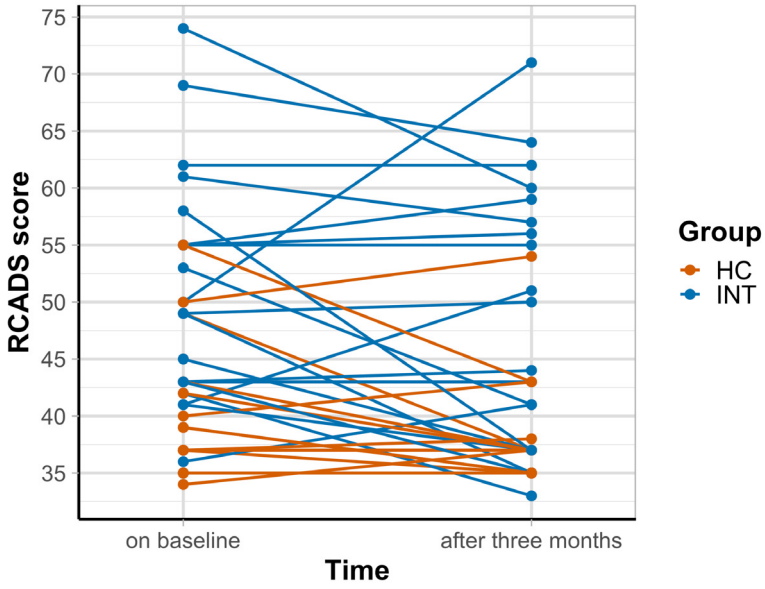

Figure S4 Longitudinal changes in anxiety symptoms within adolescents with depression and comorbid anxiety and healthy control participants. RCADS: revised child anxiety and depression scale; HC: healthy control participants; INT: adolescents with depression and comorbid anxiety.

Table S1a Overview of literature; demographic information.

Study	Author	Contrast	Patients	Age (years)	Anxiety symptoms	Depressive symptoms	Illness (months)	HC	Age (years)	Anxiety symptoms	Depressive symptoms
1	Bessette et al. [7]	MDD vs. HC	7M, 24F	17.1±1.9	n.a.	BDI-II: 26.3±11.2	n.a.	12M, 19F	17.0±2.4	n.a.	BDI: 3.1±3.0
2	Aghajani et al. [9]	MDD vs. HC	4M, 21F	15.6±1.4	RCADS anx: 31.5±5.7	CDI: 18.6±9.7	n.a.	3M, 18F	14.7±1.6	RCADS anx: 14.4±9.7	CDI: 4.6±3.3
3	LeWinn et al. [10]	MDD vs. HC	21M, 31F	16.2±0.2	RCADS tot: 65.8±1.0	CDRS: 72.5±11	n.a.	16M, 26F	16.0±0.2	RCADS tot: 41.7±1.2	CDRS: 33.0±0.7
4	Liao et al. [11]	GAD vs. HC	13M, 11F	17.0±0.7	PSWQ: 54.6±8.9	n.a.	n.a.	13M, 11F	16.6±0.8	SCARED: < 25	n.a.
5	Cullen et al. [8]	MDD vs. HC	4M, 10F	16.8±1.2	n.a.	BDI-II: 29.0±11.8	33.9±20.2	6M, 8F	16.8±1.5	n.a.	n.a.

Results are reported in mean ± SD. HC: healthy control. MDD: major depressive disorder. GAD: generalized anxiety disorder. F: female. M: male. BDI: Beck's Depression Inventory. RCADS: Revised Child Anxiety and Depression Scale. CDRS: Children's Depression Rating Scale. SCARED: Child Anxiety Related Emotional Disorders. PSWQ: The Penn State Worry Questionnaire.

Table S1b Overview of literature; findings.

Study	Variables	CC			PLIC		EC		ALIC		CR		ACR		PCR		SCR		SLF		ILF		IFOF		UF		ATR		PTR		PC		MFPC	
		G	B	S	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R
1	FA TBSS, MDD vs HC	↓	↓	ø	↓	↓	↓	ø	↓	↓	ø	ø	↓	↓	ø	ø	↓	↓	ø	ø	↓	ø	↓	↓	↓	ø	ø	↓	↓	ø	↓	ø	↓	↓
2	FA TBSS, MDD vs HC	ø	↓	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø
	FA TOI, MDD vs HC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	-	-	-	-	-	-	-	-	-
	FA TBSS, MDD vs HC	↓	↓	ø	ø	ø	↓	↓	↓	↓	ø	ø	↓	↓	ø	ø	↓	↓	ø	ø	ø	ø	↓	↓	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø
3	Tractography, MDD vs HC	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	↓	↓	ø	ø	ø	ø	ø	ø	ø	ø
	FA TBSS, GAD vs HC	ø	ø	ø	ø	↓	ø	ø	↓	ø	↓	↓	ø	ø	ø	ø	ø	ø	↓	ø	↓	↓	↓	↓	↓	↓	ø	ø	ø	ø	ø	ø	ø	ø
4	FA TBSS, ass w/ PSWQ	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø
5	FA TBSS, MDD vs HC (uncorr)	↓	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	↓	ø	ø	ø	↓	↓	↓	↓	ø	ø	ø	ø	ø	ø	ø	ø

CC: corpus callosum; G: genu; B: body; S: splenium; PLIC: posterior limb of internal capsula; EC: external capsula; ALIC: anterior limb of internal capsula; CR: corona radiata; ACR: anterior corona radiata; PCR: posterior corona radiata; SCR: superior corona radiata; SLF: superior longitudinal fasciculus; ILF: inferior longitudinal fasciculus; IFOF: inferior fronto-occipital fasciculus; UF: uncinate fasciculus; ATR: anterior thalamic radiation; PTR: posterior thalamic radiation; PC: posterior cingulum; MFPC: medial frontal pregenual cingulate.

Table S2 Overview of treatments received by participants.

Combination of treatments	Number of participants (n)
CBT individual	5
CBT group	1
CBT individual + systemic family therapy	7
CBT individual + systemic therapy, parents only	4
CBT individual+ EMDR	1
CBT individual + CBT group + systemic family therapy	1
CBT group + systemic family therapy	1
Creative therapy + systemic therapy, parents only	1
None started	1

CBT: cognitive behavioral therapy. EMDR: Eye Movement Desensitization and Reprocessing. Creative therapy was received in a group setting.

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3

Longitudinal development of resting-state functional connectivity in adolescents with and without internalizing disorders

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Neurosci Appl 2024 Vol. 3

Abstract

Objective: Longitudinal studies using resting-state functional magnetic resonance imaging (rs-fMRI) focused on adolescent internalizing psychopathology are scarce and have mostly investigated standardized treatment effects on functional connectivity (FC) of the full amygdala. The role of amygdala subregions and large resting-state networks had yet to be elucidated, and treatment is in practice often personalized. Here, longitudinal FC development of amygdala subregions and whole-brain networks are investigated in a clinically representative sample.

Methods: Treatment-naïve adolescents with clinical depression and comorbid anxiety who started care-as-usual (n = 23; INT) and healthy controls (n = 24; HC) participated in rs-fMRI scans and questionnaires at baseline (before treatment) and after three months. Changes between and within groups over time in FC of the laterobasal amygdala (LBA), centromedial amygdala (CMA) and whole-brain networks derived from independent component analysis (ICA) were investigated.

Results: Groups differed significantly in FC development of the right LBA to the postcentral gyrus and the left LBA to the frontal pole. Within INT, FC to the frontal pole and postcentral gyrus changed over time while changes in FC of the right LBA were also linked to symptom change. No significant interactions were observed when considering FC from CMA bilateral seeds or within ICA-derived networks.

Conclusion: Results in this cohort suggest divergent longitudinal development of FC from bilateral LBA subregions in adolescents with internalizing disorders compared to healthy peers, possibly reflecting nonspecific treatment effects. Moreover, associations were found with symptom change. These results highlight the importance of differentiation of amygdala subregions in neuroimaging research in adolescents.

Introduction

Internalizing disorders like anxiety and depression often start in adolescence and are highly prevalent in this stage of life. Sadly, recent studies imply that comorbid anxiety disorders are rather the rule than the exception in clinically depressed adolescents, as more than 50% of adolescents with a clinical depression have one or more comorbid anxiety disorders [1]. These adolescents often display more severe symptoms, poorer response to treatment and increased risk of suicidal behaviors compared to clinically depressed adolescents without comorbid psychopathology [1]. Models suggest that complex interactions between environment, genes, neurobiological characteristics, and the timing of developmental stages contribute to the development of these mental illnesses [2-4]. To attain insights in neurobiological underpinnings of adolescent internalizing disorders, investigation of function and structure of neural networks is vital.

Activity of neural networks at rest, also called functional connectivity (FC), is widely investigated by use of resting-state functional MRI (rs-fMRI) [5, 6]. Rs-fMRI focuses on spontaneous, low-frequency fluctuations (<0.1 Hz) in the blood oxygenation level-dependent (BOLD) signal that occur in the absence of a task or stimulus, which can be used to identify functional neural networks [7-9]. Two common methods to identify these networks are seed-based correlation analysis (SCA), a hypothesis-driven approach to investigate the primary signal of a region-of-interest (ROI) to the whole brain [7], and independent component analysis (ICA), a fully data-driven, exploratory approach to extract resting-state networks using a multivariate exploratory analysis, which divides the brain into independent spatial regions according to their signal fluctuations [10]. To our knowledge, fully data driven approaches are still scarce in current literature despite their advantages, like being unbiased to a priori selected regions. When comparing SCA and ICA, secondary signals expressed by the brain are missed in SCA [7], whereas distinct signals occurring in a particular region can go unnoticed due to the limited number of components that is being extracted in ICA [10]. Therefore, SCA and ICA can be seen as complementary methods to investigate FC of neural networks and brain regions, such as the amygdala.

As one of the most extensively investigated brain regions in humans and animals, the function and structure of the amygdala is quite well known [11, 12]. It consists of several subregions, like the laterobasal amygdala (LBA), involved in regulation of fear and the perception hereof, and centromedial amygdala (CMA), mainly concerned with acute stress-reactions [11-13]. Recently, it has been hypothesized that these subregions each contribute to different aspects of mental disorders [12, 14], but their role in adolescent internalizing disorders has yet to be elucidated. Involvement of either or both subregions is likely, as meta-analyses of cross-sectional studies reported changes of full amygdala FC in depressed and anxious adolescents and adults when compared to healthy peers [15, 16], such as hypoconnectivity between the amygdala and the medial PFC [16], and decreased amygdala FC within the executive control network, which has been found in regulating emotions and initiating goal-directed responses [17] although a recent meta-analysis did not replicate these findings in depressed adolescents [18]. These alterations may underly the characteristic imbalance between affective-cognitive networks in adolescent internalizing disorders [19-21]. To expand the understanding of the course of these alterations, development of amygdala FC and its subregions needs to be investigated. Unfortunately,

longitudinal studies in this population are scarce and have reported inconsistent and isolated findings on full amygdala FC [2, 22-27], like increased FC of the amygdala to the right medial and middle frontal gyrus and decreased FC of the amygdala and the right posterior cingulate cortex, which were associated with decreases in depressive symptoms [23, 27]. These studies suggest a normalizing or compensatory effect of treatment on amygdala FC [2, 22-27], although this must be interpreted cautiously due to the scarcity of studies and their inconsistent, isolated results. Altogether, even though longitudinal studies are inconclusive, alterations in FC of amygdala subregions are highly suspect to be involved in adolescent internalizing disorders.

Current models suggest that several whole-brain resting-state networks are involved in internalizing disorders as well (for a comprehensive overview, see Table S2 and Figure 1 in [28]). Cross-sectional alterations in FC within and between these networks have been reported in depressed adolescents and anxious adults when compared to healthy peers, such inter- and intranetwork alterations in the limbic, default mode, central executive, and salience networks [15, 18, 29]. It is therefore likely that, in addition to the changes in amygdala FC, complex and widely spread changes in FC of other networks are involved in the development and course of adolescent internalizing disorders.

Besides the need to explore the longitudinal trajectory of FC of these regions and networks in this population, outcomes of prior longitudinal studies on FC development in adolescents with internalizing disorders may not reflect practical applicability in clinical settings, as they predominantly investigated treatment effects using standardized protocols [23-27]. In clinical practice, however, especially adolescents typically receive more personalized treatments tailored to their individual needs, adjusted to their level of emotional and cognitive development and in collaboration with their family [30-32].

To gain more insight in the development of neural networks, we have conducted the *Emotional Pathways' Imaging Study in Clinical Adolescents* (EPISCA) study [33]. In EPISCA, we used a study design in which participants received care-as-usual and were followed over the course of six months without study interventions, thus reflecting real world development of neural networks in a clinically heterogeneous group as close as possible. Using this longitudinal dataset, we have previously demonstrated that baseline measures of white matter microstructure are associated with changes in depressive symptoms over time in adolescents with internalizing disorders [34].

In the present study, FC in adolescents with internalizing disorders and healthy controls over a period of three months was investigated to obtain more insight in the development of FC of amygdala subregions and whole brain networks between and within these groups, possibly reflecting treatment effects. Based on previous findings, we expected FC differences between groups over time in regions involved in emotion processing, such as limbic structures, and emotion regulation, like frontal regions.

Methods

Participants

Data for this study were derived from the EPISCA study [33]. Briefly, two groups were investigated over time and in both groups, the majority of participants identified themselves as being female. The first group consisted of treatment-naïve adolescents with clinical depression and comorbid anxiety (internalizing disorders group, 'INT', n = 30 at baseline). They were included in the study before start of care-as-usual and were diagnosed with clinical depression and at least one clinical anxiety disorder as assessed by categorical measures of DSM-IV depressive or anxiety disorders. The second group consisted of healthy control peers (HC, n = 32 at baseline). All participants were investigated three times in a six-month timespan, during which the INT group received personalized variants of cognitive behavioral therapy (CBT) and family-based interventions. Extensive information about recruitment of participants, in- and exclusion criteria and demographic data of the full sample can be found in previous publications [33-56] and in the supplemental information. Detailed information about personalized treatments, MRI data acquisition, preprocessing of the rs-fMRI data and statistical analyses of demographical data, including multiple comparison corrections, is also included in supplemental information.

In the present study, rs-fMRI data obtained at baseline (before treatment; T1) and after three months (T2) were analyzed, as loss-to-follow-up was substantial after six months (loss-to-follow-up after three months: 16%, loss-to-follow-up after six months: 24%). At baseline, two participants (n = 1 INT, n = 1 HC) were excluded due to an anomaly on the structural T1-scan. Furthermore, one HC participant was excluded as criteria for clinical psychopathology were met. Two HC participants were excluded due to technical issues and six participants (n = 4 INT, n = 2 HC) did not follow-up after the first visit, leaving data from 25 INT and 26 HC participants for initial analysis. In addition, data from several questionnaires was included if available on both timepoints. To assess severity of depressive and anxious symptoms, total scores on the Children's Depression Inventory (CDI) [57] and on the anxiety subscale of the Revised Child Anxiety and Depression Scale (RCADS) [58] were included. Pubertal stage was taken into account by using baseline scores on the self-report Pubertal Development Scale (PDS) [59], according to the following categories: 1) prepubertal, 2) early pubertal, 3) midpubertal, 4) late pubertal, and 5) postpubertal.

General preprocessing of resting-state data

Image pre-processing and analyses were performed using the Oxford Centre for Functional Magnetic Resonance Imaging of the Brain (FMRIB) Software Library (FSL), version 6.0.3 (RRID:SCR_002823). Data underwent several preprocessing steps following the procedures described in [60-62]. First, non-brain removal was performed on T1 and high-resolution EPI data using the Brain Extraction Tool (BET) [63]. Then, BOLD data were preprocessed using FEAT (FMRI Expert Analysis Tool) [64], including motion correction using MCFLIRT [65], spatial smoothing using a Gaussian kernel of full-width half- maximum (FWHM) 6.0 mm and grand-mean intensity normalization of the entire 4D dataset by a single scaling factor to enable higher-level analyses and registration. Resting- state fMRI scans were

first registered to high-resolution EPI images, which were registered to T1 images, which in turn were registered to the Montreal Neurological Institute (MNI) T1-template brain (resolution 2 mm) using FNIRT nonlinear registration [65-67]. These transformation matrices were then combined to obtain a native-to-MNI-space transformation matrix per participant.

Next, ICA-AROMA (ICA-based Automatic Removal Of Motion Artifacts) was used to remove motion-related artefacts [60, 61]. Afterwards, native T1-weighted images were segmented into white matter (WM), central spinal fluid (CSF) and grey matter (GM) probability maps using FSL FAST [68]. Without extensive erosion of WM and CSF masks, these signals can be correlated with the GM signal [69]. Therefore, we applied 2 erosion cycles for the CSF mask and 4 erosion cycles for the WM mask to prevent partial voluming. If a mask contained fewer than 5 voxels after a given erosion cycle, the previous cycle was selected. Then, these masks were linearly aligned into native resting state space using FSL's FLIRT [65-67]. BOLD data were then submitted to FEAT to perform high-pass temporal filtering (Gaussian-weighted least-squares straight line fitting, cutoff 0.01 Hz) and subsequently nuisance regression with WM and CSF masks was performed. The processed resting-state fMRI data of each participant were then warped to MNI space using the combined native-to-MNI transformation matrix.

Data quality was assessed by inspecting each participant's processed EPI data as a time series (i.e., "carpet plot") alongside framewise displacement (FD), as calculated by FSL_motion_outliers. Participants were excluded if mean FD over time was > 0.2 mm, or > 20% suprathreshold FDs, or any FD > 5.0 mm [62].

Identification of resting-state networks

Rs-fMRI data were investigated twofold using the Oxford Centre for Functional Magnetic Resonance Imaging of the Brain (FMRIB) Software Library, version 6.0.3 (FSL; RRID:SCR_002823). First, SCA was used to investigate FC of two bilateral amygdala subregions, creating seeds derived from the Juelich histological atlas [44]. In short, voxelwise bilateral regions-of-interest (ROIs) of the laterobasal amygdala (LBA) and centromedial amygdala (CMA) were created by including voxels only if the probability of the voxel belonging to either subregion was higher than 40%. Each voxel was exclusively assigned to one region, overlapping voxels were assigned to the region with the greatest probability. Using these ROIs as seeds, FC of each subregion to the whole brain was investigated. First, we explored FC of each subregion for every individual participant on each timepoint. These results, containing data from both timepoints and all participants, were subsequently used as input to generate group-level FC maps of each subregion to the whole brain, using a cluster-forming threshold of $Z > 3.1$ and a cluster-corrected extent threshold $p < 0.05$ (Figure 1B and Table S2). The activated networks were successively binarized and used as a mask for the statistical analyses below, ensuring that analyses were confined within the respective networks.

Second, to determine group-level data-driven resting-state networks, Probabilistic Independent Component Analysis (PICA) [70] and dual regression analysis [71] were used. First, PICA was applied to separate 4D functional data into spatial maps with an associated time course for each map, starting with temporally concatenating all preprocessed subjects' data for both timepoints in Montreal Neurological

Institute (MNI)-standard space into one dataset. Subsequently, this dataset was decomposed in 20 independent components, consisting of independent vectors describing signal variation across the spatial (maps) and temporal (time-course) domain. This number of components results in a representative set of functional connectivity networks [72] in line with previous work in our group [73]. Afterwards, dual regression was used to generate subject-level maps for each of the 20 group components. This resulted in a set of maps (one for each original group-level ICA component) that describe the network structure based on the data from each participant. The 20 spatial maps at group-level were visually inspected. Components with spatial similarity to the networks of interest were selected based on descriptions of functional networks in previous work: default mode, dorsal attention, frontoparietal (separated in a left and right component), executive control, salience and affective network (Figure 1A and Table S2) [72, 74-76].

Statistical analysis of resting-state networks

After investigating FC of the LBA and CMA seeds (Figure 1B) and confirming the seven networks as defined by ICA (Figure 1A), further statistical analyses were conducted. First, differences in head motion between groups were investigated by comparing individual mean relative and absolute motion parameters created by FSL's MCFLIRT using the Mann-Whitney U test in R (Table 1; R Project for Statistical Computing (RRID:SCR_001905)) [65, 77].

Then, several analyses were conducted. First, SCA was applied to explore group x time interactions in connectivity of two bilateral amygdala subregions, namely the LBA and CMA. To underline the importance of investigating subregions, we conducted additional analyses using bilateral whole amygdala seeds. Second, exploratory group x time interactions were investigated in seven resting-state networks derived from ICA: default mode, frontoparietal (separated in a left and right component), executive control, dorsal attention, salience and affective network [72-76, 78]. Specifically, multi-level block permutation analyses were conducted in FSL's PALM [79, 80] (RRID:SCR_017029) to examine voxel-wise changes in FC in the networks of interest (four networks related to bilateral amygdala subregions; seven whole-brain networks) over time between INT and HC, using 5000 permutations and threshold-free cluster enhancement (TFCE) [81]. Two contrasts were investigated in each time direction: i) group x time interaction HC (T2 > T1) > INT (T2 > T1) and ii) group x time interaction INT (T2 > T1) > HC (T2 > T1). In other words, the first interaction explores for each voxel within the functional networks whether HC had a greater change in FC after three months than INT, while the second interaction investigates whether the change in FC after three months was greater in INT compared to HC (design is provided in Table S3). Age at the time of scanning was accounted for by subject-specific regressors. To assure these assumptions, additional analyses were conducted to control the effect of age on group x time interactions by adding age at baseline as a fixed, non-time varying covariate. Two extra contrasts were added to the main design to test positive and negative effects of age on changes in FC. Results are compared to the main design in the Supplemental Text (Table S7 and Figure S1). Correction for multiple testing considered the fact that we investigated both directions for interaction effects [82]. Analyses across seeds and hemispheres were corrected for multiple comparisons at a false discovery

rate (FDR) of 5% [83]. In case of a significant interaction, directionality of the interaction within each group was explored (Table S3) and a separate design was used to investigate whether changes in FC were associated with symptom change within INT (Table S4). Multi-level block permutation analyses were conducted in PALM with 5000 permutations and TFCE to investigate associations with changes in anxiety symptoms, as measured by the RCADS, and changes in depressive symptoms, as measured by the CDI. An F-test was added to assess their overall effects on FC change, which reflects significance without providing directionality or a joint score of both symptom scores. INT-participants were included in these association analyses if the questionnaire (RCADS and/or CDI) was filled out on baseline (before treatment) and after three months.

Results

Exclusion of participants

After motion control, four participants ($n = 2$ INT, $n = 2$ HC) were excluded, leaving 23 INT participants and 24 HC participants for further analysis. Motion parameters were not significantly different between INT and HC (Table 1).

Table 1 Motion parameters.

	INT (n = 23)	HC (n = 24)
Head motion (median ± SD)		
mean absolute head motion T1	0.18 ± 0.10	0.16 ± 0.08
mean relative head motion T1	0.07 ± 0.02	0.08 ± 0.02
mean absolute head motion T2	0.22 ± 0.12	0.17 ± 0.19
mean relative head motion T2	0.08 ± 0.02	0.08 ± 0.02

Demographics

Characteristics of the final sample are summarized in Table 2. Groups did not differ in demographic measurements. As expected, patients experienced significantly higher levels of depression and anxiety on both timepoints compared to healthy controls ($p < 0.000$). Within INT, depression and anxiety severity significantly decreased over time ($p = 0.017$, $p < 0.001$, resp.). There were no significant symptom changes within HC.

Table 2 Demographic characteristics of participants with and without clinical depression and comorbid anxiety.

	INT (n = 23)	HC (n = 24)
Demographics at baseline		
Age in years (mean ± SD; range)	15.86 ± 1.47 (13 - 18)	15.12 ± 1.74 (12 - 20)
Male / Female (n)	2 / 21	3 / 21
IQ (mean ± SD)	105.30 ± 8.35	107.08 ± 7.92
Puberty stage (median ± SD; range) [a]	4.00 ± 0.73 (2 – 5)	4.00 ± 0.73 (3 – 5)
Days between visits (median ± SD)	100.00 ± 11.76	98.00 ± 12.59
Ethnicity (% white)	91.30	95.83
Primary diagnoses [b] (n)		
MDD + GAD	15	
MDD + SAD	13	
MDD + specific phobia	8	
MDD + panic disorder	4	
MDD + separation anxiety	7	
Secondary (externalizing) diagnoses [b] (n)		
ADHD; inattention	1	
ADHD; hyperactive	1	
Oppositional Defiant Disorder	1	
Behavioral Disorder	1	
Self-report measures (median ± SD)		
CDI on baseline [c]	20.00 ± 9.20	4.00 ± 3.62
CDI after three months	11.50 ± 9.40	2.00 ± 3.31
RCADS on baseline	33.0 ± 14.70	10.00 ± 11.14
RCADS after three months [d]	25.00 ± 15.56	10.00 ± 8.60

MDD: Major Depressive Disorder; GAD: Generalized Anxiety Disorder; SAD: Social Anxiety Disorder; ADHD: Attention Deficit Hyperactivity Disorder; CDI: Children's Depression Inventory; RCADS: Revised Child Anxiety and Depression Scale; SD: Standard Deviation. [a] Diagnoses are not mutually exclusive. [b] Three healthy controls and two patients did not complete the questionnaire. [c] One patient did not complete the questionnaire. [d] Two patients did not complete the questionnaire.

Voxelwise changes in FC over time between patients and controls

Significant group x time interactions were found in FC of the left and right LBA (Table 3; Figure 1C/1D). Specifically, development of FC between the left LBA and frontal pole (Figure 1C; Table 3) was significantly different between INT and HC. Furthermore, significant differences were found in development of FC between the right LBA and right postcentral gyrus between INT and HC (Figure 1D; Table 3). However, results did not stand when corrections for multiple testing were applied. No

significant interactions were observed within the ICA-defined networks or in FC of the CMA or whole amygdala bilateral seeds.

Voxelwise changes in FC over time within patients and controls

Additional analyses concerning the left and right LBA further revealed effects of time within INT (Table S6). Figure 1E/1F illustrates the change in z-score between and within the groups, indicating an increase of FC in the INT group and a decrease in the HC group of the bilateral LBA seeds over time. Results were not significant at the FDR-corrected level. At the uncorrected level, FC of the left LBA significantly increased to the frontal pole (Figure 1E) and decreased to the postcentral gyrus, but FC of the right LBA did not significantly change. FC did not significantly change over time within HC.

Associations with symptom changes in significant clusters

Within INT, associations between changes in symptom scores and changes in FC of each LBA seed were further explored. There were no significant associations at the corrected significance level, but an association was found between changes of FC between the right LBA and the postcentral gyrus and the overall effect of changes in anxious and depressive symptoms at the uncorrected level (Table S6) ($p = 0.032$, 30 voxels; $p = 0.037$, 13 voxels; $p = 0.049$, 3 voxels. TFCE and FWE corrected at p-values < 0.05). Figure 2 illustrates the associations between changes in symptoms and changes in FC of the right LBA, indicating an association between a decrease of symptoms and an increase of FC of right LBA to the postcentral gyrus. This association was only significant when analyzing the joint effect of anxious and depressed symptoms. Correlation analyses between changes in FC of LBA seeds and changes in anxious or depressive symptoms separately did not reveal any significant associations.

Table 3 Coordinates of significant clusters in group x time interaction.

	Voxels	p-value	MNI coordinates			Regions involved
			X	Y	Z	
Left LBA seed	581	0.003	0	50	-6	Frontal Pole; Frontal Medial Cortex; Paracingulate Gyrus; Anterior Cingulate Gyrus
Right LBA seed	74	0.021	60	-12	18	Postcentral Gyrus; Central Opercular Cortex

LBA: laterobasal amygdala. Threshold-free cluster enhancement (TFCE) and family-wise error (FWE) corrected at p-values < 0.05.

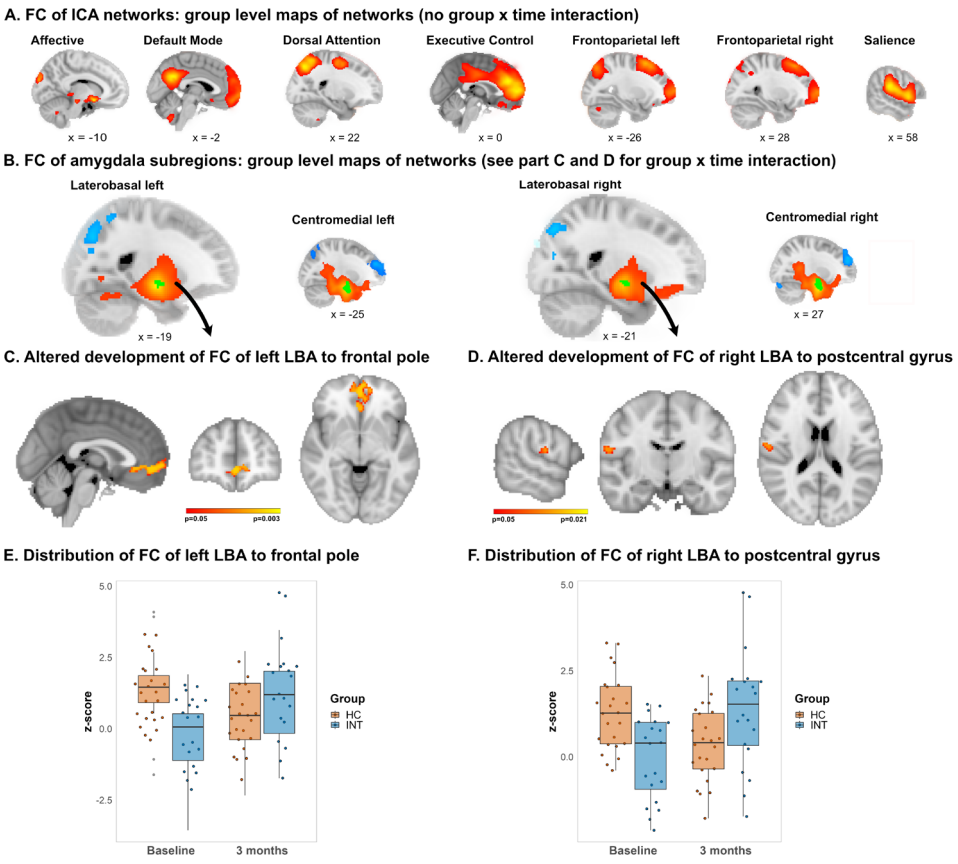

Figure 1 Different development of LBA FC in adolescents with internalizing disorders compared to healthy peers. (a) FC of networks derived from data-driven ICA analysis. (b) Positive (red) and negative (blue) FC of networks derived from SCA using amygdala subregions as seeds (green). (c) Significant group x time interaction in left LBA FC to the frontal pole (INT (T2 > T1) > HC (T2 > T1)), coordinates of displayed slices (MNI, x, y, z): 0, 50, -6. (d) Significant group x time interaction in right LBA FC to the postcentral gyrus (INT (T2 > T1) > HC (T2 > T1)), coordinates of displayed slices (MNI, x, y, z): 60, -12, 18. (e) Distribution of mean z-scores for INT and HC on baseline and after three months in the significant cluster in the frontal pole. Dots represent individual z-scores. (f) Distribution of mean z-scores for INT and HC on baseline and after three months in the significant cluster in the postcentral gyrus. Dots represent individual z-scores. Images are displayed according to radiological convention: right in image is left in the brain. LBA: laterobasal amygdala; INT: internalizing group; HC: healthy controls; MNI: Montreal Neurological Institute.

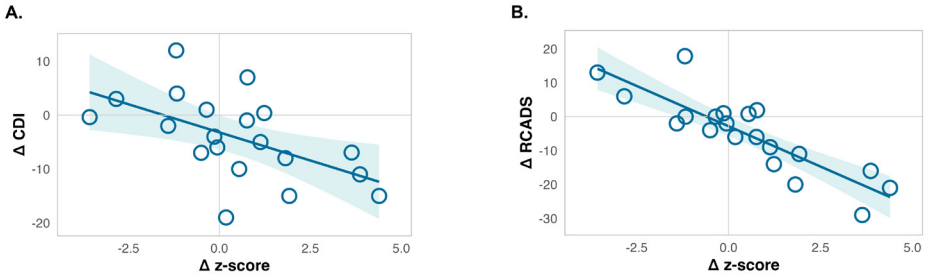

Figure 2 Associations between longitudinal changes in anxious and depressive symptoms and changes in functional connectivity between the right LBA and the postcentral gyrus within adolescents with depression and comorbid anxiety. (a) Association between changes in depressive symptoms (D CDI) and changes in functional connectivity (D z-score). Changes are calculated as score after three months minus score on baseline. Dots represent individual associations between changes in z-score and changes in depressive symptoms. Individual z-scores are derived from mean timeseries extracted from significant clusters. (b) Association between changes in anxious symptoms (D RCADS) and changes in functional connectivity (D z-score). Changes are calculated as score after three months minus score on baseline. Dots represent individual associations between changes in z-score and changes in anxious symptoms. Individual z-scores are derived from mean timeseries extracted from significant clusters. CDI: Children's Depression Inventory. RCADS: Revised Child Anxiety and Depression Scale.

Discussion

In this study, we investigated longitudinal development of FC in a cohort of adolescents with internalizing disorders (e.g. with clinical depression and at least one comorbid anxiety disorder) who started care-as-usual and their healthy peers over the course of three months. Specifically, FC of amygdala subregions, the whole amygdala, and large resting-state networks derived from data-driven analysis was examined. Initial evidence was found for different FC development of the left and right laterobasal amygdala (LBA) in adolescents with internalizing disorders compared to healthy peers. Moreover, change in FC of the right LBA was associated with the overall effect of changes in anxious and depressive symptoms in INT.

To start, development of FC between the left LBA and frontal pole was significantly different between patients and healthy peers (Figure 1C). Further analyses revealed that over time, FC increased in patients but did not change in healthy peers (Figure 1E). There were no significant associations between changes in FC of the left LBA and changes in anxiety symptoms (measured with the RCADS), depressive symptoms (measured with the CDI) or an overall effect of these symptom changes in the INT group. This difference in FC development of the left LBA to the frontal pole between INT and HC fits nicely with current models of corticolimbic network dysregulation in affective disorders, in which abnormal processing within and between regions involved in cognitive control (i.e. prefrontal cortices) and emotions (i.e. limbic structures) are thought to be part of neurobiological mechanisms underlying depression and anxiety disorders [3, 4]. The frontal pole, a distinct segment within the frontal cortex, maintains both functional and structural connections with the LBA, thereby positing its likely involvement in the regulation of affective and social-cognitive abilities [84]. Indeed, changes in function and structure of the LBA and the frontal pole have frequently been reported in cross-

sectional studies on adolescent and adult internalizing disorders and thus support models concerning corticolimbic dysregulation [2, 85, 86]. This finding is also generally in line with previous longitudinal studies, which have reported increases in FC between the entire amygdala and frontal regions over time in depressed adolescents. For example, three studies demonstrated an increase in FC of the amygdala to the bilateral prefrontal cortex (PFC) [24], left medial PFC [25] and the dorsal anterior cingulate cortex [26] after several weeks of psychotherapy (either five weeks or at least six sessions of CBT or 16 weeks of interpersonal psychotherapy, resp.). Interestingly, in two studies, some of these findings correlated with improvement of depression severity [24, 26], but this result was not replicated in the study by Villa et al. [25] nor in the present study. Another study in depressed adolescents reported an increase in amygdala FC to the right medial and middle frontal gyri after eight weeks of selective serotonin reuptake inhibitor (SSRI) treatment [27], which was again associated with a decrease of depressive symptoms. Considering these results, we cautiously propose that the increase in FC to the frontal pole in patients could be due to a nonspecific effect of treatment, regardless of which treatment is offered. However, the control group were healthy adolescents rather than a non-treated patient group, due to ethical concerns of withholding treatment. Therefore, we cannot conclude whether this increase was due to treatment effects or non-specific effects of improvement in symptoms related to time. Nonetheless, our hypothesis is supported by the fact that previous studies showed a similar pattern of increased FC between the amygdala and frontal regions related to treatment, while all investigated different treatment methods. The lower functional coupling to regulatory regions in the frontal cortex, such as the frontal pole, at baseline could reflect aberrant psychophysiology, while the increase in FC to the frontal pole over time possibly mirrors new skills learned in treatment, although we did not find an association between this FC change and symptom levels as measured with the RCADS and CDI. We cautiously hypothesize that the changes in FC might reflect adaptive changes or nonspecific effects of treatments. Furthermore, internalizing disorders have a wide range of symptoms, which are only partly captured by questionnaires like the RCADS and CDI. Perhaps other symptoms, which are not measured by these questionnaires, would be associated with these FC changes.

Second, using the right LBA as a seed region, a significant group x time interaction between groups was found in FC to the right postcentral gyrus (Figure 1D). Further analyses revealed no effect of time within each group (Figure 1F). However, changes in connectivity to the postcentral gyrus were associated with the overall effect of changes in anxious and depressive symptoms within INT. Other associations with only anxious or depressive symptoms were not significant. Structural and functional studies have confirmed connectivity between the amygdala and sensorimotor regions, including the postcentral gyrus [87]. Functionally, the amygdala-sensorimotor pathway is involved in multiple steps of emotion processing, among others emotion recognition and emotional regulation [9, 88, 89]. For example, high levels of activation in this pathway have been reported when attention is focused on emotional state and in response to unconscious perceived threats [88]. Previous findings concerning this pathway in internalizing disorders are inconsistent and the relation to clinical symptoms in adolescents is unknown. However, one study demonstrated increased FC of the amygdala as a whole to the postcentral gyrus in adolescents with depression [90] and increased grey matter volume of the postcentral gyrus has been

reported in adolescents with anxious depression, but not in healthy controls or depressed adolescents [91]. Because the overall effect of symptom changes was associated with FC change in the right LBA-postcentral gyrus pathway, we posit that the amygdala-sensorimotor pathway might be especially involved in internalizing psychopathology with anxiety *and* depressive components, although the specific role of the LBA remains to be investigated.

Taken together, these results indicate that FC development of amygdala subregions is different in adolescents with internalizing disorders while they receive case-as-usual compared to healthy peers. Results were specifically located in FC of the LBA, a subregion connected to, among others, frontal regions, while analyses on FC of the CMA, mainly connected to the brainstem nuclei that generate behavioral and visceral correlates of acute stress-reactions [11-13], and analyses on FC of the whole amygdala did not reveal any significant interactions. Therefore, these results provide more insight in involvement of amygdala subregions in adolescent internalizing disorders and highlight the importance of differentiation between these subregions.

These findings are generally in line with previous work on longitudinal data of the EPISCA study, where we have demonstrated greater baseline cross-sectional connectivity of the uncinate fasciculus (UF), a white matter tract connecting the amygdala to the frontal cortex [37] and greater full amygdala FC to the dorsolateral prefrontal cortex (dlPFC) in adolescents with internalizing disorders compared to healthy peers [35], although longitudinal analyses on white matter revealed no changes in the UF [34].

Interestingly, we did not find any changes in FC of large resting-state networks derived from ICA. This could be due to several reasons, such as our sample size, which might be too small relative to the complexity of our data, or the time frame, which might not be long enough to detect any differences between groups over time. Our findings are in line with a large recent population-based study in adolescents with internalizing symptoms, which reported small changes in FC from ICA derived networks over four years that did not survive multiple testing correction [92].

Strengths, limitations & future research

This study is unique in the way that it investigated FC of amygdala subregions and data-driven whole-brain networks in a heterogeneous cohort of adolescents with internalizing psychopathology. This approach offers possibilities to expand our knowledge of FC development in adolescents with internalizing disorders compared to the healthy population, while simultaneously reflecting the clinical practice in which adolescents typically receive personalized treatment, focused on their individual needs. However, a few limitations of the present study need to be mentioned. First, this study design did not use a standardized treatment protocol but followed patients over three months' time, while they received care-as-usual; thus, it does not allow to evaluate treatment efficacy and any precise hypotheses about underlying specific reasons *why* FC and disease severity change in this population are relatively hard to generate. However, this study design provides valuable information about *how* FC and symptoms change over time, regardless of specific interventions, and is representative of clinical practice. Second, the study

groups predominantly consisted of female participants, but the sex distribution was not significantly different between the clinical and healthy control group. Yet, as girls are more likely to develop internalizing psychopathology and the female sex has been associated with an increased risk of comorbid anxiety and depression, we feel our study is representative of the clinical population although the sex ratio might be more unbalanced compared to ratios reported in current literature [93, 94]. Third, due to the imbalance in sex within groups, correction for sex was not possible, even though a mediating role in FC development has been reported [95]. Fourth, sample size and study duration were modest. In this heterogeneous group, adolescents were diagnosed with at least one out of five anxiety disorders as noted in Table 1 while receiving variants of personalized treatment as noted in Supplemental Table S1, and the sample reflected the adolescence in ages ranging from 12 to 20. In the light of the small sample size, this heterogeneous group could make interpretation of results difficult. With regards to the study duration, previous longitudinal studies in adolescents with internalizing disorders have assessed longitudinal effects of cognitive behavioral therapy (CBT) over three months and reported functional brain changes in amygdalar – prefrontal connections, which in some studies were associated with improvement of clinical symptoms [25, 96-98]. Taken together, we feel our sample represents clinical practice as close as possible and is thus indicative of the development of neural networks across adolescence in the real world, and we expected three months to be sufficient to detect any changes. Fifth, interpretation of results is limited as we did not include a clinical control group due to ethical reasons. Sixth, results did not stand after FDR correction for multiple testing across seeds and hemispheres. Lastly, information about social-economic status or illness duration was not collected and therefore could not be accounted for.

Future research could focus on several aspects. First, previous studies used different interventions and timespans, complicating comparison and generalization of the results. Therefore, a project within a large consortium like the ENIGMA-Anxiety Working Group might be interesting to pool imaging data, increase sample size and improve reliability of results within a mega- or meta-analysis [16, 28, 99]. Second, future studies should consider longitudinal investigation of amygdala subregions to further explore their unique role in adolescent internalizing disorders. Third, this study provides new insights in amygdala FC development in this population, which could lead to identifying possible new targets for innovative treatments such as fMRI-based neurofeedback [100-102]. Fourth, future studies could consider more frequent assessment in a large timeframe to detect small FC changes in ICA derived networks.

Conclusion

Concluding, this study provides initial evidence that FC development over the course of three months is different in adolescents with internalizing disorders who receive care-as-usual compared to their healthy peers, possibly reflecting nonspecific treatment effects, and highlights the importance of differentiation of amygdala subregions.

Supplemental methods

Participants

The first group, adolescents with internalizing disorders (INT), were included in the study before start of care-as-usual and recruited in outpatient departments of two child-and-adolescent psychiatric clinics. They were (i) diagnosed with clinical depression and at least one clinical anxiety disorder as assessed by categorical measures of DSM-IV depressive or anxiety disorders, (ii) were being referred for cognitive behavioral therapy at an out-patient care unit, and (iii) had no current or prior use of antidepressants except for stable SSRI use during the course of the study (n = 2).

The second group consisted of healthy control peers (HC), who were recruited through local advertisements. They had (i) no current or past DSM-IV diagnoses of Axis I and/or Axis II disorders, (ii) no clinical scores on validated mood and behavioral questionnaires, (iii) no history of traumatic experiences, and (iv) no current psychotherapeutic and/or psychopharmacological intervention of any kind.

Exclusion criteria for both groups were: (i) a primary DSM-IV diagnosis of attention deficit hyperactivity disorder, oppositional defiant disorder, conduct disorder, pervasive developmental disorders, posttraumatic stress disorder, Tourette's syndrome, obsessive–compulsive disorder, bipolar disorder, and psychotic disorders, (ii) current use of psychotropic medication, (iii) current substance abuse, (iv) a history of neurological disorders or severe head injury, (v) age < 12 or > 21 years, (vi) pregnancy, (vii) left-handedness, (viii) IQ score < 80, as measured by either the Wechsler Intelligence Scale for Children (WISC; Wechsler [103]) or the Wechsler Adult Intelligence Scale (WAIS; Wechsler [104]), and (ix) general MRI contraindications (e.g. metal implants, claustrophobia).

At the first visit, participants underwent clinical assessment by a child and adolescent psychiatrist. Afterwards, the Anxiety Disorders Interview Schedule (ADIS; Silverman *et al.* [105]) was performed to obtain DSM-IV-based classifications of anxiety and depressive disorders. To assess severity of anxious and depressive symptoms, additional self-report questionnaires were completed at each visit, including the Children's Depression Inventory (CDI; Kovacs [57]) and the Revised Child Anxiety and Depression Scale (RCADS; Chorpita *et al.* [58]). Pubertal stage was assessed using the self-report Pubertal Development Scale (PDS; Petersen *et al.* [59]), according to the following categories: 1) prepubertal, 2) early pubertal, 3) midpubertal, 4) late pubertal, and 5) postpubertal. HC-participants were excluded when criteria for a (history of) DSM-IV diagnosis or (sub)clinical scores on clinical questionnaires were met.

Ethics

The EPISCA study was approved by the medical ethics committee of Leiden University Medical Center. All participants provided informed consent according to the Declaration of Helsinki; both participants and parents signed the informed consent form. All anatomical scans were reviewed by a radiologist.

Analysis of demographic data and symptom severity

To examine differences in demographic data between the two groups, and within-subject changes of symptoms in the INT group, (paired) t-tests or two-sided Fisher exact tests were used for continuous and dichotomous data respectively. For non-normally distributed data, as defined by a significant Shapiro-Wilk test, the Mann-Whitney U test or Wilcoxon signed-rank test were used. A limited number of items were missing in the CDI questionnaire (10 items in total, 6 items for 6 participants at session 1 and 4 items for 1 participant at session 2) and in the RCADS (4 items in total, 3 items for 3 participants at session 1 and 1 item for 1 participant at session 2). Expectation maximization was allowed as Little's MCAR tests showed that data was completely missing at random for both questionnaires at both sessions.

The Bonferroni method was used to correct p-values for multiple comparisons (4 tests, being comparisons on each timepoint of scores on the RCADS and CDI between the INT and HC group, corrected p-value = 0.0125).

MRI data acquisition

Data were collected using a Philips 3.0 T Achieva MRI scanner (Philips Medical Systems, The Netherlands) with an eight-channel sensitivity encoding (SENSE) head coil. Resting-state functional MRI data were acquired for each subject using T2*-weighted gradient echo-planar imaging (EPI) with the following scan parameters: 160 whole-brain volumes; repetition time (TR) 2200 ms; echo time (TE) 30 ms; flip angle 80°; 38 transverse slices; no slice gap; field of view 220 mm; in-plane voxel size 2.75 x 2.75 mm; slice thickness 2.72 mm; total duration of the resting-state run 6 minutes.

Participants were instructed to lie still with their eyes closed and not to fall asleep. Wakefulness during acquisition was confirmed after the scan. In addition, a sagittal 3-dimensional gradient-echo T1-weighted image was acquired for registration purposes with the following scan parameters: TR 9.8 ms; TE 4.6 ms; flip angle=8°; 192 x 152 matrix; FOV 224 x 177 x 168 mm, 140 sagittal slices; no slice gap; 1.16 x 1.16 x 1.20 mm voxels. Lastly, a high-resolution EPI scan was acquired for registration purposes with the following scan parameters: TR 2200 ms; TE 30 ms; flip angle = 80°; 112 x 109 matrix; FOV 220 x 220 x 168 mm, 84 sagittal slices; no slice gap; 1.96 x 1.96 x 2 mm voxels. Prior to scanning, all participants were introduced to the scanning situation by lying in a dummy scanner and hearing scanner sounds.

Supplemental results

Table S1 Overview of treatments received by participants.

Combination of treatments	Number of participants (n)
CBT	8
CBT + family therapy	12
CBT + EMDR	1
Creative therapy + family therapy	1
None started	1

CBT: cognitive behavioral therapy. EMDR: Eye Movement Desensitization and Reprocessing. Creative therapy was received in a group setting.

Table S2 Peak coordinates of intrinsic functional connectivity using amygdala subregions (SCA).

Seed	Clusters	Cluster size	z-score	X	Y	Z	Regions in cluster
Left CMA	1	31372	15.4	-26	-6	-26	Thalamus, putamen, pallidum, hippocampus, amygdala, accumbens, temporal pole (bilateral)
	2	12144	7.32	4	20	42	Paracingulate gyrus, ACC, superior frontal gyrus
	3	5409	6.39	36	56	22	Frontal pole (right)
	4	3883	6.36	-36	46	26	Frontal pole (left)
Right CMA	1	26793	14.3	26	-4	-26	Amygdala, hippocampus, temporal pole, parahippocampal gyrus (bilateral)
	2	584	4.74	-24	32	-8	Frontal orbital cortex, frontal pole (right)
	3	7309	6.22	-8	-64	66	Lateral occipital cortex, precuneous cortex (left)
	4	2558	6.49	40	52	28	Frontal pole (right)
	5	2311	5.58	-38	52	14	Frontal pole (left)
Left LBA	1	13435	15.6	-22	-10	-10	Amygdala, hippocampus, putamen, superior temporal gyrus (bilateral)
	2	4547	4.94	-8	-76	46	Precuneous cortex, lateral occipital cortex (bilateral)
	3	820	4.65	4	-38	38	Posterior cingulate cortex, postcentral gyrus, precentral gyrus (bilateral)
Right LBA	1	11294	15.9	26	-10	-8	Amygdala, pallidum, putamen, hippocampus (bilateral)
	2	1897	4.56	-48	-52	56	Supramarginal gyrus, angular gyrus (left)
	3	1411	4.74	14	-72	48	Precuneal cortex, lateral occipital cortex (right)

CMA: centromedial amygdala. LBA: laterobasal amygdala.

Table S3 Peak coordinates of ICA derived networks.

Network	Function	Clusters	Cluster size	z-score	X	Y	Z	Regions within cluster
Default mode	Ruminative, negative self-referential processes [106]	1	5637	9.15	-2	66	-4	Frontal pole
		2	4597	15	0	-54	30	PCC, precuneal cortex
		3	1991	10.6	-44	-66	30	Lateral occipital cortex (superior division), angular cortex (right)
		4	1052	7.39	54	-62	28	Lateral occipital cortex (superior division), angular cortex (left)
		5	1038	6.82	-66	-14	-16	Postcentral gyrus
Affective	Emotion processing and regulation [107].	1	2882	5.57	-10	8	-12	Accumbens (bilateral), thalamus, amygdala
		2	826	4.66	26	-36	-4	Hippocampus (bilateral)
Executive control	Emotion regulation and goal-directed response initiation [17]	1	22723	16.8	0	40	24	Paracingulate gyrus, ACC, frontal pole
		2	361	5.02	44	-54	-44	Cerebellum (right)
		3	259	5.9	-40	-56	-42	Cerebellum (left)
Dorsal Attention	Goal directed and visuospatial attention [108]	1	14797	11.3	22	-70	48	Lateral occipital cortex (superior division), precuneus (bilateral)
		2	1322	11	28	-6	56	Superior & middle frontal gyrus, precentral gyrus (right)
		3	722	6.16	-24	-10	54	Precentral gyrus, superior & middle frontal gyrus (left)
Frontparietal right	Attention processing [109]	1	9141	10.6	28	26	52	Middle frontal gyrus, superior frontal gyrus
		2	4258	15.5	46	-56	44	Angular gyrus, lateral occipital cortex
		3	2451	9.54	-38	-66	-42	Cerebellum
		4	2122	7.45	66	-28	-6	Middle tempral gyrus, posterior division
Frontoparietal left	Attention processing [109]	1	11542	10.6	-26	14	56	Superior frontal gyrus, middle frontal gyrus
		2	5169	13.4	-46	-52	46	Angular gyrus, supramarginal gyrus
		3	2776	10.3	30	-66	-32	Cerebellum
		4	2098	8.79	-56	-42	-14	Inferior temporal gyrus

Table S3 (continued)

Salience	External stimulus detection and processing of emotionally salient information [110]	1	8465	12.3	58	-28	22	Insula, putamen, thalamus, parietal operculum cortex, supramarginal gyrus (right)
		2	7561	11.4	-56	-28	16	Insula, putamen, thalamus, parietal operculum cortex, supramarginal gyrus (left)
		3	1737	7.63	6	-2	48	ACC, juxtapositional lobule cortex (bilateral)
		4	111	4.95	-22	-46	60	Superior parietal lobule, postcentral gyrus (right)

Table S4 Design modeling multilevel block permutations to investigate voxelwise group x time interactions and effects of time within groups.

Table S5 Design modeling multilevel block permutations to investigate voxelwise associations between FC changes and symptom changes within INT group.

Due to their size, these tables are available using the QR code below.

Table S6 Effects of time on FC of significant seeds within groups.

	Cluster	Voxels	p-value	MNI coordinates			Regions involved
				X	*Y*	*Z*	
Left LBA seed							
Positive effect of time within INT group [a]	1	347	0.009	-6	64	-10	Frontal Pole, Paracingulate Gyrus, Medial Frontal Cortex
Negative effect of time within INT group [a]	1	1288	0.002	48	-42	60	Supramarginal Gyrus, Postcentral Gyrus, Angular Gyrus, Superior Parietal Lobule
	2	6	0.048	32	-54	66	Superior Parietal Lobule
Effect of time within HC group [a]	Æ	Æ	Æ	Æ	Æ	Æ	
Right LBA seed							
Effect of time within INT group [a]	Æ	Æ	Æ	Æ	Æ	Æ	
Effect of time within HC group [a]	Æ	Æ	Æ	Æ	Æ	Æ	
Association with symptom change [b]	1	30	0.032	-56	-4	34	Precentral Gyrus, Postcentral Gyrus
	2	13	0.037	66	-8	10	Postcentral Gyrus, Central Opercular Cortex, Precentral Gyrus
	3	3	0.049	54	-14	30	Postcentral Gyrus

LBA: laterobasal amygdala. INT: internalizing group. HC: healthy control group. Æ: no significant difference. [a] Positive and negative effects of time within groups are investigated using design 1 provided in Supplemental Spreadsheet 1. [b] Associations with symptom changes are investigated using design 2 provided in Supplemental Spreadsheet 2. Threshold-free cluster enhancement (TFCE) and family-wise error (FWE) corrected at p-values < 0.05

Table S7 Coordinates of significant clusters in group x time interaction in sensitivity analyses including age as covariate.

	Cluster	Voxels	p-value	MNI coordinates			Regions involved
				X	*Y*	*Z*	
Left LBA seed without age covariate	1	581	0.003	0	50	-6	Frontal Pole; Frontal Medial Cortex; Paracingulate Gyrus; Anterior Cingulate Gyrus
Left LBA seed with age covariate	4	50	0.038	0	50	-6	Frontal Medial Cortex; Paracingulate Gyrus
	3	19	0.044	-8	60	2	Frontal Pole
	2	12	0.047	4	34	-6	Anterior Cingulate Gyrus
	1	1	0.05	6	28	-8	Subcallosal Cortex

LBA: laterobasal amygdala. Threshold-free cluster enhancement (TFCE) and family-wise error (FWE) corrected at p-values < 0.05.

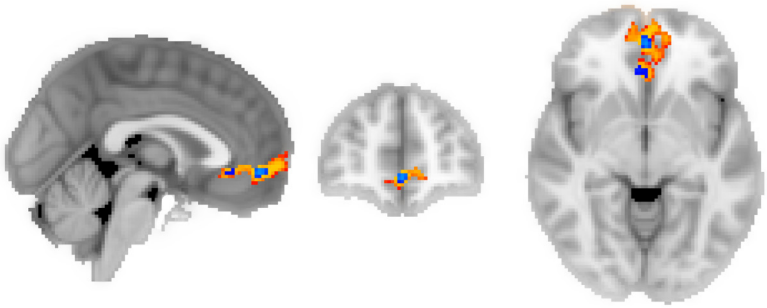

Figure S1 Different development of left LBA FC in adolescents with internalizing disorders compared to healthy peers in sensitivity analyses including age as covariate. Significant group x time interaction in left LBA FC to the frontal pole (INT (T2 > T1) > HC (T2 > T1)), coordinates of displayed slices (MNI, x, y, z): 0, 50, -6. Red represents results without age as covariate, blue represents results including age as covariate.

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Part 2

4

Investigating microstructure of white matter tracts as candidate endophenotypes of Social Anxiety Disorder – findings from the Leiden Family Lab study on Social Anxiety Disorder (LFLSAD)

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Neuroimage Clin 2020 Vol. 28 Pages 102493

Abstract

Background: Social anxiety disorder (SAD) is a mental illness with a complex, partially genetic background. Differences in characteristics of white matter (WM) microstructure have been reported in patients with SAD compared to healthy controls. Also, WM characteristics are moderately to highly heritable. Endophenotypes are measurable characteristics on the road from genotype to phenotype, putatively reflective of genetically based disease mechanisms. In search of candidate endophenotypes of SAD we used a unique sample of SAD patients and their family members of two generations to explore microstructure of WM tracts as candidate endophenotypes. We focused on two endophenotype criteria: co-segregation with social anxiety within the families, and heritability.

Methods: Participants (n = 94 from 8 families genetically vulnerable for SAD) took part in the Leiden Family Lab Study on Social Anxiety Disorder (LFLSAD). We employed tract-based spatial statistics to examine structural WM characteristics, being fractional anisotropy (FA), axial diffusivity (AD), mean diffusivity (MD) and radial diffusivity (RD), in three a-priori defined tracts of interest: uncinate fasciculus (UF), superior longitudinal fasciculus (SLF) and inferior longitudinal fasciculus (ILF). Associations with social anxiety symptoms and heritability were estimated.

Results: Increased FA in the left and right SLF co-segregated with symptoms of social anxiety. These findings were coupled with decreased RD and MD. All characteristics of WM microstructure were estimated to be at least moderately heritable.

Conclusion: These findings suggest that alterations in WM microstructure in the SLF could be candidate endophenotypes of SAD, as they co-segregated within families genetically vulnerable for SAD and are heritable. These findings further elucidate the genetic susceptibility to SAD and improve our understanding of the overall etiology.

Introduction

Social anxiety disorder (SAD) is a highly prevalent mental illness with a typical onset during late childhood and early adolescence [1]. Patients with SAD experience an excessive fear of negative evaluation in social situations, which are either avoided or endured with intense fear or anxiety [2]. SAD often has a chronic course and high comorbidity rates with other mental disorders [3-8]. Unfortunately, the wide range of cognitive behavioral and pharmacological therapies available proves to be insufficient in 30 – 40% of patients [9]. To improve current therapies and develop new interventions, the etiology of SAD should be further elucidated [10, 11].

In the past decades, research into the neurobiological background of mental disorders has increased and evolved, with a special interest in neuroimaging of the brain. Indeed, several mental disorders, including SAD, have been associated with altered brain functioning [11, 12]. For example, previous research in patients with SAD has reported abnormalities in the activity of the anxiety circuitry and its regulatory networks, such as hyperactivation of the amygdala and insula in relation to negative emotional stimuli [13] and decreased functional coupling between the amygdala and regulatory prefrontal cortical regions such as the orbitofrontal cortex [14]. In addition, altered resting state functional connectivity has been reported, such as decreased connectivity between the amygdala and frontal regions such as the medial, dorsolateral and ventrolateral prefrontal cortex (PFC) and between regions implicated in the default mode network like the medial PFC and the lateral parietal cortex (reviewed in MacNamara *et al.* [15]).

A recent neurofunctional model on SAD suggested disturbed emotion regulation networks in patients with SAD, with an imbalance between hyperactive parietal and medial occipital brain regions and fear circuitry on the one hand, and impairments in regulatory control networks in frontal areas on the other hand. This imbalance might be partly attributed to impaired communication between these areas due to decreased structural connectivity, such as abnormal microstructure of several white matter (WM) tracts [11].

A method to study characteristics of WM microstructure of the brain is diffusion tensor imaging (DTI), which in turn can be utilized to model four commonly used scalar measures of diffusivity: fractional anisotropy (FA), axial diffusivity (AD), radial diffusivity (RD) and mean diffusivity (MD). Differences in microstructure of several WM tracts, such as the uncinate fasciculus (UF), superior longitudinal fasciculus (SLF) and inferior longitudinal fasciculus (ILF), have been reported frequently in Magnetic Resonance Imaging (MRI) studies in SAD. For an overview of current literature on DTI studies in SAD, see Supplemental Table 1a and 1b, summarizing the work by Jenkins *et al.* [12], Tukel *et al.* [16], Qiu *et al.* [17], Phan *et al.* [18], Liao *et al.* [19], [20], Baur *et al.* [21]. The UF connects the prefrontal cortex with the anteromedial temporal lobe and is thought to be, among others, involved in social emotional processing [22]. In voxelwise analyses, decreased FA of the left and right UF has been previously reported in SAD patients compared to healthy controls [17, 18, 21] and one study replicated these results using tractography analyses [20]. The SLF is roughly divided in three subparts (I, II and III) and is the major tract connecting parietal cortices to prefrontal areas [23]. It is thought to subserve visuo-spatial attention

and language functionality [24], and in voxelwise studies decreased FA of the left SLF has been reported in patients with SAD [16, 21]. Interestingly, this finding was replicated in a voxelwise meta-analysis of FA [12]. The ILF originates from medial and lateral anterior temporal areas and terminates in the occipital lobe. This tract is suggested to play a role in facial recognition [25-27] and one voxelwise study reported decreased FA in patients with SAD compared to healthy controls [16]. Interestingly, when using a tract-of-interest (TOI) approach, an increase in average FA of the bilateral UF and right ILF has been reported after 10 weeks of cognitive behavioral group therapy [28] and higher values of a combined tractography measure of AD, MD, RD and FA of the right ILF has been found to predict better clinical response to cognitive behavioral therapy in individual and group settings [29]. Also, an inverse voxelwise relation between FA in a putative amygdala-prefrontal pathway and trait anxiety has been reported [30, 31]. Interestingly, the three WM tracts mentioned above are also thought to be involved in several resting-state functional brain regions and networks in which altered connectivity has been reported in SAD patients, such as regions involved in the DMN, the ventral attention network and the fronto-parietal network, [15, 32, 33]. These results suggest that abnormal WM microstructure, together with other underlying neurobiological processes, could be linked to SAD.

It is thought that the overall vulnerability to develop SAD is based on complex interactions between genetic (dis)advantages and liabilities, epigenetic factors and environmental factors [1, 34, 35]. For example, studies using twins and families indicated that genetic and non-shared environmental factors explained roughly equally most of the individual differences in SAD [36] and a recent genome wide association study (GWAS) analysis confirmed a heritable basis of SAD [37]. To study the genetic vulnerability to SAD more closely, an endophenotype approach could be used. Endophenotypes are defined as biological or psychological markers of a disorder, which are thought to be in the causal chain between genetic contributions to a disorder and diagnosable symptoms of psychopathology [38, 39]. The endophenotype approach assumes that underlying measurable components of a mental illness are heritable and present before the development of clinical symptoms and include, for example, neurobiological changes in brain structure and function. It is thought that endophenotypes are theoretically capable of providing greater statistical power to localize and identify disease related biomarkers than affection status alone [40]. To be considered an endophenotype, a candidate psychoneurobiological characteristic has to be associated with the disorder (criterion 1), state-independent (criterion 2), heritable (criterion 3) and co-segregate with the disorder within families of probands whilst already present in a preclinical state (criterion 4) [38, 39, 41, 42].

Several neurobiological candidate endophenotypes for SAD have been proposed, such as WM and grey matter (GM) characteristics, amygdala and prefrontal brain function and alterations in functional connectivity networks [43]. The Leiden Family Lab study on Social Anxiety Disorder (LFLSAD) is to our knowledge the first comprehensive two-generation family neuroimaging study on SAD and has been designed specifically to examine the heritability and first part of the co-segregation criteria of candidate endophenotypes of SAD [44]. Previous results of this study suggest that several characteristics of brain structure and function, like cortical and subcortical GM characteristics, increased and

prolonged amygdala activation, and increased brain activity whilst processing unintentional social norm violations, could be endophenotypes of SAD [45-49]. However, it still remains to be elucidated whether characteristics of WM microstructure could be candidate endophenotypes of SAD. Results of previous studies suggest that this could indeed be the case, as they were found to be associated with the disease in case-control studies (endophenotype criterion 1) and are at least moderately ($h^2 \geq 0.2$) to highly ($h^2 \geq 0.6$) heritable as shown by multiple studies in healthy twins (endophenotype criterion 3) [50, 51]. However, to our knowledge the criterion of co-segregation within families of probands (criterion 4, first element) has not been examined in previous work.

Using DTI data from the LFLSAD, the present study investigated characteristics of WM microstructure as candidate endophenotypes of SAD, with a focus on: 1) co-segregation within families genetically enriched for SAD and 2) estimation of heritability of WM characteristics. We employed tract-based spatial statistics (TBSS; [52]) to examine the association between measures of WM microstructure and measures of social anxiety. Our main parameter of interest was FA, while we used other parameters, being AD, MD and RD, to gain more insight into underlying WM microstructure (cf. van der Werff *et al.* [53], Aghajani *et al.* [54]). Based on previous research we expected to find a negative association between the level of social anxiety symptoms and FA and AD in the UF, SLF and ILF, coupled with a positive association between clinical symptoms and RD and MD. Furthermore, we expected estimates of all WM parameters to be at least moderately heritable. We employed a-priori defined TOI analyses in these three tracts using two types of analyses: a voxelwise analysis and an analysis of the averaged values of WM parameters over the whole TOI. In addition, we performed an exploratory voxelwise analysis of the whole WM skeleton to investigate WM microstructure outside the a-priori defined regions.

Methods and Materials

Participants

The LFLSAD is a multiplex (families were selected based on a minimum of two (sub)clinical SAD cases within one nuclear family) and multigenerational (multiple nuclear families encompassing two generations from the same family participated) family study on SAD (total sample: n = 132, from nine families, MRI participants n = 113), designed to investigate candidate neurobiological endophenotypes of SAD. A design like this is especially powerful to investigate environmental and genetic influences on SAD-related characteristics [44]. The background, objectives and methods as well as clinical characteristics of the sample and an a priori power analysis are described in more detail elsewhere [44]. Preregistration of the study is available on https://osf.io/e368h. The LFLSAD sample consists of families selected on presence of a primary diagnosis of SAD in a parent (25 – 55 years old; 'proband'), with a child who met the criteria for clinical or subclinical SAD (living at home, 8 – 21 years old; 'proband's SA child'). Furthermore, the partner of the proband, other children of this nuclear family (≥ 8 years of age), siblings of the proband and their partners and children (≥ 8 years of age) were invited to participate. Thus, the sample consisted of two generations of family members: the generation

of the proband (generation 1) and the generation of the proband's SA-child (generation 2; see Figure 1). Exclusion criteria for the proband or proband's SA child were comorbidity other than internalizing disorders; other family members were included regardless of the presence of psychopathology. Exclusion criteria for all participants for the MRI experiment were general MRI contraindications, such as metal implants or pregnancy.

MRI data from one family (n = 3 family members) had to be excluded from the present analysis as the proband from this family was not able to participate in the MRI experiment due to an MRI contraindication. DTI data from two subjects (9.4 y and 18.5 y) were not available due to an early stop of data acquisition during the MRI experiment, which consisted of several structural and functional scans as described elsewhere (total duration of the MRI protocol: 54 min 47 s) [44]. Thus, data from n = 108 participants was available for initial DTI analysis.

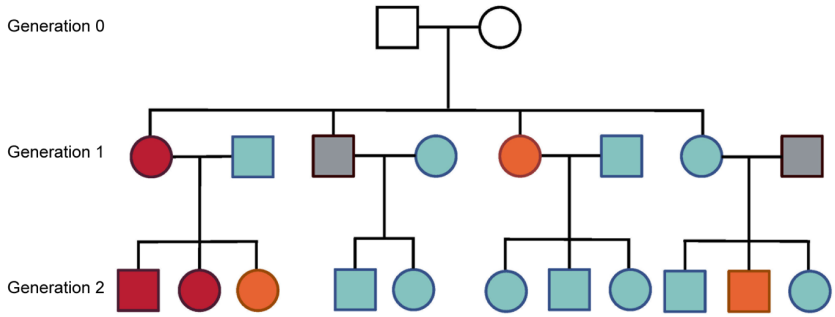


Figure 1 Family structure in the LFLSAD. Example of a family within the Leiden Family Lab study on Social Anxiety Disorder. Families were included based on the combination of a parent with social anxiety disorder (SAD; "proband": depicted in red) and a proband's child with SAD (red) or (sub)clinical SAD (orange). In addition, family members of two generations were invited, independent from the presence of SAD within these family members (no SAD: light blue; did not participate: gray). Grandparents (Generation 0; white) were not invited for participation. This family is slightly modified to guarantee anonymity; however, the number of family members and the frequency of (sub)clinical SAD are depicted truthfully. Squares and circles represent men and women, respectively. This figure is a reprint of the figure published in [44].

Ethics

The LFLSAD study was approved by the Medical Ethical Committee of the Leiden University Medical Center (P12.061). All participants provided informed consent according to the Declaration of Helsinki; both parents signed the informed consent form for their children, while children between 12 and 18 years of age signed the form themselves as well.

Phenotyping

Confinement of diagnosis

To determine the presence of DSM-IV diagnoses, with special attention to SAD, the Mini-International Neuropsychiatric Interview (MINI)-Plus (version 5.0.0) or MINI-Kid (version 6.0) was used by experienced clinicians and was voice-recorded for review [55-58]. Diagnosis of clinical SAD was

determined using the DSM-IV-TR criteria for the generalized subtype of SAD, but a clinician verified that DSM-5 criteria were also met to establish the diagnosis. Subclinical SAD was diagnosed when DSM-5 criteria were met, but important areas of functioning were not impaired (criterion G) [2].

Questionnaires

All participants completed self-report questionnaires regarding anxiety-related symptoms. If applicable, age-appropriate questionnaires were used. Among others, we measured social anxiety symptoms, using the Liebowitz Social Anxiety Scale (LSAS-SR) for adults (≥ 18 years of age) [59, 60] and the Social Anxiety Scale for Adolescents (SAS-A) for younger participants (< 18 years of age) [61], the intensity of fear of negative evaluation using the revised Brief Fear of Negative Evaluation (BFNE – II scale) [62, 63] and the level of trait anxiety using the State-Trait Anxiety Inventory (STAI) [64]. Furthermore, depressive symptoms were evaluated by the self-report Beck Depression Inventory (BDI-II) for adults [65, 66] or the Children's Depression Inventory (CDI) for adolescents [67]. To analyze the scores of the age-appropriate self-report questionnaires, z-scores were computed for the level of social anxiety symptoms and depressive symptoms as described previously [44].

Incidental missing values on the self-report questionnaires were replaced by the mean value of the completed items. Data on the BFNE-II was missing for one participant. Differences in scores on self-report questionnaires between participants with (sub)clinical SAD and non-affected relatives were assessed by fitting regression models in R [68]. Within these models, outcomes from self-report questionnaires (levels of social anxiety symptoms (z-score; z-SA), intensity of fear of negative evaluation (FNE), depressive symptoms (z-score) and trait anxiety) were modelled as dependent variables and (sub) clinical SAD as independent variable. Genetic correlations between family members were accounted for by including random effects in the models, and gender and age (centered) were added as covariates. The Bonferroni method was used to correct p-values for multiple comparisons (4 tests, corrected p-value = 0.0125).

MRI data acquisition

Scanning was performed at Leiden University Medical Center using a 3.0T Philips Achieva MRI scanner (Philips Medical Systems, Best, The Netherlands), equipped with a 32-channel Sensitivity Encoding (SENSE) head coil. The MRI session consisted of several structural and functional scans, as described elsewhere (total duration of the MRI protocol: 54 min 47 s) [44]. Of interest for the present study are two diffusion weighted imaging (DWI) scans with the following characteristics: repetition time (TR) 7316 ms, echo time (TE) 69 ms, field of view (FOV) 240 x 240 x 150 mm, acquisition matrix 128 x 128 with 75 slices, slice thickness 2 mm, voxel size 1.9 x 2.4 mm. DWI scans (b = 1000 s/mm^2) were acquired in 30 directions with two additional non-DWI scans (b = 0 s/mm^2) serving as reference scans. The two DWI scans were collected with reversed phase-encode blips, resulting in pairs of images with distortions going in opposite directions (anterior-posterior and posterior-anterior). Scan duration for each scan was about 4 min 30 s. Furthermore, one high-resolution T1-weighted structural scan was acquired with the following characteristics: 140 slices, resolution 0.875 mm × 0.875 mm × 1.2 mm, FOV = 224 mm × 168

mm × 177.333 mm, TR = 9.8 ms, TE = 4.59 ms, flip angle = 8°. All structural MRI scans were inspected by a neuroradiologist. No clinically relevant characteristics were reported in any of the participants.

Data analysis

DTI processing

DTI models diffusivity of water molecules across the brain, using tensors. These tensors consist of three main eigenvalues (λ_1, λ_2 and λ_3) which in turn can be used to calculate the four most commonly used characteristics of WM microstructure: fractional anisotropy (FA), axial diffusivity (AD), radial diffusivity (RD), and mean diffusivity (MD) [69]. FA provides a relative difference between the largest eigenvalue as compared to the others, reflecting the tendency of water molecules to diffuse in one direction as opposed to all others and could therefore be described as a general indicator for WM microstructure (e.g. myelin thickness, membrane integrity) [69, 70]. AD is defined as the first eigenvalue (l_1) and reflects water diffusion along the principal direction of the fiber, displaying fiber bundle coherence and axonal integrity [71]. RD is defined as the average of the second and third eigenvalue (λ_2 and λ_3) and reflects water diffusion perpendicular to the principal direction of the fiber, thus being more indicative of the level of myelinization [72]. MD is defined as the average of the three eigenvalues and hence reflects average water diffusion in all directions within a fiber, thus putatively reflective of a degree of myelination [73]. In general, decreased FA is coupled with decreased AD and / or increased RD and MD and vice versa [69, 74].

Image pre-processing and analyses were performed using the Oxford Centre for Functional Magnetic Resonance Imaging of the Brain (FMRIB) Software Library (FSL) [75]. The susceptibility-induced off-resonance field from the two pairs of DWI images was estimated using a method similar to that described in [76] and the two images were combined into a single corrected one. Afterwards, the Brain Extraction Tool (BET) was used to remove non-brain tissue from the non-diffusion images [77]. Image distortion and motion artefacts induced by eddy currents or inter-volume head motions were corrected [78] and image quality was statistically evaluated afterwards [79]. No outliers were detected. Individual FA images and primary (l_1), secondary (l_2) and tertiary (l_3) eigenvalues were created by fitting a tensor model to the raw diffusion data using FMRIB's Diffusion Toolbox (FDT) [80]. Individual maps of other diffusivity measures were calculated out of eigenvalues, defining AD as l_1, RD as $l_{23} = (l_2 + l_3) / 2$ and MD as $l_{123} = (l_1 + l_2 + l_3)/3$. The individual vector and raw FA images were visually and statistically evaluated for alignment on WM tracts according to standardized protocols, designed to facilitate harmonized image analysis across multiple sites (http://enigma.ini.usc.edu/protocols/dti-protocols/).

Then, a study-specific custom FA template was created. All subjects' FA data were slightly eroded and aligned into a common space using the nonlinear registration tool FNIRT [81, 82], which uses a b-spline representation of the registration warp field [83]. Afterwards, a mean FA image and distance map to the masked template were created. Individual FA and non-FA (AD, MD and RD) images were then projected onto the template. Subsequently, quality control was performed twofold: we visually inspected the registered images for misalignment onto the skeleton, and individual projection distances of the

extracted skeletons onto the template were calculated to detect outliers (defined as individual projection distance to the template exceeding the threshold of 3.8 mm), which could represent bad alignment to the template [84]. All images were well aligned, and no outliers were detected.

Investigation of WM candidate endophenotypes

We focused on two endophenotype criteria: (i) co-segregation of the candidate endophenotypes with the disorder within families (the first element of criterion 4) and (ii) heritability estimation (h^2) of characteristics of WM microstructure (criterion 3). Co-segregation of WM characteristics within families was examined by exploring the relationship between parameters of WM microstructure and two-dimensional measures of social anxiety (z-SA and FNE) using three a priori defined WM tracts based on previous literature: the SLF, ILF and UF (see Supplemental Figure 1 for TOIs, for an overview of current literature, see Supplemental Table 1a and 1b). To investigate WM microstructure outside the a-priori defined regions, we also examined the association between WM parameters and a diagnosis of (sub)clinical SAD (see Supplemental section).

Selection of tracts

Binary unilateral masks of the SLF, UF and ILF were created using the probabilistic Johns Hopkins University (JHU) white-matter tractography atlas [85] provided by FSL, thresholded at a conventional 20% [52]. Using the mean FA skeleton, each tract was unilaterally masked to include only voxels comprised in both the tract and the skeleton. This confines the statistical analysis to voxels from the center of the tract, thereby minimizing anatomic intersubject variability, deviations in registration and partial volume effects [86].

Co-segregation

To examine co-segregation of WM characteristics, voxelwise and average TOI analyses were conducted. We report findings uncorrected for the number of tracts because of the use of a priori defined TOIs, which are possibly also functionally related [87] and because of the innovative and more explorative nature of the present study (to the best of our knowledge, this is the first comprehensive family study on SAD). To investigate WM microstructure outside the a-priori defined regions, an exploratory analysis was conducted to investigate voxelwise associations between FA in the WM skeleton and the level of self-reported symptoms of SAD was conducted using NINGA (see Supplemental section for more details and results).

For the voxelwise and average TOI analyses, sensitivity analyses were conducted to control for (i) psychopathology other than SAD and (ii) severity of depressive symptoms. Therefore, all participants with past and/or present (comorbid) psychopathology other than SAD were excluded (sensitivity analysis 1); or the z-score of the level of depressive symptoms was added as a covariate in the analyses (sensitivity analysis 2). Details and results of these analyses are included in the Supplemental section.

Voxelwise TOI analysis

First, we performed voxelwise analyses within each of the TOIs to examine subtle localized differences which could disappear in average TOI analyses due to the size of the TOI. In these voxelwise TOI analyses, FA was used as principal outcome measure as this is a general indicator of WM microstructure. Additional WM parameters, being AD, MD and RD, were examined for significant clusters only, to provide complementary information about WM microstructure and to aid interpretation of FA changes. The voxelwise TOI analyses were conducted by performing multiple nonparametric regression analyses using the NeuroImaging Nonparametric Genetic Analysis (NINGA) toolbox [88, 89] as methods previously used in analyses of fMRI data of the LFLSAD could, due to the specific structure of TBSS data and assumptions regarding the random field theory, not account for family-wise errors (FWE). NINGA implements linear mixed effect for covariate inference in presence of family relatedness using an approximate non-iterative random effect estimator based on restricted maximum likelihood function. It uses permutation test to provide essential spatial statistics inference tools for uncorrected and family-wise error (FWE) corrected p-values. Individual levels of self-reported social anxiety (z-SA and FNE) were modelled as independent variables and voxelwise FA values as the dependent variable. Covariate inference was incorporated in the model to account for nuisance kinship, gender and age. We used Threshold-Free Cluster Enhancement (TFCE) statistics to define significant clusters, and permutation testing to provide FWE corrected p-values at a conventional threshold of a= 0.05.

To gain more insight in the direction of FA in clusters displaying significant associations with SA-symptoms, RD, MD and AD were examined by extracting and binarizing the significant cluster from the previous analysis. This mask was then used to comprise only the relevant voxels in individual skeletons of AD, MD and RD. Next, individual levels of self-reported social anxiety symptoms (z-SA and FNE) were modelled as independent variables and values of AD, MD or RD in the relevant cluster as the dependent variable. Covariate inference was incorporated to account for nuisance kinship, gender and age.

Average TOI analysis

Second, we conducted conventional TOI analyses using average individual values of WM parameters over the whole tract to allow comparison with previous literature ("average TOI analyses") and to ensure continuity with previous analyses performed on data from the LFLSAD [45].That is, following methods previously described [45-47, 49], associations between individual average values of WM parameters (FA, MD, RD and AD over the whole tract) per tract and clinical symptoms of SAD (z-SA and FNE) were examined by performing multiple regression analyses using linear mixed models in R [68]. Average values of FA, AD, MD and RD were extracted for each individual unilaterally per tract. Per TOI, mean WM parameters were modelled as dependent variables and the outcomes of self-report questionnaires as independent variables. Correlations between family members were accounted for by including random effects in the models [90]. Both age (centered) and gender (centered) were included as covariates. As most of the dependent variables were non-normally distributed, the robustness of the linear mixed model used was confirmed by checking the distribution of the residuals of the phenotypes with the Shapiro-

Wilk normality test and visual inspection, which showed all residuals followed an approximate normal distribution.

Heritability

Next, general heritability (h^2) of the WM microstructure characteristics was estimated, using methods previously used in analyses of the LFLSAD sample to ensure consistency [45-49]. This method estimates heritability by jointly modelling SAD status and the individual average values of FA, AD, MD, and RD within all TOIs in a multivariate-mixed probit model, by which the familial relationship and ascertainment of the families (based on SAD in the proband and (sub)clinical SAD in the proband's SA-child) were taken into account [90]. To adjust for age and gender, these variables were included as covariates (both centered) in the marginal regression models. Variance of the random effects was determined using maximum-likelihood estimates; subsequently, heritability was estimated [90].

Results

Sample characteristics

Sample characteristics are summarized in Table 1. Participants with (sub)clinical SAD reported significantly higher levels of social anxiety (self-reported social anxiety symptoms (z-SA) and FNE), depressive symptoms and trait anxiety compared to their non-SAD relatives but did not differ with respect to gender-distribution, generation, age or IQ. For a more elaborate description of this sample, including diagnostic information and details on quality checking and data availability, we refer to the Supplemental section and previous publications on the LFLSAD in general [44] and the MRI sample in particular [45-47, 49].

Table 1 Characteristics of participants with and without (sub)clinical SAD.

	(Sub)clinical SAD (n = 31)	No SAD (n = 57)	Statistical analysis
Demographics			
Male / Female (n)	13 / 18	28 / 29	$c^2 = 0.42$, p = .52
Generation 1 / Generation 2 (n)	19 / 12	27 / 30	$c^2 = 1.56$, p = .21
Age in years (mean ± SD); range	33.7 ± 15.5 (9.2 - 59.6)	32.9 ± 14.8 (9.6 - 61.5)	b ± SE = 0.8 ± 3.3, p = .80
Estimated IQ (mean ± SD)	102.2 ± 12.2	105.5 ± 10.8	b ± SE = -3.1 ± 2.4, p = .21
Diagnostic information (n)			
Clinical SAD	15	0	$c^2 = 33.3$, p < .001**
Self-report measures (mean ± SD)			
Social anxiety symptoms (z-score)	2.4 ± 3.3	0.7 ± 1.3	b ± SE = 1.9 ± 0.5, p < .001**
FNE	23.0 ± 12.4	12.0 ± 7.6	b ± SE = 10.6 ± 2.2, p < .001**
Depressive symptoms (z-score)	0.05 ± 0.9	-0.6 ± 0.6	b ± SE = 0.6 ± 0.2, p = .001**
Trait anxiety	38.0 ± 9.8	33.2 ± 8.6	b ± SE = 5.0 ± 2.0, p = .01*

SAD: social anxiety disorder; FNE: fear of negative evaluation; STAI: state-trait anxiety inventory; SD: standard deviation. Sample for dimensional analysis: n = 94 for z-SA, n = 93 for FNE. Data on the presence of subclinical SAD were, due to technical reasons, lost for six family members (remaining sample for categorical analysis: n = 88). ** significant at Bonferroni corrected p-value of 0.0125; * significant at uncorrected p-value of 0.05.

Co-segregration

Three bilateral TOIs were examined in voxelwise and average TOI analyses to explore the association between WM microstructure and clinical symptoms, as measured by self-reported levels of social anxiety (z-SA) and intensity of FNE. Both analyses revealed that higher levels of z-SA and FNE were significantly associated with higher FA values and lower MD and RD values in the left and right SLF. These significant findings will be discussed more in depth in the following paragraphs. We did not find any significant correlations between levels of social anxiety or FNE and WM microstructure in the bilateral UF or ILF.

Voxelwise TOI analysis

Significant associations are summarized in Table 2 and illustrated in Figure 2. A cluster in the left SLF was significantly positively associated with levels of social anxiety and FNE (b = 0.147, p = 0.006 and b = 0.039, p = 0.002 resp.). In addition, a cluster in the right SLF was significantly positively associated with levels of FNE (b = 0.027, p = 0.04). In both clusters, these findings were coupled with significant negative associations between the level of clinical symptoms and MD and RD. AD was not significantly associated with clinical symptoms.

Table 2 Significant associations between measures of SA and voxelwise TOI analyses of fractional anisotropy in the SLF; post-hoc analyses of additional WM parameters within the clusters.

Clinical measure	Side	WM parameter	Voxels (mm^3)	Peak MNI coordinates			b	p
				x	y	z		
z-SA	L	FA	207	-32	-39	29	0.147	.006
		AD					0.071	.37
		MD					-0.012	.03
		RD					-0.076	< .001
FNE	L	FA	178	-33	-38	29	0.039	.002
		AD					0.017	.49
		MD					-0.004	.01
		RD					-0.021	< .001
FNE	R	FA	51	34	-30	32	0.027	.04
		AD					0.013	.50
		MD					-0.023	< .001
		RD					-0.023	< .001

Threshold-free cluster enhancement (TFCE) and family-wise error (FWE) corrected at p-values < 0.05. ß-values and p-values represent the outcome of the analyses on mean values of white matter integrity over all voxels. z-SA: social anxiety (z-score); FNE: fear of negative evaluation; FA: fractional anisotropy; AD: axial diffusivity; MD: mean diffusivity; RD: radial diffusivity; L: left; R: right.

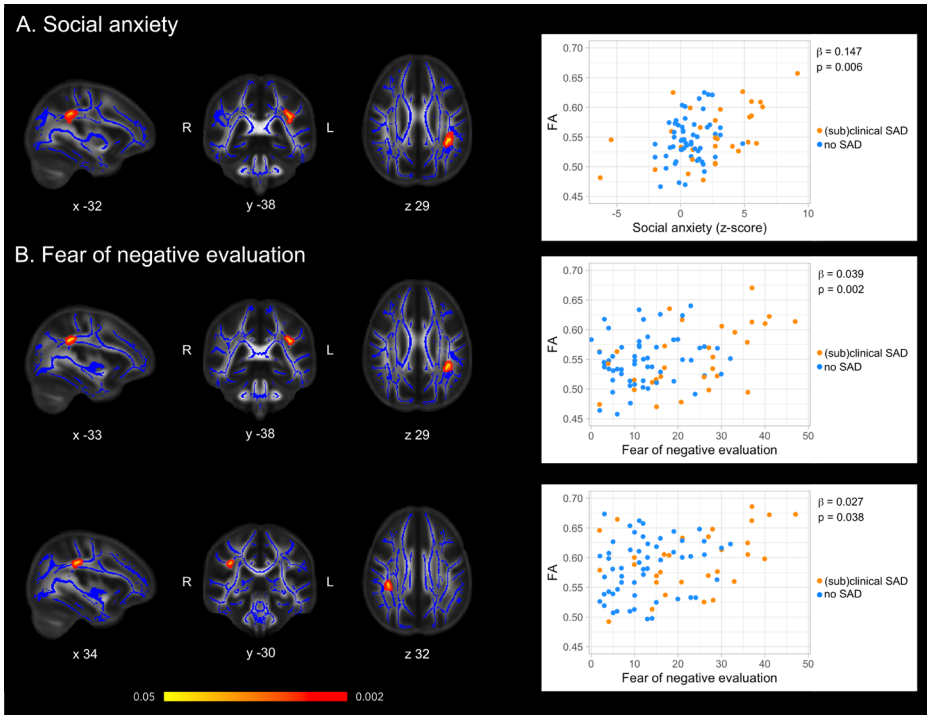


Figure 2 Significant clusters from voxelwise TOI analyses of fractional anisotropy in the superior longitudinal fasciculus. Sagittal, coronal and axial sections of the WM skeleton (blue), with subregions of the superior longitudinal fasciculus (SLF) showing significant associations of fractional anisotropy (FA) with levels of A) social anxiety (z-score) and B) fear of negative evaluation within families genetically enriched for social anxiety disorder (SAD) ($p < 0.05$, threshold-free cluster enhancement (TFCE) and family-wise error (FWE) corrected (yellow/orange)). The color bar indicates p-values.

Average TOI analysis

For all TOIs and all four parameters of WM microstructure, associations with clinical symptoms can be found in Table 3. In accordance with findings from the voxelwise TOI analyses, mean FA in the left SLF demonstrated a significant positive association with levels of self-reported social anxiety (b = 0.002, p = 0.03) and a near-significant association with levels of FNE (b = 3.67E-04, p = 0.06). In addition, mean FA in the right SLF showed a significant positive association with levels of intensity of FNE (b = 3.58E-04, p = 0.04). This finding was coupled with a significant negative association with mean RD values (b = -4.39E-07, p = 0.04) and a marginally significant, negative association with mean MD values (b = -3.15E-07, p = 0.09).

Heritability

Per TOI, heritability of every WM parameter was estimated over its average value. All results displayed at least moderate heritability (0.2 – 0.4), extending to very high heritability (0.9 – 1.0) for some WM parameters [91]. Results are summarized in Table 3.

Table 3 Associations between measures of SA and average values of white matter parameters in tracts of interest.

TOI	Side	WM parameter	Effect of social anxiety (z-score)			Effect of FNE			Heritability estimate	
			β	SE	p	β	SE	p	h^2	SE
ILF	L	FA	0.001	0.001	.58	3.44E-04	2.18E-04	.11	0.30	0.02
		AD	-4.46E-07	1.98E-06	.82	-1.37E-07	4.21E-07	.74	0.99	0.48
		MD	-9.58E-07	1.52E-06	.53	-3.38E-07	3.25E-07	.30	0.90	0.13
		RD	-1.20E-06	1.45E-06	.41	-4.17E-07	3.11E-07	.18	0.66	0.34
	R	FA	0.001	0.001	.28	1.89E-04	1.77E-04	.29	0.41	0.09
		AD	-3.46E-07	1.29E-06	.79	-1.15E-07	2.61E-07	.66	0.87	0.07
		MD	-1.09E-06	9.70E-07	.26	-2.57E-07	1.95E-07	.19	0.79	0.05
		RD	-1.35E-06	1.05E-06	.20	-2.66E-07	2.19E-07	.23	0.57	0.00
SLF	L	FA	0.002	0.001	.03	3.67E-04	1.92E-04	.06	0.41	0.05
		AD	1.15E-06	1.33E-06	.39	2.76E-08	2.75E-07	.92	0.73	0.21
		MD	-7.33E-07	1.13E-06	.51	-3.05E-07	2.34E-07	.19	0.70	0.01
		RD	-1.68E-06	1.21E-06	.17	-4.57E-07	2.55E-07	.07	0.59	0.03
	R	FA	0.001	0.001	.07	3.58E-04	1.76E-04	.04	0.31	0.02
		AD	1.21E-07	1.14E-06	.92	2.03E-08	2.26E-07	.93	0.84	0.05
		MD	-1.02E-06	9.14E-07	.27	-3.15E-07	1.84E-07	.09	0.78	0.05
		RD	-1.56E-06	1.04E-06	.13	-4.39E-07	2.16E-07	.04	0.58	0.04
UF	L	FA	0	0.001	.79	2.68E-04	3.19E-04	.40	0.37	0.05
		AD	1.16E-06	2.14E-06	.59	-1.43E-07	4.67E-07	.76	0.53	0.00
		MD	2.66E-07	1.56E-06	.86	-2.84E-07	3.38E-07	.40	0.51	0.01
		RD	-5.01E-08	1.68E-06	.98	-3.58E-07	3.59E-07	.32	0.51	0.10
	R	FA	0	0.001	.67	1.69E-04	2.48E-04	.50	0.61	0.02
		AD	1.05E-06	1.74E-06	.55	2.69E-07	3.77E-07	.48	0.26	0.01
		MD	-1.53E-07	9.36E-07	.87	-6.65E-09	1.91E-07	.97	0.73	0.01
		RD	-4.98E-07	1.11E-06	.65	-1.12E-07	2.27E-07	.62	0.72	0.14

TOI: tract of interest; WM: white matter; ILF: inferior longitudinal fasciculus; SLF: superior longitudinal fasciculus; UF: uncinate fasciculus; L: left; R: right; FA: fractional anisotropy; AD: axial diffusivity; MD: mean diffusivity; RD: radial diffusivity; FNE: fear of negative evaluation; SE: standard error; h^2: heritability estimate.

Discussion

In the present study we investigated whether characteristics of WM microstructure could be candidate endophenotypes of SAD. To our knowledge, this is the first comprehensive family study on WM microstructure in SAD, which enabled us to specifically examine co-segregation of WM characteristics in families of probands, as affected and non-affected family members have participated in this study (Bas-Hoogendam et al., 2018a). As recently stated by Glahn *et al.* [40], a multiplex, multigenerational family design like the LFLSAD is particularly powerful to investigate candidate endophenotypes as "[..] Reduced environmental variation among family members can reduce noise, improving statistical power to observe genotype-phenotype associations. [..] Designs that require multiple affected individuals in a family may result in a more severe phenotypic profile and a different underlying genetic architecture as compared to simplex families. [..] Family selection also impacts the distribution of phenotypes among unaffected family members, with members of multiplex families generally having greater endophenotype impairment than simplex family members."

In the present work, we focused on two endophenotype criteria, namely co-segregation of WM characteristics with social anxiety within participating families and estimation of heritability of these WM characteristics. Voxelwise and average tracts of interest (TOI) analyses were used to examine associations between measures of self-reported social anxiety and WM characteristics in the UF, SLF and ILF. For all three TOIs, heritability of WM characteristics was estimated.

Our analyses revealed that increased FA in the left and right SLF co-segregated with social anxiety within families enriched for SAD. These findings were coupled with decreased MD and RD and were consistent across both TOI analyses. The voxelwise results suggest that significant clusters are located in the SLF II. Furthermore, and in line with previous literature, all WM characteristics were estimated to be at least moderately heritable [50, 92].

The SLF II is the major part of the SLF and is mostly concerned with visuospatial attention and processing. Structurally it connects the caudal part of the inferior parietal lobule (IPL) and intraparietal sulcus with the posterior part of the prefrontal cortices. Functionally, the SLF II is thought to connect the parietal part of the ventral attention network with the prefrontal component of the dorsal attention network and is involved in the default mode network (DMN) [23, 24, 93-98].

Interestingly, previous studies that reported decreased FA and increased RD in the SLF described clusters in a different subpart of the SLF, namely SLF III, in patients with SAD compared to healthy controls (see Supplementary Table 1a and 1b; Tukel et al. [16], Qiu et al. [17], Baur et al. [21]. These findings were replicated in a meta-analysis using these three studies [12]. It should be noted that the SLF III differs from the SLF II as it extends from the supramarginal gyrus to the ventral premotor regions and is thought, among others, to be involved in language processing Schmahmann et al. [23], [24, 94]. In addition, the studies mentioned above had a different study design: they examined WM in SAD patients versus healthy controls in a case-control design, whilst we investigated WM microstructure in families

genetically enriched for SAD using a unique family study design. To the best of our knowledge, only one other study examining WM in SAD, using a case-control design and tractography analyses, reported increased FA [19]. In this study, the FA-increase was located in fibers passing through the genu of the corpus callosum.

Current literature suggests that increased FA coupled with decreased MD and RD and unchanged AD could imply dense axonal packaging or increased myelination [69, 99-101]. It should be noted that increased FA does not necessarily mean a better connection of the WM tract involved; instead, this could be suggestive of a compensatory mechanism, due to reduced crossing WM fibers or a more coherent alignment of fibers in the SLF II [99, 102, 103].

Our findings might seem contradictory to the neurofunctional model of the socially anxious brain described by Bruhl et al. [11], which proposed that decreased structural connectivity of, among others, the SLF could contribute to decoupling of hyperactive parietal and medial occipital brain regions from other networks involved in emotion regulation such as amygdala, limbic, salience and ventral attention networks. However, this subset of the model was based on the three DTI studies described above [16, 17, 21] and might yet be partly conceptual.

As recently reviewed by Bas-Hoogendam et al. [104], neuroimaging studies in SAD have reported multimodal changes in the brain. For example, changes in functional connectivity of the DMN have been reported. The DMN is thought to be involved in, among others, social referencing [15, 105]. In addition, cortical thickness and surface area of the IPL are positively associated with SAD [45]. Also, heightened activity in the medial temporal gyrus, superior temporal gyrus, and superior temporal sulcus during unintentional social norm processing is associated with SA [46] and a recent fMRI study by Kim et al. [106] reported heightened processing and prolonged attention during social threats in the IPL and the supramarginal gyrus. As the SLF II connects the IPL to the middle and superior frontal gyri, these results are relevant for the interpretation of our present findings.

We propose a few hypotheses for our findings, but realize that the current literature is not yet clear about the differentiation of subsets of the SLF and FA, and their involvement in SAD. First, the SLF II might be involved in the DMN as it connects different hubs of this network, and changes in the DMN have been reported in patients with SAD [15]. Second, the SLF II might be involved in abnormal visual processing as this tract is associated with posterior temporal parts and the attention network, and visual biases have been reported in SAD. Finally, FA is known to be decreased when strong fibers in multiple directions are present (e.g. in a crossing fiber area). If myelination of one fiber bundle in one of these directions is decreased, FA could be increased. Therefore, as our significant findings are in an area with a high amount of crossing fibers, we cautiously propose that increased social anxiety could be associated with lower myelination in one fiber bundle in a crossing fiber area.

Limitations and recommendations for future studies

Although this study is the first comprehensive two-generation family neuroimaging study on SAD, thus enabling investigation of WM microstructure as potential endophenotype of SAD, the findings of this study should be interpreted in light of its limitations. First, some participants have mental comorbid disorders, which thus might have influenced our results. However, as comorbidity is high in the clinical population of SAD patients [3-6], we deem our cohort as a representative sample. In addition, results of sensitivity analyses, in which we excluded participants with comorbid mental disorders, were in line with the main results. In addition, we would like to mention that although we used a continuous scale to analyze social anxiety rather than a dichotomous SAD versus non-SAD approach, we recognize our findings might not be SAD specific as changes in the SLF have also been reported in other mental emotional disorders, for example in a transdiagnostic meta-analysis of emotional disorders [12]. We therefore recommend future studies to consider transdiagnostic approaches, for example using the Research Domain Criteria framework [107].

Second, microstructure of WM tracts has been examined both in voxelwise and average TOI analyses to allow for interpretation in the light of the current literature whilst also being able to detect subtle changes in WM tracts. As we did not use tractography to analyze the DTI data, a method which is able to trace anatomical connections of WM between several brain regions and thus examine crossing fibers and different subsets of the SLF, we cannot investigate the idea that increased FA is a result of decreased myelination in one fiber bundle in a crossing fiber area. In addition, no post-hoc correction for the number of TOIs (n = 3, bilateral) has been applied thus results should be interpreted carefully. Also, analyses were done in the WM skeleton (and in TOIs), limiting it to the core of major WM tracts. As a result, more peripheral WM parameters in smaller tracts were not investigated. Therefore, future analyses should consider including tractography to investigate crossing fibers and distinguish between the different subsets of the SLF and their involvement in SAD. Also, we have only corrected linearly for age whilst the development of WM is known to peak at 28 years, followed by a slow decline [108]. In addition, the genetic data for GWAS analyses are not yet available, thus our findings cannot be linked to genetic variation yet. Finally, due to the lack of healthy control families without SAD, we could not examine the second part of the fourth endophenotype criterium (nonaffected family members show other levels of the endophenotype compared to the general population). As endophenotypes are thought to be present before the development of clinical symptoms, they should thus be measurable in unaffected family members and differ from healthy control families. This could not be investigated in the present study. In addition, as a cross-sectional design has been used, we could not examine the second endophenotype criterium (state-independency). Therefore, to further examine its potential role as an endophenotype, we advise future studies to consider including control families to compare changes in the SLF in nonaffected family members to SLF microstructure in healthy control families, and to consider a longitudinal design to study trait-stability.

Conclusion

The findings of the present work confirmed our hypothesis that altered white matter microstructure could be a candidate endophenotype of SAD. However, contradictory to our hypothesis of decreased FA, we found that increased FA in the SLF II co-segregated with SA within families genetically enriched for SAD. This was coupled with decreased RD and MD. Furthermore, all white matter characteristics were estimated to be at least moderately heritable, thus supporting the heritability criterion for endophenotypes. These findings might further elucidate the genetic susceptibility to SAD and improve our understanding of the overall etiology.

Supplemental Methods

Ethics

Every participant received €75 for participation in the LFLSAD (duration whole test procedure, including breaks: 8 hours) [44] and travel expenses were reimbursed. Furthermore, participants were provided with lunch/dinner, snacks and drinks during their visit to the lab. Confidentiality of the research data was maintained by the use of a unique research ID number for each participant.

Questionnaires

To estimate cognitive ability, we used the similarities (verbal comprehension) and block design (perceptual reasoning) subtests of the Wechsler Adult Intelligence Scale IV (WAIS-IV) [109] or Wechsler Intelligence Scale for Children III (WISC-III) [110].

Statistics

Differences in the presence of other psychopathology between (sub)clinical SAD and non-affected family members were assessed by the chi-square test in IBM SPSS Statistics for Windows [111] (10 tests, Bonferroni-corrected p-value = 0.005). Results can be found in Supplemental Table 2.

Reference values for z-scores on questionnaires

The following references were used (mean ± SD): LSAS-SR: 13.5 ± 12.7 [59]; SAS-A: 34.7 ± 2.3 [112]; BDI-II: 10.6 ± 10.9 [113]; CDI: 8.9 ± 5.4, unpublished data from the study by [112].

DTI-preprocessing

Prior to the MRI scan, all participants were informed about the MRI safety procedures and they were told that they could refrain from continuing the experiment at any time. Children and adolescents were familiarized with the MRI scanner using a mock scanner [114].

DWI data were converted to DICOM file format using dcm2niix [115].

Association between SAD diagnosis and parameters of WM integrity in TOI analyses

We examined the relationship between WM integrity and a diagnosis of (sub)clinical SAD in both voxelwise TOI analyses (using NINGA) and average TOI analyses in a similar way to the dimensional TOI analyses. As we assumed the same endophenotype to be reflected in both clinical and subclinical cases, (sub)clinical SAD was modelled as the independent variable. Covariate inference was incorporated in the model to account for nuisance kinship, gender and age. In order to obtain a reliable estimate of the main effect of (sub)clinical SAD, an interaction term assessing (sub)clinical SAD-by-age was also included in the model. Because data on the presence of subclinical SAD were, due to technical reasons, lost for six family members, data from these participants could not be used for this analysis (remaining sample: n = 88). Again, additional WM parameters were examined for significant FA clusters only.

Voxelwise analysis of whole WM skeleton

For reasons of completeness, we also explored voxelwise co-segregation of social anxiety with WM integrity in the white matter tracts in the skeleton using NINGA. Individual levels of self-reported social anxiety (z-SA and FNE) were modelled as independent variables and voxelwise FA values as the dependent variable. Covariate inference was incorporated in the model to account for nuisance kinship, gender and age. We used Threshold-Free Cluster Enhancement (TFCE) statistics to define significant clusters, and permutation testing to provide FWE-corrected p-values at a conventional threshold of a = 0.05 to correct for multiple comparisons. Additional parameters of WM integrity, being RD, MD and AD, were examined for significant clusters only by extracting and binarizing the significant FA cluster from the previous analysis. This mask was then used to comprise only the relevant voxels in individual skeletons of AD, MD and RD. Next, individual levels of self-reported social anxiety symptoms (z-SA and FNE) were modelled as independent variables and AD, MD or RD in the relevant cluster as the dependent variable. Again, covariate inference was incorporated to account for nuisance kinship, gender and age.

Supplemental Results

Sample characteristics after quality checking and data availability

The sample for the present analyses consisted of data of 110 participants from eight families (56 males (50.9 %), mean number of participating family members per family: 13.8, range 5 – 28). These family members were, according to the design, divided over two generations (generation 1: n = 51, 24 males; age (mean ± SD, range) 46.5 ± 6.7 years, 34.3 – 61.5 years; generation 2: n = 59, 32 males, age 18.1 ± 6.0 years, 9.0 – 32.2 years) who differed significantly in age ($\beta = -30.3$, $p < 0.001$), but not in male/female ratio ($\chi^2 = 0.56$, $p = 0.57$). After pre-processing, data from n = 94 participants was available for further analysis, as for two subjects BET was unable to adequately extract non-brain tissue images and an additional twelve subjects had to be excluded due to excessive head motion (defined as relative head motion with respect to the previous volume > 2.5 mm).

Association between (sub)clinical SAD diagnosis and parameters of WM integrity in TOI analyses

There were no significant correlations between a diagnosis of (sub)clinical SAD and measures of diffusivity in any of the three TOIs (see Supplemental Table 3 for results of average TOI analyses).

Voxelwise analysis of whole WM skeleton

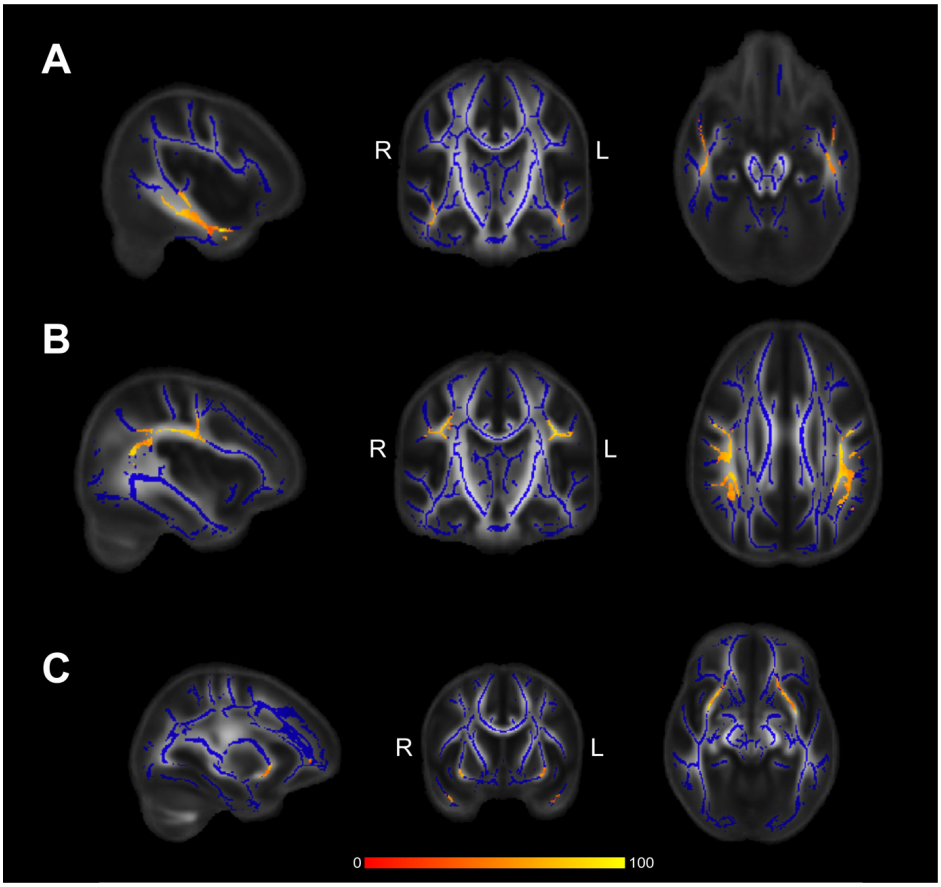
No significant clusters were found. However, when applying a liberal threshold of FWE-corrected $p < 0.1$, levels of self-reported social anxiety scores showed a near-significant positive association with one cluster of FA in the left SLF (FWE corrected TFCE value $p = 0.093$, $\beta = 0.176$). This was coupled with a negative association with RD and no significant associations of AD or MD. See Supplemental Table 4 for more details.

Sensitivity analyses

Although SAD is associated with high rates of comorbidity [5, 116], we performed a sensitivity analysis in order to examine the effects (associations) of social anxiety disorder independent from comorbidity (sensitivity analysis 1), by excluding participants with (comorbid) psychopathology. Note however, that the results of this analysis may be biased as the majority of the probands, on which the selection of the families was based, were excluded.

In the remaining sample (n = 50, SAD patients: n = 11; HC: n = 39), in the voxelwise TOI analysis we found a correlation between levels of social anxiety (z-SA) and FA in the right ILF (see Supplemental Table 5). In the average TOI analysis, near-significant positive associations with mean FA values in bilateral ILF and SLF and levels of social anxiety (z-SA) were found. This was accompanied by negative associations with levels of social anxiety and mean RD and MD values in those regions. There was no effect of FNE (see Supplemental Table 6).

When the level of depressive symptoms (z-score) was added as a covariate to the model (sensitivity analysis 2), in the voxelwise TOI analyses both levels of FNE and SA were significantly associated with a cluster of FA in the left SLF (see Supplemental Table 5). In the average TOI analyses, no association maintained conventional p-values below 0.05, although mean FA values in the left SLF reached a near-significant trend association with levels of social anxiety (b = 0.002, p = 0.08) (see Supplemental Table 7). Because of these findings, the effect of levels of depressive symptoms (z-score) on white matter integrity in all TOIs was examined as well (again corrected for age and gender; see Supplemental Table 7). No significant associations between the level of depressive symptoms and WM indices were found.



Supplemental Figure 1 Tracts (TOIs) used in analyses. Sagittal, coronal and axial sections of the WM skeleton (blue), with mean FA for tractwise analyses of resp. A) inferior longitudinal fasciculus (ILF), B) superior longitudinal fasciculus (SLF) and C) uncinate fasciculus (UF) (TOIs depicted in yellow/orange). The colour bar represents the probability of voxels belonging to the respective tract.

Supplemental Table 1a Overview of previous literature, descriptive information.

Author & journal	Contrast	Patients	Age (years)	LSAS	Depressive symptoms	Illness (months)	HC	Age (years)	LSAS	Depressive symptoms
Phan et al. [18]	SAD vs. HC	15 M / 15 F	27.2 ± 7.8	76.6 ± 17.3	BDI = 10.7 ± 6.5	n.a.	10 M / 20 F	29.9 ± 8.1	13.4 ± 11.3	BDI = 1.8 ± 2.4
Baur et al. [21] [a]	SAD vs. HC	18 M / 7 F	32.0 ± 10.4	66.0 ± 23.0	BDI = 15 ± 10.8	192	18 M / 7 F	32.0 ± 10.1	n.a.	n.a.
Liao et al. [19] [b]	SAD vs. HC	12 M / 6 F	22.7 ± 3.7	54.4 ± 12.0	HAM-D = 7.3 ± 6.2	49.2	13 M / 5 F	21.9 ± 3.7	19.1 ± 7.9	HAM-D = 1.0 ± 1.6
Baur et al. [20] [a]	SAD vs. HC	18 M / 7 F	31.6 ± 10.4	66.0 ± 23.0	BDI = 15 ± 10.8	192	18 M / 7 F	32.3 ± 10.1	n.a.	n.a.
Qiu et al. [17] [b]	SAD vs. HC	12 M / 6 F	22.7 ± 3.9	54.1 ± 11.9	n.a.	49.2	12 M / 6 F	21.8 ± 3.9	19.5 ± 8.5	n.a.
Jenkins et al. [12]	Within SAD	45 M / 28 F	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Tukel et al. [16]	SAD vs. HC	11 M / 11 F	27.7 ± 6.6	73.9 ± 28.5	HAM-D = 4.1 ± 4.5	14.4	11 M / 11 F	28.7 ± 6.6	n.a.	n.a.

Age and questionnaire data are reported as mean ± SD. [a] Study results seem derived from the same sample. [b] Study results seem derived from the same sample. M: male; F: female; SAD: social anxiety disorder; HC: healty control participants; BDI: Beck Depression Inventory; HAM-D: Hamilton Depression Rating Scale; n.a: no data available.

Supplemental Table 1b Overview of previous literature, findings.

	Main analysis method	WM alterations	CST	IFOF		ILF		SLF		UF		AF	
				L	R	L	R	L	R	L	R	L	R
[18]	Voxelwise	FA	ø	ø	ø	ø	ø	ø	ø	ø	↓		ø
[21]	Voxelwise	FA	ø	ø	ø	ø	ø	↓	ø	↓	ø		ø
		RD ROI								ø	ø		
		AD ROI								ø	ø		
		FA association with trait anxiety (SAD only)						ø	ø	↓	↓		
		AD association with trait anxiety						ø	ø	↓	ø		
		FA association with social anxiety						ø	ø	ø	ø		
[19]	Tractography	FA ROI											↑
		Fiber tracking density											↑
		FA association with social anxiety											
[20]	Tractography	FA	ø	ø	ø					↓	ø		
		Volume	ø	ø	ø					↓	↓		
		FA association with social anxiety								↓	ø		
		FA association with trait anxiety								ø	ø		
[17]	Voxelwise	FA	ø	ø	ø	ø	ø	↓	ø	↓	ø		ø
		MD	ø	↑	↑	ø	ø	↑	↑	ø	ø		ø
		FA association with social anxiety		ø	ø			↓	ø	↓	ø		
		MD association with social anxiety		ø	ø			↑	ø	ø	ø		
[12]	Voxelwise meta-analysis	FA	↓	ø	ø	ø	ø	↓	ø	↓	↓		ø
		Association with social anxiety											

Supplemental Table 1b Overview of previous literature, findings. (continued)

[16]	Voxelwise	FA	ø	ø	ø	ø	↓	ø	↓	ø	ø		ø		
		MD ROI					ø		ø						
		AD ROI					↓		↓						
		RD ROI					↑		↑						
		FA association with social anxiety					ø		↓						
		MD association with social anxiety					ø		ø						
		AD association with social anxiety					ø		ø						
		RD association with social anxiety					ø		ø						

FA: fractional anisotropy; RD: radial diffusivity; AD: axial diffusivity; MD: mean diffusivity; ROI: region of interest; ↓: decreased in SAD; ↑: increased in SAD; ø: no difference between SAD and HCs; CST: corticospinal tract; IFOF: inferior fronto-occipital fasciculus; ILF: inferior longitudinal fasciculus; SLF: superior longitudinal fasciculus; UF: uncinate fasciculus; AF: arcuate fasciculus; L: left; R: right. Empty cells indicate no data was available.

Supplemental Table 2 Additional diagnostic information of participants within the LFLSAD.

Diagnostic information (n)	(Sub)clinical SAD (n = 31)	No SAD (n = 57)	Statistical analysis
Depressive episode – present	1	1	$c^2 = 0.23$, p = .62
Depressive episode – past	10	9	$c^2 = 3.93$, p = .05*
Dysthymia – present	2	0	$c^2 = 4.03$, p = .05*
Dysthymia – past	0	1	$c^2 = 0.46$, p = .50
Panic disorder – lifetime	4	2	$c^2 = 3.14$, p = .08
Agoraphobia – present	2	1	$c^2 = 1.51$, p = .22
Agoraphobia – past	0	2	$c^2 = 1.04$, p = .31
Separation anxiety	0	1	$c^2 = 0.83$, p = .36
Specific phobia	2	3	$c^2 = 0.08$, p = .78
Generalized anxiety disorder	1	0	$c^2 = 1.99$, p = .16
Obsessive-compulsive disorder	1	0	$c^2 = 1.99$, p = .16
Alcohol dependency present	0	1	$c^2 = 0.52$, p = .47
Alcohol dependency lifetime	0	3	$c^2 = 1.59$, p = .21

SAD: social anxiety disorder.* significant at uncorrected p-value of 0.05.

Supplemental Table 3 Effects of (sub)clinical SAD on average white matter integrity per tract (TOI).

TOI	Side	WM parameter	Effect of (sub)clinical SAD		
			β	SE	p
ILF	L	FA	-0.002	0.004	.55
		AD	9.56E-07	6.24E-06	.88
		MD	4.50E-06	4.88E-06	.36
		RD	5.05E-06	5.48E-06	.36
	R	FA	0.003	0.004	.54
		AD	1.48E-06	6.24E-06	.81
		MD	5.42E-07	4.81E-06	.91
		RD	5.42E-08	5.29E-06	.99
SLF	L	FA	0.001	0.004	.74
		AD	3.28E-06	5.66E-06	.56
		MD	1.72E-06	4.92E-06	.73
		RD	8.91E-08	5.71E-06	.99
	R	FA	0.002	0.004	.61
		AD	-8.07E-07	5.57E-06	.88
		MD	8.12E-08	4.59E-06	.99
		RD	-8.63E-07	5.30E-06	.87
UF	L	FA	-0.005	0.006	.47
		AD	-9.42E-06	7.92E-06	.23
		MD	-6.96E-07	5.37E-06	.90
		RD	3.53E-06	6.73E-06	.60
	R	FA	-0.002	0.006	.79
		AD	1.77E-07	8.47E-06	.98
		MD	8.30E-07	4.65E-06	.86
		RD	1.91E-06	5.62E-06	.73

TOI: tract of interest; WM: white matter parameter; ILF: inferior longitudinal fasciculus; SLF: superior longitudinal fasciculus; UF: uncinate fasciculus; FA: fractional anisotropy; AD: axial diffusivity; MD: mean diffusivity; RD: radial diffusivity; L: left; R: right; SE: standard error.

Supplemental Table 4 Associations between measures of SA and exploratory voxelwise analyses of fractional anisotropy over the whole WM skeleton and results of post-hoc analyses of additional parameters.

Clinical measure	Anatomical region	WM parameter	Voxels (mm^3)	Peak MNI coordinates				
				x	y	z	b	p
z-SA	SLF (L)	FA	56	-33	-37	30	0.176	.093
		AD					0.107	.85
		MD					0.002	.12
		RD					-0.090	.001

Threshold-free cluster enhancement (TFCE) and family-wise error (FWE) corrected at threshold of $p < 0.1$. ß-values and p-values represent mean values of white matter integrity over all voxels. z-SA: social anxiety (z-score); L: left; WM: white matter parameter; FA: fractional anisotropy; AD: axial diffusivity; MD: mean diffusivity; RD: radial diffusivity.

Supplemental Table 5 Significant results of sensitivity analyses 1 and 2 in voxelwise TOI analyses of fractional anisotropy in the superior longitudinal fasciculus.

Sensitivity analysis	Clinical measure	ROI	Side	Voxels (mm^3)	Peak MNI coordinates				
					x	y	z	b	p
1	z-SA	ILF	R	26	38	-47	-5	0.268	.027
2	FNE	SLF	L	62	-33	-37	29	0.002	.025
2	z-SA	SLF	L	154	-33	-37	30	0.009	.016

Threshold-free cluster enhancement (TFCE) and family-wise error (FWE) corrected at $p < 0.05$. Sensitivity analysis 1: model without participants with comorbid psychopathology (e.g. patients with a single SAD diagnosis vs. healthy control participants). Sensitivity analysis 2: model with levels of depression added as additional covariate. FNE: fear of negative evaluation; z-SA: social anxiety (z-score); SLF: superior longitudinal fasciculus; ILF: inferior longitudinal fasciculus; L: left; R: right.

Supplemental Table 6 Results of sensitivity analysis 1: sample consisting of participants without (comorbid) psychopathology. Effects of levels of social anxiety and FNE on average values of white matter integrity per TOI.

			Effect of social anxiety (z-score)			Effect of FNE		
			β	SE	p	β	SE	p
ILF	L	FA	0.003	0.002	.08	3.78E-04	2.96E-04	.20
		AD	1.21E-06	2.09E-06	.56	2.45E-07	4.11E-07	.55
		MD	-2.82E-06	1.60E-06	.08	-3.48E-07	3.21E-07	.28
		RD	-4.06E-06	1.92E-06	.03*	-4.49E-07	3.81E-07	.24
	R	FA	0.003	0.002	.09	3.04E-04	3.36E-04	.37
		AD	5.93E-07	2.29E-06	.80	-1.82E-07	4.40E-07	.68
		MD	-2.67E-06	1.60E-06	.10	-4.14E-07	3.15E-07	.19
		RD	-3.84E-06	2.01E-06	.06	-3.74E-07	3.98E-07	.35
SLF	L	FA	0.003	0.002	.07	1.45E-04	3.65E-04	.69
		AD	1.50E-06	2.00E-06	.45	1.32E-07	3.88E-07	.73
		MD	-2.79E-06	1.79E-06	.12	-3.38E-07	3.54E-07	.34
		RD	-4.25E-06	2.26E-06	.06	-4.28E-07	4.46E-07	.34
	R	FA	0.004	0.002	.05	1.69E-04	3.62E-04	.64
		AD	3.50E-07	2.07E-06	.87	-8.86E-09	4.01E-07	.98
		MD	-3.24E-06	1.66E-06	.05	-4.44E-07	3.34E-07	.18
		RD	-4.44E-06	2.15E-06	.03*	-5.07E-07	4.23E-07	.23
UF	L	FA	0.002	0.002	.40	-1.91E-04	4.18E-04	.65
		AD	-5.71E-07	2.99E-06	.85	-3.08E-07	5.66E-07	.59
		MD	-1.63E-06	1.67E-06	.33	-1.77E-07	3.28E-07	.59
		RD	-2.04E-06	2.04E-06	.32	-1.29E-07	4.03E-07	.75
	R	FA	0.000	0.002	.90	-4.47E-04	3.86E-04	.25
		AD	2.45E-06	3.01E-06	.42	1.90E-08	5.80E-07	.97
		MD	2.82E-07	1.64E-06	.86	3.29E-07	3.10E-07	.29
		RD	8.77E-07	1.69E-06	.60	4.49E-07	3.32E-07	.18

ILF: inferior longitudinal fasciculus; SLF: superior longitudinal fasciculus; UF: uncinate fasciculus; FNE: fear of negative evaluation; FA: fractional anisotropy; AD: axial diffusivity; MD: mean diffusivity; RD: radial diffusivity; L: left; R: right; SE: standard error;* significant at p-value of 0.05.

Supplemental Table 7 Results of sensitivity analysis 2: effects of levels of depression on the effects of levels of social anxiety and FNE on average values of white matter integrity per TOI average values of white matter integrity per TOI.

β			Effect of social anxiety (z-score)			Effect of FNE			Effect of depression (z-score)		
			β	SE	p	β	SE	p	β	SE	p
ILF	L	FA	2.97E-04	0.001	.79	4.29E-04	2.74E-04	.12	0.002	0.003	.46
		AD	3.23E-07	2.17E-06	.88	1.46E-07	5.29E-07	.78	-5.12E-06	5.67E-06	.37
		MD	-5.05E-07	1.67E-06	.76	-2.71E-07	4.09E-07	.51	-3.82E-06	4.38E-06	.38
		RD	-8.76E-07	1.59E-06	.58	-4.42E-07	3.92E-07	.26	-3.35E-06	4.19E-06	.42
	R	FA	8.49E-04	0.001	.35	2.25E-04	2.23E-04	.31	0.001	0.002	.61
		AD	-2.07E-07	1.42E-06	.88	-9.48E-08	3.28E-07	.77	-1.17E-06	3.66E-06	.75
		MD	-9.39E-07	1.07E-06	.38	-2.50E-07	2.45E-07	.31	-2.18E-06	2.78E-06	.43
		RD	-1.19E-06	1.16E-06	.30	-2.50E-07	2.76E-07	.36	-2.50E-06	3.04E-06	.41
SLF	L	FA	0.002	0.001	.08	3.58E-04	2.43E-04	.14	0.003	0.003	.20
		AD	1.67E-06	1.46E-06	.25	1.47E-07	3.45E-07	.67	-1.56E-06	3.84E-06	.68
		MD	-3.26E-07	1.24E-06	.79	-2.67E-07	2.94E-07	.36	-3.21E-06	3.23E-06	.32
		RD	-1.28E-06	1.33E-06	.33	-4.36E-07	3.21E-07	.17	-4.30E-06	3.50E-06	.22
	R	FA	0.001	0.001	.15	3.54E-04	2.21E-04	.11	0.003	0.002	.21
		AD	4.77E-07	1.25E-06	.70	1.70E-07	2.81E-07	.54	-1.81E-06	3.22E-06	.58
		MD	-5.68E-07	1.00E-06	.57	-2.32E-07	2.31E-07	.32	-3.74E-06	2.59E-06	.15
		RD	-1.04E-06	1.13E-06	.36	-3.57E-07	2.72E-07	.19	-4.77E-06	2.98E-06	.11
UF	L	FA	4.13E-04	0.002	.80	4.29E-04	3.99E-04	.28	0.000	0.004	.95
		AD	2.13E-06	2.34E-06	.36	4.91E-08	5.91E-07	.93	-3.79E-06	6.19E-06	.54
		MD	8.40E-07	1.72E-06	.63	-2.36E-07	4.26E-07	.58	-3.31E-06	4.52E-06	.46
		RD	-1.04E-06	1.13E-06	.36	-3.57E-07	2.72E-07	.19	-4.77E-06	2.98E-06	.11
	R	FA	9.79E-04	0.001	.45	4.18E-04	3.08E-04	.17	-0.002	0.003	.53
		AD	1.81E-06	1.92E-06	.35	6.26E-07	4.73E-07	.19	-2.74E-06	5.05E-06	.59
		MD	-2.32E-07	1.03E-06	.82	-2.85E-08	2.40E-07	.91	2.57E-07	2.69E-06	.92
		RD	-9.41E-07	1.22E-06	.44	-3.13E-07	2.83E-07	.27	1.87E-06	3.17E-06	.56

ILF: inferior longitudinal fasciculus; SLF: superior longitudinal fasciculus; UF: uncinate fasciculus; FNE: fear of negative evaluation; FA: fractional anisotropy; AD: axial diffusivity; MD: mean diffusivity; RD: radial diffusivity; L: left; R: right; SE: standard error

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5

White matter microstructure alterations in social anxiety disorder – a mega-analysis across 12 cohorts in the ENIGMA-Anxiety Working Group

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Abstract

Background: Studies investigating social anxiety disorder (SAD) have reported inconsistent alterations in white matter (WM) microstructure. The ENIGMA-Anxiety Working Group investigated differences in microstructure of 25 WM tracts between individuals with SAD and healthy controls in a mega-analysis.

Methods: We analyzed data from 487 individuals with SAD and 1,604 healthy controls (HC) (age 8 – 65) from twelve cohorts worldwide. Analyses and quality control were performed using standardized ENIGMA diffusion tensor imaging (DTI)-protocols. We primarily examined fractional anisotropy (FA) as the main parameter of WM microstructure. Linear mixed-effects analyses were conducted to compare individuals with SAD with HC in the full sample. Next, adult (age > 21) and adolescent (age ≤ 21) samples were analyzed separately. In sensitivity analyses, additional effects of sex, medication, symptom severity and comorbid psychiatric disorders were investigated.

Results: In the full sample, individuals with SAD showed lower FA in several tracts, including the corpus callosum and fornix, when compared to HC. Widespread sex-by-diagnosis interactions were observed, mostly driven by lower FA in SAD females. Adults with SAD showed lower FA in multiple tracts, while age-by-diagnosis interactions were observed in adolescents.

Conclusions: Using a mega-analytic approach, several differences in WM microstructure were found between individuals with SAD and HC, both in the full sample and in age-group specific sensitivity analyses. Some neurobiological changes in WM tracts in individuals with SAD may vary with age and sex, whereas others might relate to broader transdiagnostic neurobiological features underlying psychopathology. Further research should investigate these issues in more detail.

Introduction

Social anxiety disorder (SAD) is one of the most prevalent and debilitating psychiatric disorders with a lifetime prevalence rate between 4 – 13 % and a typical onset in childhood or early adolescence, often with high psychiatric comorbidity and a chronic course [1, 2]. The pathogenesis of SAD involves complex interactions among biological, environmental, genetic, and temperamental factors [2, 3]. Neurobiological factors may help to inform current treatments and identify possible targets for new interventions.

Various modalities of magnetic resonance imaging (MRI) uncovered altered brain structure, functioning and connectivity in individuals with SAD [4, 5]. A review of prior work examined white matter (WM) microstructure assessed with diffusion tensor imaging (DTI) in small groups of individuals with a primary diagnosis of SAD compared to healthy controls (HC) [6]. These DTI studies revealed that WM microstructure differed from HC in several regions, mostly using fractional anisotropy (FA) as a general measure of WM microstructure. Moreover, lower FA of the uncinate fasciculus (UF) in SAD compared to HC has been reported in some studies using whole brain voxel-wise analysis or fiber tractography [7-9], although this finding has not been replicated in other studies [10-13]. Additionally, some studies reported negative correlations between anxiety symptoms and FA in the right inferior fasciculus (ILF) and superior longitudinal fasciculus (SLF) in individuals with SAD [10, 12, 13]. Taken together, there is little consistency between affected WM regions reported, and most of these studies had low sample sizes (ranging from n = 36 to n = 88 for the total sample).

Within the framework of the Enhancing NeuroImaging Genetics through Meta Analysis (ENIGMA)-Anxiety Working Group [14], we initiated a worldwide multi-site analysis to investigate WM microstructure in SAD. The present study consisted of a mega-analysis of harmonized pooled individual participant data (IPD) [15], including 487 individuals with SAD and 1,604 HC from twelve samples worldwide. Our main objective was to explore differences in WM microstructure between individuals with SAD and HC in major tracts of the brain. In addition, we explored age-specific SAD-related alterations in WM microstructure by conducting sensitivity analyses in adults and adolescents separately, as WM maturation is nonlinear and heterochronicity in this maturation has been associated with psychiatric disorders [16, 17]. Furthermore, we investigated SAD-related WM interactions with age and sex, WM differences in relation to psychiatric comorbidity, medication use and symptom severity. Lastly, we investigated whether WM differences in SAD correlated with WM differences in other psychiatric disorders. As these analyses were conducted in the largest dataset on WM microstructure in SAD to date, we aim to reduce the chance of false positives and thus to increase the accuracy of our findings [18].

Methods and materials

Participants

Individual-level participant data (IPD) from previous neuroimaging studies on SAD were considered for inclusion if DTI-data were collected during MRI-scanning [15]. Cohorts were included if participants had a current or lifetime diagnosis of SAD (criterion 1), also consisted of a group of HC without any lifetime psychiatric disorder (criterion 2) and had at least collected information on sex and age for all participants (criterion 3). For the patient group, current or lifetime diagnoses of comorbid major depressive disorder (MDD), generalized anxiety disorder (GAD), panic disorder, agoraphobia, specific phobia, obsessive-compulsive disorder (OCD), post-traumatic stress disorder (PTSD), and substance use dependence were allowed. However, individuals with SAD were excluded if they had a current diagnosis or history of psychosis, schizophrenia, bipolar disorder or autism spectrum disorder of at least moderate severity. The diagnostic assessment measures and exclusion criteria for every cohort can be found in Table S1.

Local institutional review boards and ethics committees approved the individual research protocols. Written informed consent was obtained for all adult participants and parents of participants younger than 18 years old at the local research sites. In addition, principal investigators from each site signed a memorandum of understanding. This memorandum included guidelines on subject de-identification, data sharing, and confidentiality and security practices [14]. Furthermore, approval from local officials to share data was obtained by principal investigators from each site, or via research contract offices when raw data was to be shared.

Ten ENIGMA-Anxiety Working Group sites shared their IPD, after locally processing their data using the ENIGMA-DTI processing pipeline (http://enigma.ini.usc.edu/protocols/dti-protocols/), while for the Muenster cohort, raw imaging data was shared and processed subsequently at the coordinating site (Leiden). In addition, raw data from the publicly available Philadelphia Neurodevelopmental Cohort was downloaded and processed in Leiden.

Total sample size of IPD after processing and quality control consisted of 487 individuals with SAD and 1,604 HC. More specifically, the adult sample (age 22 – 65 years) contained data from 230 individuals with SAD and 1301 HC; the adolescent sample (age 8 – 21 years) included 257 individuals with SAD and 303 HC. Demographic information and clinical characteristics for every cohort are summarized in Table 1, Table 2 and Table S2.

Image (pre)-processing

Information on scanner and acquisition parameters is presented in Table S3. At each site, preprocessing of diffusion weighted images was performed, which included eddy current correction, echo-planar imaging (EPI) induced distortion correction and tensor fitting. After preprocessing, data were processed according to the ENIGMA-DTI protocol (available at https://enigma.ini.usc.edu/protocols/dti-protocols/) using the FMRIB Software Library (FSL). Tract-based spatial statistics (TBSS) were applied

as follows. First, individual tensor images were analyzed, resulting in individual level images of FA and additional measures of WM microstructure, being mean diffusivity (MD), radial diffusivity (RD) and axial diffusivity (AD). Then, quality control procedures were conducted, including visual inspection of FA and registration and projection onto the standardized ENIGMA skeleton. Finally, individual mean values of FA, MD, AD and RD were obtained for 25 regions of interest (ROI), covering most WM regions in the brain. These IPD-results from the local sites were sent to the coordinating site in Leiden. Here, final quality control measures were performed following the ENIGMA-DTI protocol, including inspection of histograms of each WM parameter per ROI per cohort to assess normality and FA outliers (> 5 SD), using an automated protocol. No outliers were detected. Afterwards, results were merged into one dataset. As no lateralized effects were hypothesized, bilateral averaged measures were used in the primary analysis, by combining all ROIs across both hemispheres (mean of the left and right hemisphere regions weighted by the number of voxels). Lastly, we optimized harmonization of FA by applying eHarmonize, a newly developed python package, to match site-specific FA to lifespan reference curves [19].

Regions of interest

In total, 25 ROIs were investigated. We examined the average FA of 24 WM ROIs, of which 21 are illustrated in Figure 1, and the average FA over all WM regions. Three additional regions not illustrated in Figure 1 comprise several subregions described in the figure, being 1) the corpus callosum (CC), consisting of the body (BCC), genu (GCC) and splenium of the corpus callosum (SCC); 2) corona radiata (CR), consisting of an anterior (ACR), posterior (PCR) and superior (SCR) part; and 3) the internal capsule (IC), consisting of the anterior limb (ALIC), posterior limb (PLIC) and the retrolenticular limb (RLIC). The inferior longitudinal fasciculus (ILF) was included as part of the sagittal striatum (SS), in line with the ENIGMA-DTI protocol.

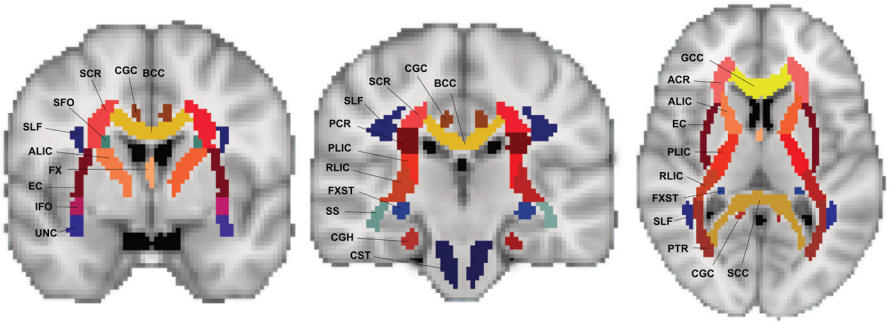


Figure 1 Illustration of white matter ROIs used to investigate differences in microstructure between individuals with SAD and HC participants. *Abbreviations:* ACR: anterior corona radiata (left and right); ALIC: anterior limb of internal capsule (left and right); BCC: body of corpus callosum; CGC: cingulum (cingulate gyrus; left and right); CGH: cingulum (hippocampal portion; left and right); CST: corticospinal tract (left and right); EC: external capsule (left and right); FX: fornix; FXST: fornix (crues) / stria terminalis (left and right); GCC: genu of corpus callosum; IFO: inferior fronto-occipital fasciculus (left and right); PCR: posterior corona radiata (left and right); PLIC: posterior limb of internal capsule (left and right); PTR: posterior thalamic radiation (left and right); RLIC: retrolenticular part of internal capsule (left and right); SCC: splenium of corpus callosum; SCR: superior corona radiata (left and right); SFO: superior fronto-occipital fasciculus (left and right); SLF: superior longitudinal fasciculus (left and right); SS: sagittal stratum (left and right); UNC: uncinate fasciculus (left and right).

Linear mixed-effects models

This study was preregistered in December 2023 at https://osf.io/5ycag/. In light of additional insights gained during the analysis process, minor refinements were made to the analytic approach (Note S1). Using linear mixed-effects models (cf. previous work from the ENIGMA-Anxiety Working Group in SAD) [20], we investigated FA for each of the 25 WM ROIs as primary outcome variable, with SAD diagnosis (dichotomous factor) or symptom severity (continuous factor) as main regressor. We used age, sex, age^2, age-by-sex and age^2-by-sex as covariates. Age was centred throughout. Mixed-effects *d* effect sizes were calculated from the t-values for diagnostic factor, and mixed-effects *r* estimates were calculated for relevant interaction and continuous variables of interest. These are similarly scaled as Cohen's *d* estimates and *r* estimates, but include a correction for non-independence in the aggregated dataset [21]. For all analyses, samples were only included for between-group contrasts when at least one observation per group was available for each of the WM regions. The threshold for significance was set at a false-discovery rate (FDR) corrected $p < 0.05$, adjusting for 25 WM regions in all analyses, in line with previous ENIGMA-DTI projects [22]. Because development of WM regions is known to peak during childhood and adolescence [16], we explored FA within the full sample and in adults (> 21 years old) and adolescents (≤ 21 years old) separately. Additional WM parameters, being MD, AD and RD, were only investigated if a significant difference in FA was found.

First, we investigated if average FA in each of the 25 predefined WM ROIs was different between individuals with SAD and HC. Then, diagnosis-by-age, diagnosis-by-age^2 and diagnosis-by-sex interactions were investigated. Next, we conducted sensitivity analyses to investigate possible confounders. In these analyses, individuals with SAD were excluded if they had comorbid lifetime PTSD, OCD, or when current SAD criteria were not met. Furthermore, we compared subgroups of individuals with SAD to HC to investigate individuals with SAD with and without comorbid lifetime MDD, with and without any comorbid anxiety disorders and with and without psychotropic medication use at the time of scanning, specifically investigating individuals with SAD using selective serotonin reuptake inhibitors (SSRI) and serotonin-noradrenaline reuptake inhibitors (SNRI), as their pharmacological mechanisms overlap. In addition, we investigated associations between WM characteristics and clinical symptoms within the SAD group, using the Liebowitz Social Anxiety Scale (LSAS) to assess severity of social anxiety, the Beck Depression Inventory II (BDI-II) to assess severity of depressive symptoms and the State-Trait Anxiety Inventory (STAI) for symptoms of trait anxiety, when available [23-25].

Lastly, as symptoms of social anxiety are common comorbidities across many psychiatric illnesses [26, 27], we performed correlation analyses between mega-analytical effect sizes observed in this study and the regional patterns of patient-control deficits derived using the same ENIGMA-DTI workflow for five other psychiatric illnesses, distributed as part of ENIGMA RVI package 28. These illnesses include schizophrenia, bipolar disorder, MDD, PTSD and OCD; findings of these studies are published before [22, 28-31].

Selection of mega analytic approach

We selected the model for our mega-analytic approach as previously reported by Groenewold and colleagues [20]. All linear mixed-effects models were fitted with a random-intercept to account for data clustering within samples. Next, models with a random slope for diagnosis per sample (complex model) and without random slope (reference model) were fitted, and fit was compared using the Likelihood Ratio Test (LRT; cf. Boedhoe and colleagues) [32], where $p < 0.05$ indicates improved model fit in complex relative to reference model. The mega-analytic model with the best model fit for most WM regions was selected, based on the full sample. All mega-analysis models were fitted with restricted maximum likelihood (ReML) in R version 4.3.0 and mixed-effects *d* and *r* effect sizes were computed (Table S4) [33].

Results

Participants

Demographic and clinical characteristics for each cohort are presented in Table 1 and Table 2.

Table 1 Demographic information for samples included in current mega-analyses.

	Cohort	Country	Full sample						Adult samples (age > 21)						Adolescent samples (age ≤ 21)					
			Total no.		Age (mean ± SD)		% Female		Total no.		Age (mean ± SD)		% Female		Total no.		Age (mean ± SD)		% Female	
			HC	SAD	HC	SAD	HC	SAD	HC	SAD	HC	SAD	HC	SAD	HC	SAD	HC	SAD	HC	SAD
1	Istanbul	TR	22	22	28.7 ± 6.6	28.7 ± 6.6	50.0	50.0	22	20	28.7 ± 6.6	29.7 ± 6.2	50.0	45.0		2		19.5 ± 0.7		100.0
2	LFLSAD	NL	10	10	47.2 ± 12.9	45.3 ± 5.3	30.0	70.0	9	10	50.4 ± 8.3	45.3 ± 5.3	22.2	70.0	1		17.9 ± NA		100.0	
3	FOR2107-MR	DE	511	31	36.6 ± 13.2	32.7 ± 10.2	62.8	74.2	476	27	37.9 ± 12.8	34.5 ± 9.7	61.8	77.8	35	4	19.7 ± 1.3	20.5 ± 0.6	77.1	50.0
4	FOR2107-MS	DE	342	57	31.3 ± 12.1	35.2 ± 12.7	67.5	70.2	298	49	33.0 ± 12.0	37.7 ± 12.1	66.1	67.3	44	8	19.9 ± 1.0	20.3 ± 0.9	77.3	87.5
5	Muenster	DE	24	22	25.9 ± 6.0	28.7 ± 8.3	62.5	54.5	22	21	26.6 ± 5.8	29.0 ± 8.3	59.1	52.4	2	1	18.5 ± 0.7	21.0 ± NA	100.0	100.0
6	MNC	DE	466	58	37.0 ± 11.8	32.9 ± 11.1	56.4	79.3	429	54	38.5 ± 11.0	34.0 ± 10.8	56.2	79.6	37	4	19.7 ± 1.6	18.8 ± 1.7	59.5	75.0
7	TIP	DE	37	39	26.2 ± 9.3	25.1 ± 6.7	73.0	74.4	22	24	30.3 ± 10.1	28.3 ± 6.8	63.6	70.8	15	15	20.1 ± 0.9	19.9 ± 0.9	86.7	80.0
8	NIMH-SDAN	US	43	24	12.8 ± 2.6	11.9 ± 2.6	48.8	62.5							43	24	12.8 ± 2.6	11.9 ± 2.6	48.8	62.5
9	PNC	US	113	185	13.8 ± 3.5	15.5 ± 3.0	46.9	57.8	2	1	22.0 ± 0.0	22.0 ± NA	50.0	100.0	111	184	13.6 ± 3.4	15.4 ± 2.9	46.8	57.6
10	MRC-SU	SA	14	16	30.4 ± 8.0	29.9 ± 9.4	50.0	68.8	11	14	33.2 ± 6.7	31.4 ± 9.2	54.5	64.3	3	2	20.3 ± 0.6	20.0 ± 1.4	33.3	100.0
11	Vanderbilt	US	5	6	23.0 ± 2.0	22.0 ± 1.5	60.0	50.0	3	3	24.3 ± 1.2	23.0 ± 1.7	66.7	66.7	2	3	21.0 ± 0.0	21.0 ± 0.0	50.0	33.3
12	West China Hospital	CN	17	17	21.9 ± 4.0	21.9 ± 4.0	29.4	29.4	7	7	25.4 ± 4.0	25.4 ± 4.0	14.3	14.3	10	10	19.4 ± 0.8	19.4 ± 0.8	40.0	40.0
	Total across all samples		1604	487	32.7 ± 13.5	24.1 ± 6.5	59.9	63.4	1301	230	36.4 ± 5.7	33.3 ± 9.2	60.1	67.0	303	257	16.5 ± 4.0	16.0 ± 2.5	58.4	60.3

Abbreviations: HC: healthy control; SAD: social anxiety disorder; SD: standard deviation; TR: Turkey; NL: Netherlands; DE: Germany; US: United States of America; SA: South-Africa; CN: China; NA: not applicable.
Notes: Sites were excluded from analysis if $n = 0$ for either diagnostic group.

Table 2 Clinical characteristics for individuals with SAD in samples included in mega-analyses.

	Cohort	Full sample						
		Lifetime comorbidity		Psychotropic medication		LSAS	STAI-T	BDI-II
		MDD (%)	ANX (%)	% any use	% SSRI / SNRI	mean ± SD	mean ± SD	mean ± SD
1	Istanbul	0.0	0.0	0.0	-	73.9 ± 28.5	-	-
2	LFLSAD	50.0	40.0	10.0	100.0	60.6 ± 25.6	42.7 ± 9.2	13.5 ± 10.4
3	FOR2107-MR	98.2	28.1	64.9	89.2	0.0 ± 0.0	60.2 ± 10.3	21.7 ± 10.3
4	FOR2107-MS	96.8	41.9	83.9	80.8	0.0 ± 0.0	57.8 ± 8.9	20.7 ± 10.3
5	Muenster	-	-	-	-	-	-	-
6	MNC	46.6	12.1	24.1	100.0	60.8 ± 19.7	55.3 ± 11.6	16.6 ± 11.1
7	TIP	82.1	0.0	28.2	100.0	65.2 ± 21.6	51.6 ± 10.3	13.8 ± 10.4
8	NIMH-SDAN	0.0	83.3	0.0	-	-	42.2 ± 6.8	-
9	PNC	15.1	42.6	58.9	0.0	-	-	-
10	MRC-SU	50.0	0.0	0.0	-	88.3 ± 28.5	-	-
11	Vanderbilt	0.0	66.7	0.0	-		25.7 ± 5.8	6.3 ± 4.6
12	West China Hospital	0.0	0.0	0.0	-	50.2 ± 12.8	0.0 ± 0.0	0.0 ± 0.0
	Total across all samples	39.9	30.9	44.7	40.4	65.1 ± 21.9	21.9 ± 53.2	53.2 ± 9.7

Abbreviations: -: information not available for sample; LSAS: Liebowitz Social Anxiety Scale, total score; STAI-T: State-Trait Anxiety Inventory – Trait subsection; BDI-II: Beck Depression Inventory, 2nd edition; ANX: any comorbid anxiety disorder; MDD: major depressive disorder; SSRI: selective serotonin reuptake inhibitor; SNRI serotonin-norepinephrine reuptake inhibitor.

Mega-analytic results for comparisons in SAD vs HC

Model fit comparisons were conducted on the full dataset (n = 487 SAD, n = 1,604 HC). For all ROIs and average FA over the whole WM skeleton, the complex model with random intercept (scan site) and random slope (SAD diagnosis per scan site) did not show a significant improvement in model fit when compared to the random intercept (scan site) reference model (Table S4). Thus, all subsequent analyses were conducted with the random intercept (scan site) model.

Mega-analytic results for comparisons of SAD vs HC and relations with comorbidity in SAD subgroups

To enhance readability, significant results are summarized in Table 3 and described below, where we report our findings by clustering significant regions displaying similar patterns. Additionally, Figure 2 displays results from the main analysis in the full sample as well as adolescent and adult samples separately. For a systematic and comprehensive overview of all analyses, we refer to Note S2 and Tables S5- S34.

In our main analyses of the full sample, lower FA was found in several regions. First, individuals with SAD had lower average FA when compared to HC, as well as lower FA in the FX, BCC and CC (Figure 2B). This pattern remained throughout several sensitivity analyses excluding individuals with SAD and comorbid PTSD (Table S8), OCD (Table S7) or other anxiety disorders (Table S12) and when including only individuals with SAD who had a current SAD diagnosis (Table S6; Note S2). We found a largely similar pattern in the adult only sample (Table S9) and in sensitivity analyses in adult individuals with SAD with (Table S17) and without comorbid depression (Table S18) and without comorbid anxiety disorders (Table S16). Moreover, findings in the CC and BCC were coupled with higher RD in the adult sample (Table 3).

Furthermore, individuals with SAD had lower FA in the SFO when compared to HC, an association also seen in adult individuals with SAD (Table S9) and in SAD patients with comorbid anxiety disorders (Table S11). Lastly, lower FA was found in the CR (Figure 2B). This finding seemed to be driven by adult individuals with SAD (Table S9).

Separate analyses on the adult sample revealed additional ROIs displaying SAD-related alterations in FA, namely lower FA in GCC, CGC and PTR (Table S9), even though we did not find significant age-by-diagnosis interactions in these regions in the full sample.

Analyses in the adolescent samples separately did not reveal significant differences in any of the ROIs when correcting for multiple comparisons (Table S10). There were no significant results when investigating the role of psychotropic medication or associations with symptom severity in the full sample, adult or adolescent samples separately (Tables S23 – S34).

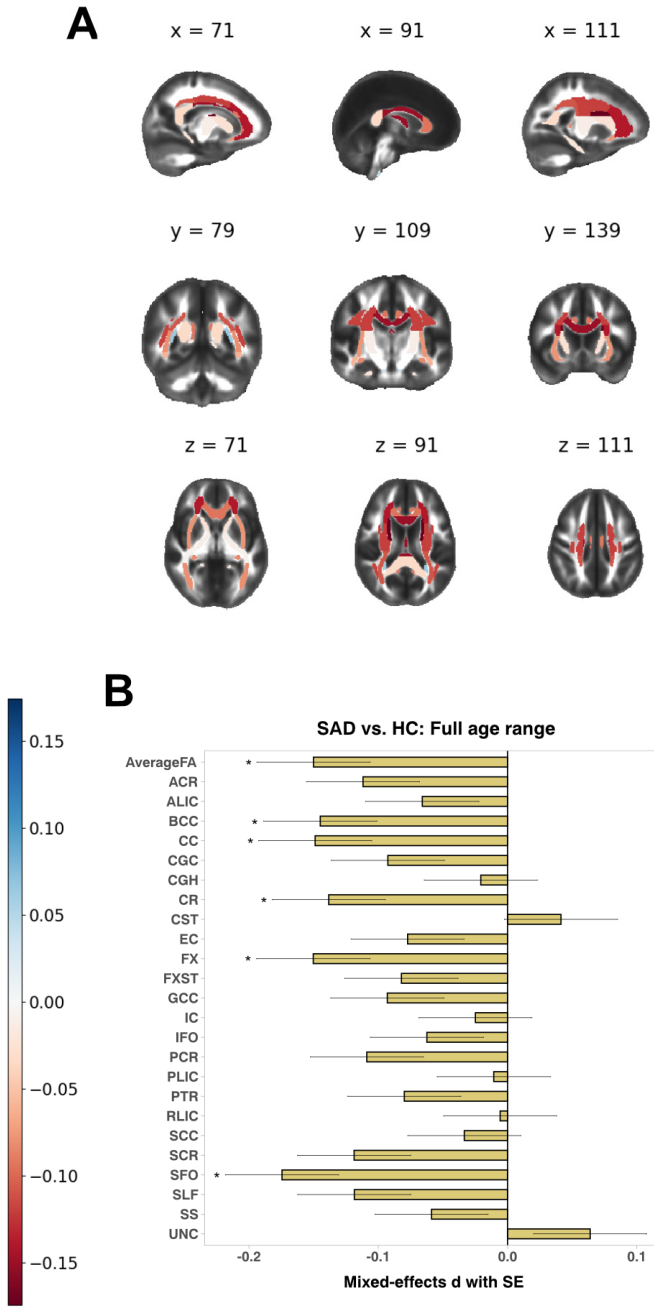


Figure 2 Differences in white matter microstructure between individuals with social anxiety disorder (SAD) and healthy controls (HC) in 25 white matter tracts.

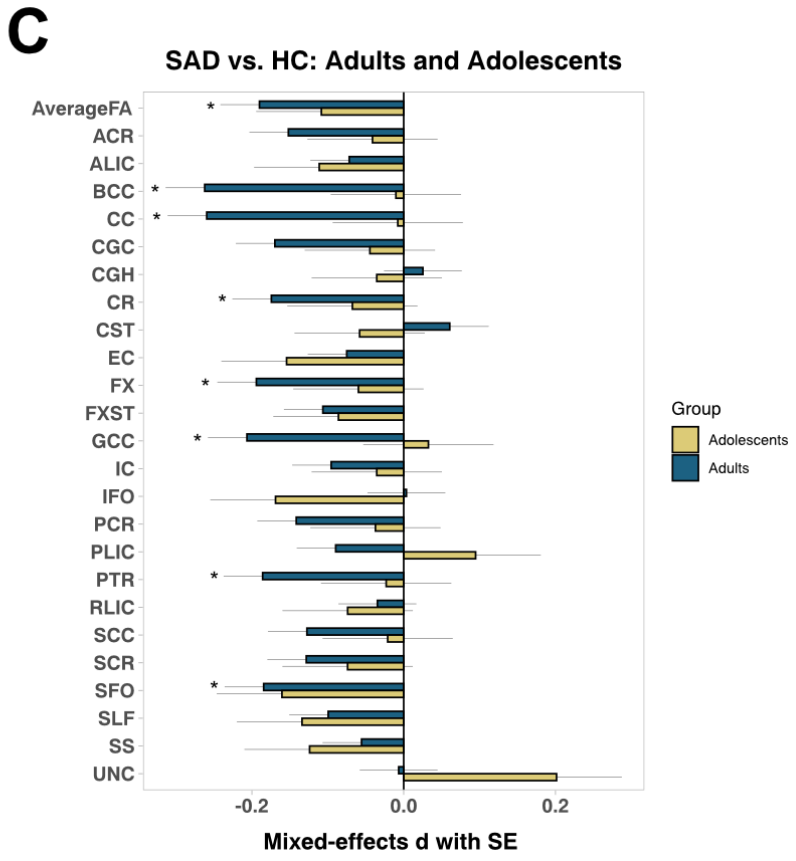


Figure 2 (continued)

Notes: A. Illustration of differences in FA between patients with SAD and healthy controls in 25 white matter regions. Color bar represents mixed-effects *d* effect size. Blue represents greater FA in SAD compared to HC. Red represents greater FA in HC compared to SAD. B. Mixed-effects *d* effect size in the full age range. Error bars represent standard error (SE). C. Mixed-effects *d* effect size stratified according to adult (> 21 years old) and adolescent age group (≤ 21 years old). Error bars represent standard error (SE). $^{*}p \leq 0.05$ after false-discovery rate adjustment for multiple comparisons.

Table 3 Overview of mega-analytic results for main diagnostic group comparisons and clinical subgroup comparisons.

Group comparison	Sens	SAD	HC	averageFA[a]		BCC		CC		GCC		CR	
	Age	n	n	mixedeff.d	pFDR[bc]	mixedeff.d	pFDR	mixedeff.d	pFDR	mixedeff.d	pFDR	mixedeff.d	pFDR
SAD vs HC	-	487	1605	-0.15	0.026	-0.15	0.026	-0.15	0.026	ns	ns	-0.14	0.032
SAD vs HC	Adu	230	1301	-0.19	0.036	-0.26[d]	0.004	-0.26[d]	0.004	-0.21	0.033	-0.17	0.047
SAD vs HC	Ado	255	292	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
SAD ANX vs. HC	-	142	1490	-0.23	0.074	ns	ns	ns	ns	ns	ns	-0.22	0.074
SAD ANX vs. HC	Adu	36	1217	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
SAD ANX vs. HC	Ado	106	262	-0.23	0.273	ns	ns	ns	ns	ns	ns	ns	ns
SAD wo ANX vs. HC	-	317	1559	-0.14	0.129	-0.20	0.032	-0.19	0.032	ns	ns	ns	ns
SAD wo ANX vs. HC	Adu	170	1277	-0.23	0.036	-0.34[d,e]	0.001	-0.33[d]	0.001	-0.24	0.025	-0.18	0.076
SAD wo ANX vs. HC	Ado	145	269	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
SAD MDD vs. HC	-	184	1493	ns	ns	-0.21	0.100	-0.19	0.101	ns	ns	-0.19	0.101
SAD MDD vs. HC	Adu	130	1245	-0.19	0.144	-0.30	0.032	-0.25	0.046	ns	ns	-0.22	0.087
SAD MDD vs. HC	Ado	54	232	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
SAD wo MDD vs. HC	-	277	1403	-0.16	0.167	ns	ns	ns	ns	ns	ns	ns	ns
SAD wo MDD vs. HC	Adu	76	1112	-0.26	0.068	-0.29	0.068	-0.35	0.039	-0.33	0.039	ns	ns
SAD wo MDD vs. HC	Ado	199	221	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Table 3 (continued)

Group comparison	Sens	SAD	HC	CGC		FX		PTR		SFO	
	Age	n	n	mixedeff.d	pFDR	mixedeff.d	pFDR	mixedeff.d	pFDR	mixedeff.d	pFDR
SAD vs HC	-	487	1605	ns	ns	-0.15	0.026	ns	ns	-0.17	0.019
SAD vs HC	Adu	230	1301	-0.17	0.050	-0.19	0.036	-0.19	0.036	-0.19	0.036
SAD vs HC	Ado	255	292	ns	ns	ns	ns	ns	ns	ns	ns
SAD ANX vs. HC	-	142	1490	ns	ns	-0.21	0.088	ns	ns	-0.28	0.034
SAD ANX vs. HC	Adu	36	1217	ns	ns	ns	ns	ns	ns	-0.51	0.069
SAD ANX vs. HC	Ado	106	262	ns	ns	ns	ns	ns	ns	ns	ns
SAD wo ANX vs. HC	-	317	1559	ns	ns	-0.14	0.129	ns	ns	-0.16	0.077
SAD wo ANX vs. HC	Adu	170	1277	-0.22	0.041	-0.19	0.074	-0.20	0.069	ns	ns
SAD wo ANX vs. HC	Ado	145	269	ns	ns	ns	ns	ns	ns	ns	ns
SAD MDD vs. HC	-	184	1493	ns	ns	-0.24	0.069	-0.17	0.101	-0.16	0.124
SAD MDD vs. HC	Adu	130	1245	ns	ns	-0.26	0.046	-0.25	0.046	ns	ns
SAD MDD vs. HC	Ado	54	232	ns	ns	ns	ns	ns	ns	ns	ns
SAD wo MDD vs. HC	-	277	1403	ns	ns	ns	ns	ns	ns	-0.18	0.150
SAD wo MDD vs. HC	Adu	76	1112	-0.33	0.039	ns	ns	ns	ns	-0.26	0.068
SAD wo MDD vs. HC	Ado	199	221	ns	ns	ns	ns	ns	ns	ns	ns

Abbreviations: Sens: sensitivity analysis by age group; SAD: social anxiety disorder; HC: healthy control; N: number of participants; BCC: body of corpus callosum; CGC: cingulum (cingulate gyrus); CR: corona radiata; FX: fornix; GCC: genu of corpus callosum; PTR: posterior thalamic radiation; SCR: superior corona radiata; SFO: superior fronto-occipital fasciculus; Mixeff.d: mixed-effects; FDR: false discovery rate correction; ns: non- significant (uncorrected $p < 0.05$); Adu: adults; Ado: adolescents; ANX: any comorbid anxiety disorder; wo: without. *Notes:* [a] Results presented for all regions with an FDR significant finding in one of the full age range mega-analyses. [b] Bold text highlights significant result after FDR multiple comparison correction. [c] Plain text indicates uncorrected significant result. [d] This decrease in FA was coupled with higher RD. [e] This decrease in FA was coupled with higher MD.

SAD interactions with age

In the full sample, significant interaction effects between diagnosis and age were observed in FA for the CST and IFO (Table S35). Additional analyses revealed interaction effects for AD in the CST, but there were no significant interaction effects for MD or RD. There were no significant interactions between diagnosis and age in the adult sample (Table S36). When adolescent participants were considered, significant diagnosis-by-age interaction effects were observed for the CST, IFO and PTR (Table S37). Follow-up analyses highlighted significant effects for RD in IFO and PTR. No significant interaction effects were found for AD or MD. Illustrating post-hoc plots showed no clear consistent direction of these interactions (Figure S1). There were no significant diagnosis-by-age^2 interaction effects in the full, adult or adolescent samples (Tables S38-40).

SAD interactions with sex

Significant diagnosis-by-sex interactions for FA were observed in 19 out of the 25 ROIs across the full sample (Table S41). The largest effects were found across the WM skeleton, SLF, CR, SCR, CST, FX, ALIC, IC, PCR, ACR and PTR. Post-hoc tests revealed that in most regions, effects were driven by lower FA in females with SAD, although in some regions, effects were driven by lower FA in males with SAD (Figure 3 and Figure S2). Other significant interactions, but with smaller effect sizes, were found in the CGC, SFO, CGH, RLIC, EC, PLIC, CC and GCC (Table S41). Significant interactions were also observed for MD in the whole WM skeleton, SCR, FXST, SS, CGC, CGH, CR, EC, PCR and SLF. In addition, significant interactions were observed in the SCR and the SLF for RD. These diagnosis-by-sex interactions were not present when we investigated adult participants (Table S42) and adolescent participants (Table S43) separately.

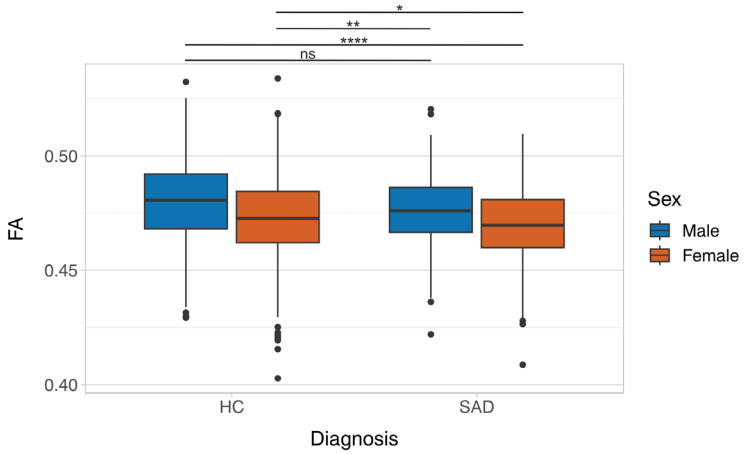


Figure 3 Sex-by-diagnosis interactions in average fractional anisotropy (FA) over all tracts included in the present study between individuals with social anxiety disorder (SAD) and healthy controls (HC) in the full sample. *Notes:* HC male: n = 651; HC female: n = 967; SAD male: n = 178; SAD female: n = 309. Significance after false-discovery rate adjustment for multiple comparisons: ns = not significant; * $p \leq 0.05$; ** $p \leq 10^{-2}$; *** $p \leq 10^{-3}$; **** $p \leq 10^{-4}$.

Overlap of SAD-related WM alterations with WM patterns in with other psychiatric disorders.

When we compared the effect sizes of WM alterations in participants with SAD with those obtained in ENIGMA-studies of other psychiatric disorders, being OCD, PTSD, schizophrenia, MDD and bipolar disorder, the effect sizes in participants with SAD were lower than these reported in these conditions (all beta < 1). There were, however, remarkable similarities between the patterns of WM alterations (Figure 4). The highest correlation was observed between the WM patterns in participants with SAD and participants with schizophrenia ($r = 0.68$, $p < 0.001$; data from [28]). In addition, significant correlations were observed for participants with MDD ($r = 0.52$, $p = 0.009$; data from [22]) and bipolar disorder ($r = 0.49$, $p = 0.015$; data from [29]). There were no significant correlations between SAD and PTSD or SAD and OCD. Detailed results are reported in Table S44.

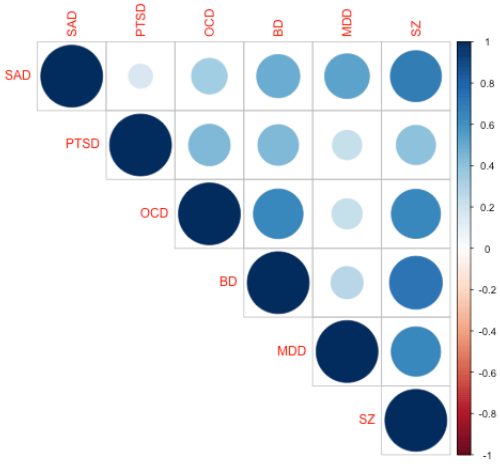


Figure 4 Graphical depiction of correlation analyses between effect sizes of white matter microstructure in social anxiety disorder and five psychiatric disorders. *Abbreviations:* SAD: social anxiety disorder; PTSD: post-traumatic stress disorder; OCD: obsessive compulsive disorder; MDD: major depressive disorder; SZ: schizophrenia disorder. Color bar represents Pearson *r* correlation coefficient.

Discussion

This present study by the ENIGMA-Anxiety Working Group used a mega-analytic approach to investigate WM microstructure in adults and adolescents with SAD compared to healthy participants, combining data from twelve cohorts from research sites worldwide. We found several differences in WM microstructure between individuals with SAD and healthy controls, in the largest sample available for analysis to date. More specifically, individuals with SAD had lower average FA over all WM regions and lower FA in several regions of interest (ROIs): the corpus callosum, fornix, corona radiata and superior fronto-occipital fasciculus. A similar pattern was found when analyses were restricted to adult participants (age > 21 years). In addition, we found multiple significant interactions between sex and diagnosis as well as between age and diagnosis across the full sample. In most regions, these interactions with sex seem to be driven by lower FA in females with SAD, whereas interactions with age seem to be driven by differences in FA in adolescents with SAD. Thus, our results indicate WM differences between individuals with SAD and healthy controls as well as moderation by sex- and age-specific changes in FA in individuals with SAD.

When investigating additional parameters of WM microstructure in significant ROIs, lower FA was coupled with higher RD and MD in analyses restricted to the adult sample and in some sensitivity analyses in the full sample. Current literature suggests that this coupling could indicate demyelination [34]. However, the precise role and interpretation of additional WM parameters in SAD remains to be further investigated, as previous studies on WM in SAD report contradictory results on these parameters [7, 9, 10, 12].

As the largest WM region of the brain, the corpus callosum facilitates interhemispheric communication by connecting cortical and subcortical areas, involved in memory, attention, language, emotional states and intelligence, across the hemispheres [35]. Located at the center of the limbic system, the fornix structurally connects brain regions such as thalamus, nucleus accumbens and hippocampus that are important for emotion regulation, memory performance and reward processing [36, 37]. Notably, lower FA in the corpus callosum and fornix has been previously reported in studies on other psychiatric disorders, among others in work from ENIGMA groups on adults with MDD, bipolar disorder and schizophrenia [22, 28, 29]. However, our findings in the corpus callosum and fornix differ from findings in previous studies on WM in SAD, which did not show lower FA in these regions but reported lower FA in regions such as the UF [7-9].

As part of the ENIGMA consortium, in which preprocessing and analytical pipelines are harmonized [38], this mega-analysis allowed us to perform post-hoc investigation into the patterns of regional deficits in WM that we found in individuals with SAD relate to recent work of other ENIGMA working groups. (Figure 4). The patterns of the effect sizes of the patient-control differences in SAD showed strong correlations in regional effects sizes with three psychiatric disorders, namely schizophrenia, MDD and bipolar disorder. This overlap was observed in patterns derived from non-overlapping samples,

thus supporting the transdiagnostic nature of the observed WM deficits and underscoring the need for further exploration [39].

Our study is among the first to report widespread sex-by-diagnosis interactions in SAD, with lower FA primarily observed in females with SAD. Prior research on sex differences in WM microstructure in anxiety disorders is limited. However, a mega-analysis on preadolescent children with high and low trait anxiety reported sex-specific alterations in the uncinate fasciculus and inferior fronto-occipital fasciculus, with lower FA observed in anxious boys [40]. One study by Kim et al. [41] has reported an inverse relationship between FA and trait anxiety in right hemisphere amygdala-frontal pathways in females, while Montag and colleagues [42] found positive associations between FA and trait anxiety in left hemisphere pathways in males. This contrasts with our findings, suggesting that sex differences in WM microstructure in SAD may emerge differently in adulthood with lower FA in females with SAD. Sex differences in FA and brain ageing have been reported in the healthy population, with males typically exhibiting higher FA than females [43, 44]. However, results on sex differences in WM maturation are not uniform, and longitudinal studies in childhood, adolescence and adulthood show different trajectories for females and males [43]. These discrepancies highlight the complexity of sex-related differences in WM microstructure. Yet, as reviewed by Parsaei et al, previous studies on SAD investigated a limited number of regions in cohorts with limited age ranges [6]. This study is among the first to investigate a large number of WM regions in a substantial sample of individuals with SAD with a wide age range, thus emphasizing the need for further longitudinal investigations into how sex-specific developmental trajectories contribute to anxiety-related brain changes.

Strengths and limitations

Strengths of this multi-site study are the use of harmonized protocols, analyses of IPD (rather than performing meta-analyses on statistics derived from previous publications) [32] and the largest sample size of individuals with SAD to date. Furthermore, we re-used data that was previously acquired and as such DTI acquisition was not harmonized across sites. On the one hand this may have affected our findings, as DTI measurements and derived parameters are susceptible to variations in hardware [45]; on the other hand, this method variance strengthens the generalizability of our findings. Lastly, being part of the ENIGMA consortium allowed us to conduct transdiagnostic correlation analyses, which revealed overlapping WM deficits across disorders.

We also acknowledge some limitations of the present work. First, the limitations of source datasets are also applied to the pooled dataset, including cross-sectional designs and missing information about age-of-onset or ethnicity for most samples, thus disallowing causal inference and additional sensitivity analyses. Second, we used a cutoff of 21 years to categorize adolescent and adult samples. Although this is in line with previous ENIGMA studies [20, 22], alternative definitions of adolescence and adulthood could affect our findings. Third, although we corrected our analyses for site effects, a selection bias might be present as most adult studies were conducted in Europe, specifically Germany, whereas most of the adolescent samples were of North American origin. Thus, findings in the adult samples only might not be

generalizable to populations in other continents. Fifth, by averaging each ROI over the two hemispheres, we were note in the position to evaluate the precise location of findings and laterality effects. Future studies are needed to investigate voxelwise alterations in significant regions, explore generalizability of our results to other internalizing disorders and examine the role of sex on WM alterations in SAD.

Conclusion

In this first large-scale mega-analysis investigating WM microstructure in SAD, several differences in WM regions were found between individuals with SAD and healthy controls in the full sample and in adults and adolescents separately. Our findings suggest that some WM abnormalities in SAD may not be disorder-specific but relate to transdiagnostic mechanisms underlying psychopathology. The observed sex-by-diagnosis interactions underscore the need for future research to investigate sex-specific neurodevelopmental and neurobiological factors in anxiety disorders.

Supplemental Note S1 Deviations and Additions to the Preregistered Analyses.

The following analyses reflect minor deviations from the preregistered plan (https://osf.io/5ycag/), made to better align with the observed data structure. First, harmonization of data was not originally included in the preregistration, as the package was still under development and ready for use a few months after preregistration. Second, correction for multiple comparisons was too strict when using the pre-registered Bonferroni method. After careful consideration, we set the threshold for significance at a false-discovery rate (FDR) corrected $p < 0{\cdot}05$, adjusting for 25 WM regions in all analyses, in line with previous ENIGMA-DTI projects [22]. Third, we only present results from additional white matter parameters, being axial, radial and mean diffusivity, if significant changes FA was found in the respective region, to avoid inflation of Type I errors and because the interpretation of these parameters is often dependent on the presence of corresponding FA alterations. Fourth, we explored sex-specific and age-specific changes only after significant effects were found in sex-by diagnosis and age-by-diagnosis analyses in several regions. Fifth, we performed correlation analyses between mega-analytical effect sizes observed in this study and the regional patterns of patient-control deficits derived using the ENIGMA-DTI workflow for five other neuropsychiatric illnesses distributed as part of the regional vulnerability index (RVI) package (https://cran.r-project.org/web/packages/RVIpkg/index.html).

Note S2 Systematic review of mega-analytic results for comparisons in SAD vs HC and relations with comorbidity in SAD subgroups.

Mega-analytic results for comparisons in SAD vs HC

After FDR correction, significant lower FA in average FA over all tracts, the BCC, CC, CR, FX, and SFO was observed in SAD patients (n = 487) compared to HC (n = 1604) in the full sample (Table S5). In these tracts, AD, MD or RD were further explored, but these additional WM parameters did not show significant group differences. These FA findings remained in three sensitivity analyses, investigating subsets of patients with a current SAD diagnosis (n = 250), without comorbid OCD (n = 447) or without comorbid PTSD (n = 424; Tables S6-8).

Significantly lower FA was observed in adult patients with SAD (n = 230), compared to adult HC (n = 1301) in the whole WM skeleton, BCC, CC, CGC, CR, FX, GCC, PTR, SFO (Table S9). Follow-up analyses revealed higher RD in the BCC and CC. No significant effects were found for MD or AD. No significant differences were observed for FA in the full analysis between adolescent patients with SAD (n = 255) patients and HC (n = 292) in the same age-range (8 - 21 years old, Table S10).

Social anxiety disorder subgroups: relations with comorbidity

In the full sample, SAD patients with comorbid anxiety disorders with (n = 142) showed lower FA in the SFO compared to healthy controls (n = 1490; Table S11). Furthermore, SAD patients without comorbid anxiety disorders (n = 317) displayed lower FA in the BCC and CC compared to healthy controls (n = 1559; Table S12). Follow-up analyses on AD, RD or MD for in these tracts did not show any significant effects. We found no significant effects when considering SAD patients with comorbid MDD (n = 184) or without comorbid MDD (n = 277; Tables S13-14).

Within the adult sample, several subgroups revealed lower FA in SAD patients when compared to HCs. Specifically, patients without comorbid anxiety disorders (n = 170) showed lower FA compared to HCs (n = 1277) in average FA, the BCC, CC, and GCC, coupled with higher RD in these regions and higher MD in the BCC only (Table S16). Additionally, SAD patients with comorbid MDD (n = 130) showed lower FA in the BCC, FX and PTR compared to HC (n = 1245; Table S17). No significant effects were observed for RD, AD or MD. There were no significant differences in FA between SAD patients with comorbid anxiety disorders (n = 36) or without comorbid MDD (n = 98) and HCs (Tables S15,18).

No significant effects were found when considering the adolescent participants (Tables S19 – 22).

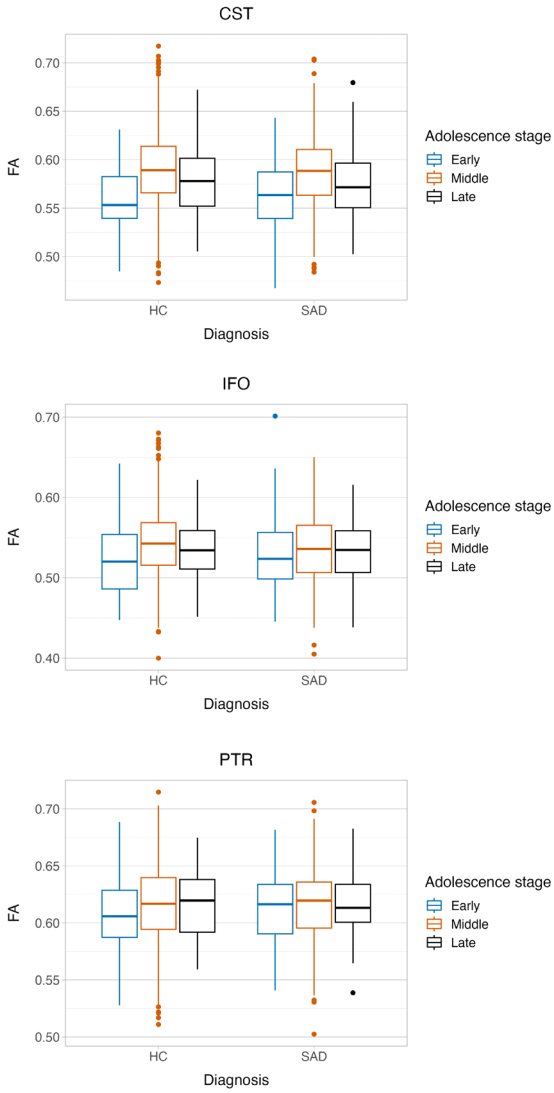
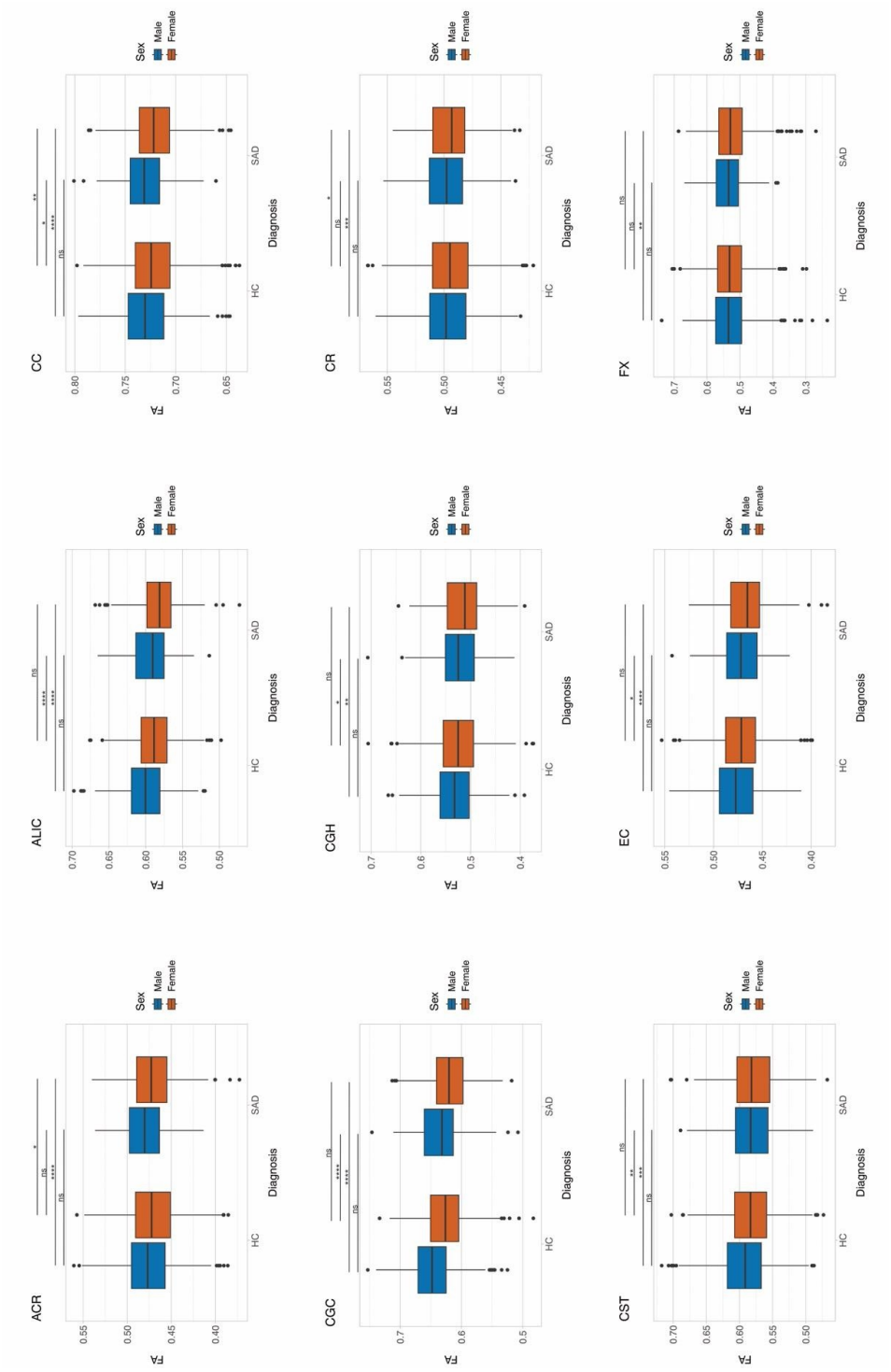


Figure S1 Post-hoc plots illustrating diagnosis-by-age interactions when comparing adolescent patients with SAD (n = 257) to HC participants (n = 317). *Abbreviations:* FA: fractional anisotropy; HC: healthy control; SAD: social anxiety disorder. *Note:* Adolescence stage based on age. Early: ≤ 14 years old. Middle: > 14 and < 18 years old. Late: ≥ 18 and ≤ 21 years old.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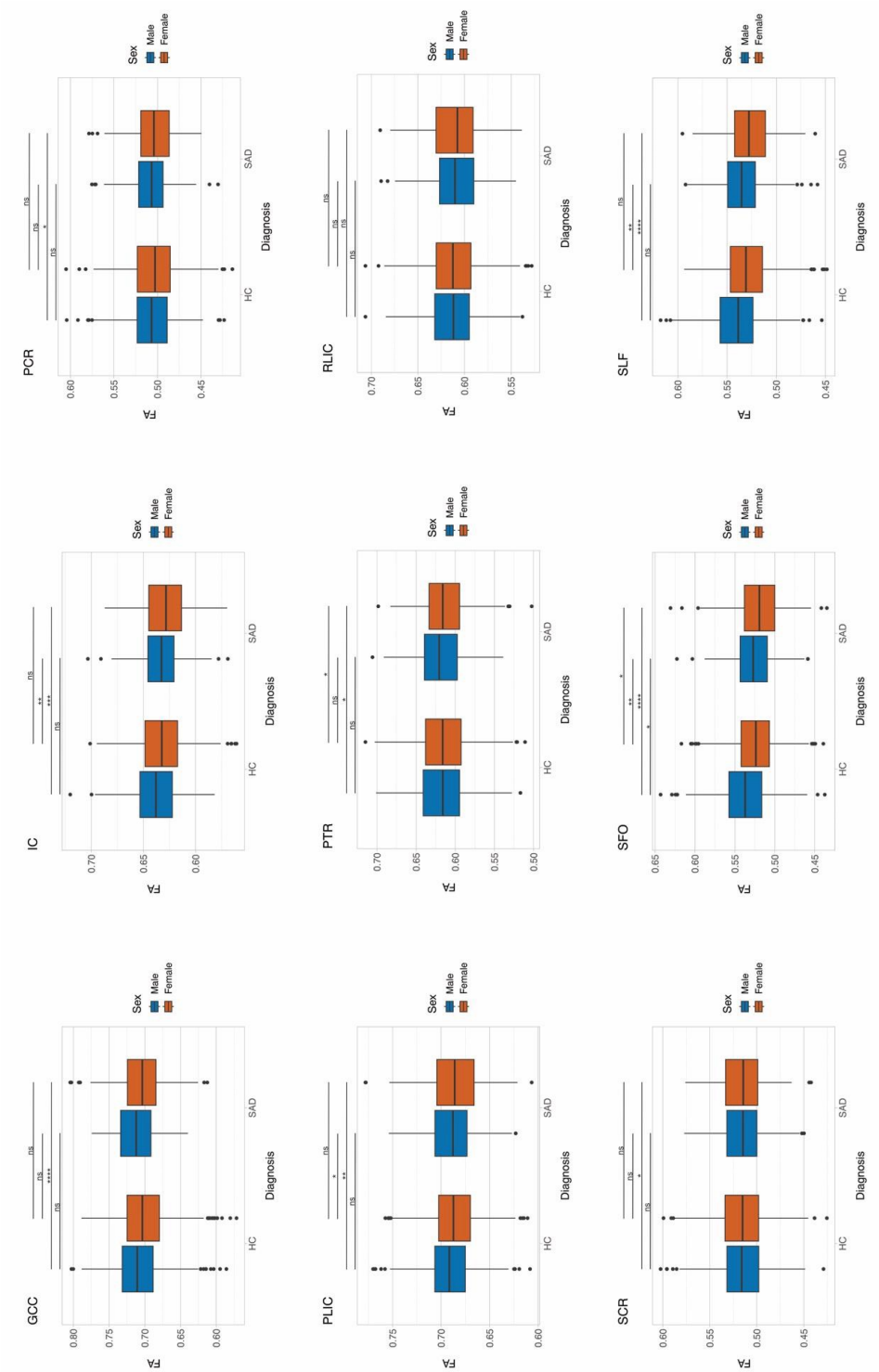

Figure S2 Post-hoc plots illustrating significant diagnosis-by-sex interactions when comparing patients with SAD (n = 487) to HC participants (n = 1604) in the full sample. *Note:* HC male: n = 651; HC female: n = 967; SAD male: n = 178; SAD female: n = 309. Significance after false-discovery rate (FDR) adjustment for multiple comparisons: ns = not significant; * $p \leq .05$; ** $p \leq 10^{-2}$; *** $p \leq 10^{-3}$; **** $p \leq 10^{-4}$.

Table S1 Study information per sample on assessment measures used for SAD diagnosis and exclusion criteria.

Site		Instrument	Exclusion criteria
1	Istanbul	SCID-CV	1) any current psychiatric disorder other than SAD diagnosed with the SCID-I/CV; 2) history of alcohol or drug abuse/dependence; 3) any serious concomitant general medical condition or neurologic disease; 4) pregnancy or lactation. Exclusion criteria for the patients and control subjects also included any contraindication for magnetic resonance imaging, and any history of neurodegenerative disease, seizure, central nervous system infection, cerebrovascular disease, diabetes mellitus, and head trauma causing loss of consciousness that lasted more than 30 min or that required hospitalization.
2	LFLSAD	MINI or MINI-Kid	Both groups: general MRI contra-indications. Controls: any psychiatric diagnosis. Patients: any other psychiatric diagnosis other than internalizing disorders.
3	FOR2107-MR	SCID	Any MRI contraindications; any neurological abnormalities. Controls: any current or former psychiatric disorder. Patients: substance dependence or current benzodiazepine treatment (wash out of at least three half-lives before study participation).
4	FOR2107-MS	SCID	Any MRI contraindications; any neurological abnormalities. Controls: any current or former psychiatric disorder. Patients: substance dependence or current benzodiazepine treatment (wash out of at least three half-lives before study participation).
5	Muenster	SCID	Psychotropic medication, presence or history of neurological, psychotic or bipolar disorders, drug dependence or abuse within the last 10 years, suicidal ideations, and fMRI contraindications.
6	MNC	SCID	Both groups: any neurological abnormalities, MRI contra-indications, presence or history of major internal or neurological disorder, dependence on or recent abuseof alcohol or drugs, hypertension, and general MRI contraindications. Patients: presence of bipolar disorder, schizoaffective disorders and schizophrenia; substance related disorders or current benzodiazepine treatment (wash out of at least three half-lives before study participation), and former electroconvulsive therapy. Controls: any current or former psychiatric disorder.
7	TIP	SCID	Both groups: any MRI contraindications; any neurological abnormalities. Controls: any current or former psychiatric disorder. Patients: substance dependence or current benzodiazepine treatment (wash out of at least three half-lives before study participation.
8a	NIMH-SDAN	KSADS/SCID	-

Table S1 (continued)

9	PNC	GOASSESS	(a) unable to provide signed informed consent (for participants under age 18 assent and parental consent were required); (b) no English proficiency; (c) physically and cognitively unable to participate in an interview and computerized neurocognitive testing.
10	MRC-SU	SCID	Any psychiatric comorbidity.
11	Vanderbilt	SCID	Both groups: unable to pass MRI safety screen, psychoactive medications in past 6 months, brain trauma. Controls: any psychiatric diagnosis. Patients: any other psychiatric diagnosis other than anxiety disorders.
12	West China Hospital	SCID	(1) the existence of a neurological disorder or other axis I psychiatric disorders; (2) axis II antisocial or borderline personality disorders (identified using the Structured Clinical Interview for DSM-IV criteria); (3) a history of drug dependence or abuse; (4) pregnancy; (5) major physical illness such as cardiovascular disease or hepatitis, as assessed by clinical evaluations and medical records; (6) any other DSM-IV axis I comorbidity.

Abbreviations: SCID-CV: Structured Clinical Interview for DSM-5 Disorders–Clinician Version. MINI: Mini-International Neuropsychiatric Interview. MINI-Kid: Mini-International Neuropsychiatric Interview for Children and Adolescents (MINI-KID). KSADS: Schedule for Affective Disorders and Schizophrenia for School-Aged Children (K-SADS). GOASSESS: Grand Opportunity Assessment [46]. SCID: Structured Clinical Interview for DSM-5 Disorders. *Notes:* Information not received (-).

Table S2 Detailed clinical characteristics of the cohorts included for mega-analysis (selectively reported for social anxiety disorder patients).

	Site	Comorbid psychiatric disorders (%)									Medication use (%)	
		PD	AG	GAD	SPH	Other-ANX	MDD	OCD	PTSD	OtherDx	Benzo	Oth-erMed
1	Istanbul	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	LFLSAD	30.0	10.0	10.0	10.0	0.0	50.0	10.0	0.0	0.0	0.0	0.0
3	FOR2107-MR	7.0	7.0	7.0	15.8	0.0	98.2	0.0	19.3	8.8	0.0	24.6
4	FOR2107-MS	19.4	19.4	12.9	3.2	0.0	96.8	6.5	3.2	29.0	0.0	32.3
5	Muenster	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.0	0.0
6	MNC	3.4	0.0	0.0	8.6	0.0	46.6	1.7	3.4	1.7	0.0	1.7
7	TIP	0.0	0.0	0.0	0.0	0.0	82.1	0.0	0.0	0.0	0.0	5.1
8	NIMH-SDAN	0.0	20.8	66.7	0.0	41.7	0.0	0.0	0.0	18.2	0.0	0.0
9	PNC	0.5	7.6	3.3	34.2	7.0	15.1	4.3	12.1	41.6	0.0	0.0
10	MRC-SU	0.0	0.0	0.0	0.0	0.0	50.0	0.0	0.0	NA	0.0	0.0
11	Vanderbilt	0.0	33.3	16.7	50.0	0.0	0.0	33.3	16.7	0.0	0.0	0.0
12	West China Hospital	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Abbreviations: PD: Panic Disorder; AG: Agoraphobia; GAD: Generalized Anxiety Disorder; SPH: Specific Phobias; OtherANX: Other comorbid anxiety disorder; OCD: Obsessive-Compulsive Disorder; PTSD: Post-Traumatic Stress Disorder; OtherDx: Other comorbid psychiatric disorder (does not include major depressive disorder); Benzo: Benzodiazepines; OtherMed: Other psychotropic medication (does not include selective serotonin reuptake inhibitors or serotonin-norepinephrine reuptake inhibitors); NA: not available (information not recorded in this sample).

Table S3 Image acquisition in the cohorts included for mega-analysis.

Site		Scanner type	Field strength	Voxel size	Slice thickness	Number of directions & b-factor
1	Istanbul	Philips Achieva	1.5T	1.75x1.75x2mm	2mm	32 directions, b-factor = 800, 1 b0 image
2	LFLSAD	Philips Achieva	3T	1.9 x 2.4mm	2mm	30 directions, b-factor = 1000, 2 b0 images
3	FOR2107-MR	Siemens Magnetom Trio	3T	2.5 x 2.5 mm	2.5 mm	30 directions; 4b images; b-factor = 1000
4	FOR2107-MS	Siemens Prisma	3T	-	-	-
5	Muenster	Siemens Magnetom PRISMA	3T	2.3x2..3x3 mm	3mm	30 directions, b-factor = 1000, 4 b0 images
6	MNC	Philips Gyroscan Intera	3T	-	-	-
7	TIP	-	-	-	-	-
8a	NIMH-SDAN	GE MR 750	3T	1.875mm^3	2.5mm	62 directions, b-factor = 1000, 2 b0 images
8b	PNC	GE MR 750	3T	1.87mm^3	2.5mm	75 directions, b-factor = 300 (6) and 1100 (69), 5 b0 images
8c	MRC-SU	GE MR 750	3T	2x2mm	2.9mm	48 directions, b-factor = 1000, 8 b0 images
9	Vanderbilt	Siemens Trio	3T	1.8x1.8x2 mm	2mm	64 directions, b-factor = 1000, 7 b0 images
10	West China Hospital	Siemens Magnetom Allegra	3T	1.8 x 1.8 x 2.0mm	2mm	- directions, b-factor = 1000
11	Istanbul	Philips Achieva	3T	2.5mm^3	2.5mm	92 directions, b-factor = 1600, 1 b0-mage
12	LFLSAD	GE SIGNA EXCITE	3T	0.9375mm^3	3mm	16 directions

Abbreviations: GE: General Electric. *Notes:* Information not provided (-).

Tables S4 – S44 Mega-analytic results and correlation analyses.

Due to their size, these tables are available using the QR code below.

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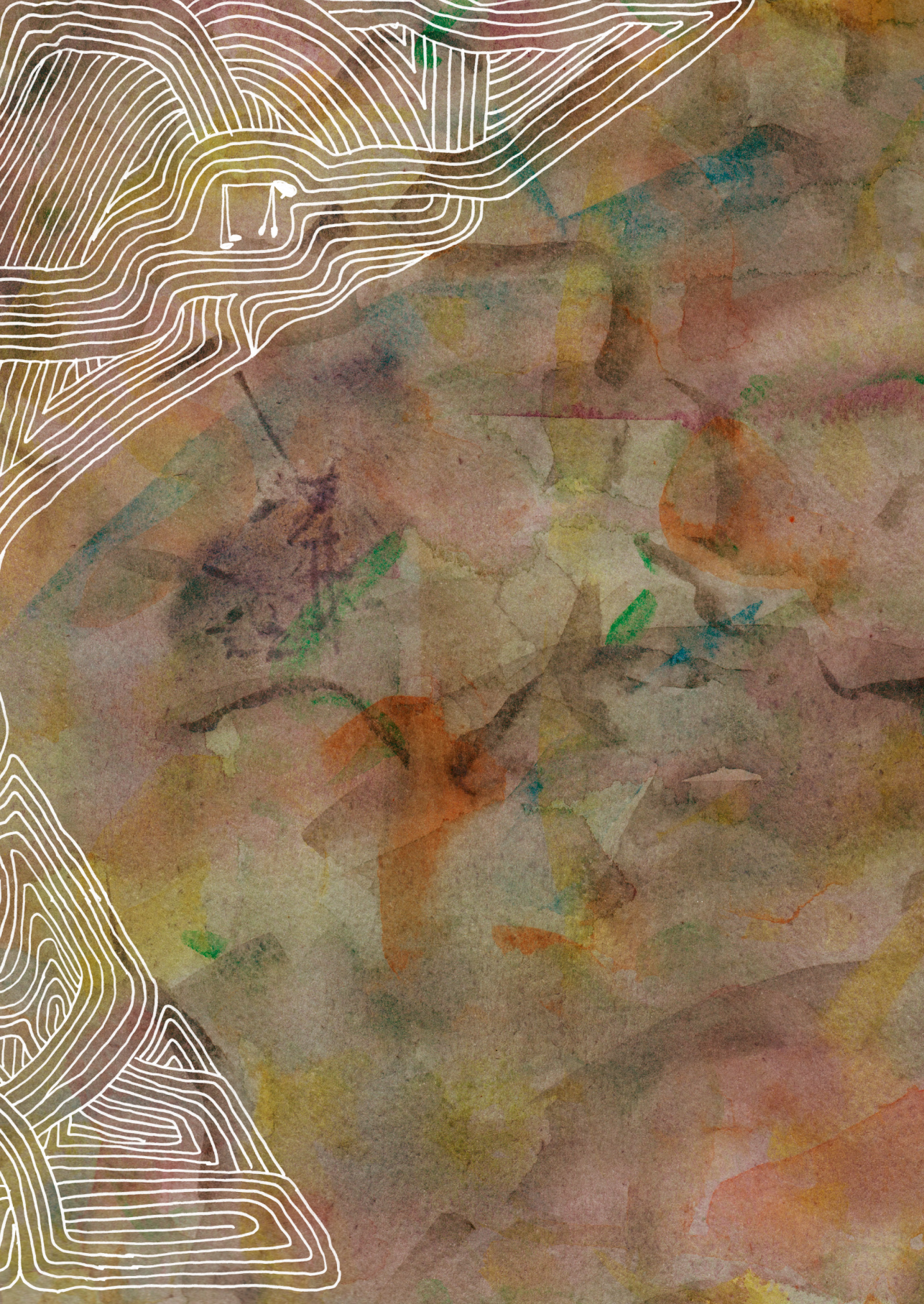


Discussion
&
Appendices

6

General discussion

Discussion

Scope of the Thesis

Internalizing disorders like anxiety and depressive disorders are prevalent, highly disabling and often comorbid with other internalizing disorders [1-3]. Internalizing disorders usually start in a crucial transitional period in life: adolescence. Previous research on adolescents with internalizing disorders has shown changes in functional and structural connectivity within and between brain regions involved in emotion processing, such as the amygdala and other parts of the limbic system, as well as in regulatory regions like the prefrontal cortices [4-6]. However, longitudinal investigations of changes in brain connectivity are lacking.

One of the most incapacitating and prevalent internalizing disorders is social anxiety disorder (SAD), a crippling fear of scrutinization by others which can lead to experiencing social situations with intense fear or avoidance of social situations [7]. Alterations in white matter microstructure and functional connectivity have been reported in a few studies on adults with SAD and a heritable base has been reported [8-10]. Considering these characteristics, SAD provides an ideal model to use innovative research approaches in order to study adolescent internalizing disorders from a new perspective.

Building on current expertise, the objective of this thesis was to expand knowledge of neurobiological networks underlying anxiety and depression in adolescents by investigating structural and functional connectivity of the brain. Two aims were addressed throughout this thesis. The first aim was to investigate longitudinal changes in structural and functional brain connectivity in adolescents with internalizing disorders. The second aim was to deepen our understanding of white matter microstructure in SAD, examining vulnerability factors to develop SAD and structural alterations in the largest cohort to date. In the following sections, I discuss the results from our studies, offer general reflections and propose directions for future research.

Part 1: Changes in brain networks over time in internalizing disorders

In the first part of this thesis (**Chapters 2 and 3**) we explored longitudinal changes in white matter microstructure and resting-state functional connectivity (RSFC) in adolescents with internalizing disorders and their healthy peers over the course of three months, using data from the *Emotional Pathways' Imaging Study in Clinical Adolescents* (EPISCA). EPISCA is a longitudinal study designed to investigate neurobiological mechanisms related to emotion processing and regulation in a clinical cohort of adolescents with stress-related psychopathology. Measurements were taken at baseline, after three months and at six months. The study consisted of three groups: adolescents with internalizing (depressive and anxiety) disorders, adolescents with trauma disorders and a healthy control group. Participants received care-as-usual and were followed over the course of six months without study interventions, thus reflecting real world development of brain networks in a clinically heterogeneous group as closely as possible. Results of the neuroimaging measurements at baseline have been previously reported [11-15].

In the studies in this thesis, only data from the internalizing group and the healthy control group on baseline and after three months were used, as loss to follow-up was too great after six months. We investigated changes in RSFC and white matter microstructure between groups over time. In addition, we explored whether functional and structural connectivity changed over time within the patient group and if these changes were associated with symptom severity. In summary, we found that development of white matter microstructure did not differ between groups or within the patient group. However, associations between baseline white matter microstructure and symptom severity were found (**Chapter 2**). With regard to functional connectivity, the groups differed in RSFC development over time, specifically in development of the laterobasal amygdala (LBA), a subsection of the amygdala, to frontal regions and to the postcentral gyrus. Moreover, in the patient group, associations between changes in LBA RSFC and symptom change were demonstrated (**Chapter 3**). In conclusion, adolescents with internalizing disorders showed different functional connectivity of the LBA compared to healthy peers, but they did not differ from healthy peers in development of white matter microstructure. To better understand these findings, I will discuss each study in detail, beginning with the investigation of structural connectivity changes over time.

Longitudinal analysis of structural connectivity

In **Chapter 2**, we focused on longitudinal changes in white matter microstructure in adolescents with internalizing disorders compared to healthy peers. No previous reports existed regarding *longitudinal* changes in white matter microstructure in adolescents with internalizing disorders. Only a few *cross-sectional* studies have been conducted in adolescent depression and only one has addressed adolescent anxiety [11, 16-19]. In all studies, lower fractional anisotropy (FA) in several tracts was found in adolescents with depression or generalized anxiety disorder when compared to healthy peers, such as in the bilateral uncinate fasciculus (UF), cingulum and corpus callosum [11, 17-20]. These tracts are thought, among others, to be involved in regulation of and communication within and between regions of the corticolimbic network [21, 22]. Thus, although inconclusive, previous results point to changes in structural connectivity in adolescents with internalizing disorders. Therefore, we hypothesized that FA development would be different in adolescents with internalizing disorders compared to healthy control participants, mainly in regions previously implied in emotion processing, such as the cingulum, UF and corpus callosum.

We explored longitudinal changes in white matter microstructure between the patient group (n = 22) and their healthy peers (n = 21), as reflected by group x time interactions. In addition, we investigated associations with symptom changes within the group of adolescents with internalizing disorders. Using tract-based spatial statistics (TBSS), we investigated structural connectivity of three tracts of interest (TOI) based on previous literature: the UF, corpus callosum and cingulum. Furthermore, we performed exploratory whole-brain voxelwise analyses.

Our analyses did not reveal differences in white matter microstructure between patients and controls. However, within the patient group, voxelwise whole-brain analyses revealed that lower baseline FA in

the right cingulum and posterior corona radiata was associated with a higher decrease of depressive symptoms over the course of three months. The corona radiata and cingulum play significant roles in various psychiatric conditions, including depression and anxiety, due to their involvement in emotional regulation and the limbic-thalamo-cortical circuitry. The corona radiata, encompassing among others the superior corona radiata (SCR) and posterior corona radiata (PCR), has been linked to psychopathology. For instance, reduced FA in the SCR is associated with a higher risk of psychopathology in adolescents with a family history of mental disorders [23]. Functional and structural connections of the PCR to the DMN and emotional regulation systems [24, 25] suggest relevance to internalizing disorders, although its precise contribution to adolescent depression and anxiety remains unclear. Similarly, the cingulum, involved in emotional regulation and communication within the limbic system [21, 24], shows reduced FA in adolescents with depression [18, 20] and delayed myelin maturation which is linked to anxiety [26, 27]. These findings suggest that the PCR and cingulum might contribute to emotional dysregulation, potentially influencing the development of internalizing disorders, though the exact nature and directionality of these microstructural changes require further investigation [28, 29]. Complementing these findings on structural connectivity, our examination of functional connectivity revealed more pronounced group differences.

Longitudinal analysis of functional connectivity

In **Chapter 3**, we described a study investigating RSFC of multiple amygdala subregions and connectivity within whole-brain resting-state networks. Several previous studies have revealed cross-sectional and longitudinal changes in RSFC in adolescents with internalizing disorders, for example in amygdala connectivity and resting-state networks.

Previous studies on *longitudinal* changes in RSFC in adolescent depression have revealed alterations over time in functional connectivity of regions involved in emotion processing, mainly in the amygdala [30-36]. Moreover, associations with changes in symptom severity were reported [32, 36]. Furthermore, alterations in resting-state networks such as the DMN have been reported in *cross-sectional* studies in depressed adolescents and anxious adults [37-39]. Recent studies have shown that amygdala subregions, being the LBA and centromedial amygdala (CMA), contribute to different aspects of emotion processing. It is thought that the LBA is involved in regulation and perception of fear, while the CMA is mainly concerned with acute stress reactions [40, 41]. However, the role of amygdala subregions and resting-state networks in mental disorders had yet to be elucidated. Moreover, previous longitudinal studies on RSFC in adolescent internalizing disorders have used standardized treatment protocols. In practice, treatment is often personalized, adjusted to the level of emotional and cognitive development and in collaboration with family [42-44].

In this study, we used rs-fMRI data of *EPISCA* to investigate longitudinal changes in RSFC in a clinically representative sample of adolescents with internalizing disorders and healthy peers. Similar to the methods described in **Chapter 2**, participants were scanned at baseline and after three months while patients received care-as-usual. Building on previous findings, we hypothesized differences over

time between the groups in RSFC, particularly in regions involved in emotion processing, such as limbic structures, and regions involved in emotion regulation, like frontal regions. We explored longitudinal changes in RSFC by combining two analysis strategies that complement each other. The first strategy was investigation of connectivity of amygdala subregions, being the LBA and CMA, to the rest of the brain, using seed-based analyses. For the second strategy we applied independent component analysis (ICA) to detect resting-state networks. Subsequently, we explored changes within these networks, being the default mode, dorsal attention, frontoparietal (separated in a left and right component), executive control, salience and affective network. We explored group x time interactions between adolescents with internalizing disorders (n = 23) and healthy controls (n = 24). Further, we investigated associations with symptom changes within the patient group.

We found significant group x time interactions of the right LBA to the postcentral gyrus and of the left LBA to the frontal pole, reflecting differences in RSFC development between adolescents with internalizing disorders and healthy peers. Within adolescents with internalizing disorders, RSFC to the frontal pole and to the postcentral gyrus changed over time, while no changes were observed in the control group. Further analyses within the patient group revealed that changes in RSFC of the right LBA were also linked to changes in internalizing symptoms. There were no significant group x time interactions when considering RSFC from CMA bilateral seeds or within ICA derived networks. Our results suggest divergent longitudinal development of RSFC from bilateral LBA subregions, which might be associated with symptom changes. Furthermore, our findings underscore the importance to investigate amygdala subregions as results were specifically located in FC of the LBA, a subregion connected to, among others, frontal regions. In contrast, analyses on FC of the CMA, mainly connected to the brainstem nuclei that generate behavioral and visceral correlates of acute stress-reactions [40, 45, 46], and post-hoc analyses on FC of the whole amygdala did not reveal any significant interactions.

General reflections on part 1

Taken together, the studies described in **Chapter 2 and Chapter 3** show that functional connectivity of the bilateral LBA diverged in adolescents with internalizing disorders from healthy peers, as reflected by significant group x time interactions, while our data did not support deviant development of structural connectivity. In addition, associations between symptom change and changes in both structural and functional connectivity were found within the patient group. These results potentially reflect nonspecific treatment effects, but could also indicate natural recovery over time, the influence of unmeasured informal interventions within care-as-usual, or compensatory brain mechanisms where the brain reorganizes its connectivity to adapt to symptom fluctuations.

It seems logical that we found changes in functional, but not structural connectivity. Functional connectivity, as reflected by RSFC, is a relatively dynamic and flexible process. Structural connectivity, on the other hand, is a slower process. Changes in functional connectivity have been reported within 12 – 24 weeks [32, 47], while development of white matter tracts usually takes years, depending on the tract

[48, 49]. As far as I am aware, no longitudinal studies on adolescent white matter microstructure have been conducted with relatively short timeframe of weeks to months.

These studies are innovative in several ways. First, we are among the first to investigate longitudinal neurobiological changes in a clinical population with internalizing disorders receiving treatment as usual and a healthy control group. Specifically, our study on white matter microstructure was the first to investigate these longitudinal changes in adolescents with internalizing disorders. Second, we were the first to investigate longitudinal changes in RSFC in subregions of the amygdala and data-driven resting-state networks in this population. A novel finding was that changes in RSFC were only present in the network originating from the LBA, not the CMA. Third, we found associations between changes in symptoms and changes in both functional and structural connectivity.

However, it should be noted that the studies conducted in this thesis used a relatively small sample size, a relatively short study duration of three months and just two study measurements. Moreover, we were only able to use data from baseline and after three months, as loss-to-follow up was too great after six months (loss-to-follow-up after three months: 16%, loss-to-follow-up after six months: 24%). Therefore, subtle effects might have been overlooked due to small sample size, too little intervals or they might not yet be present in too short a timeframe.

Because we are among the first to investigate longitudinal changes in the brain in adolescents with internalizing disorders, future studies are needed to determine whether and to what degree our results can be replicated. For example, further investigations should consider longitudinal investigations in white matter microstructure in other tracts and continue investigation of whole-brain resting-state networks. Ideally, this would be in a larger study population over a longer period of time. The inability to utilize the six-month data is a waste of resources given the substantial investment in data collection. It would be worthwhile to increase sample size, as with a higher number of participants the data of the total sample might still be useful despite a relatively high number of dropout.

Concluding, our findings contribute to an increased understanding of underlying brain networks in adolescent internalizing disorders and warrant further exploration of longitudinal changes in structural and functional connectivity.

Part 2: Structural connectivity in social anxiety disorder

The second part of this thesis (**Chapter 4 and 5**) aimed to deepen our understanding of involvement of white matter microstructure in SAD. SAD is one of the most prevalent and incapacitating psychiatric disorders [50-52]. It usually starts in late childhood or early adolescence and has a high comorbidity, mostly with other internalizing disorders [51, 53]. Previous studies have attempted to identify neurobiological factors underlying SAD by investigating structural and functional connectivity. Lower FA has been reported in patients with SAD compared to healthy controls in regions involved in emotion processing and emotion control, like the UF and the superior longitudinal fasciculus (SLF) [8]. Furthermore, changes in functional brain networks and a heritable basis for SAD have been reported [9,

54]. Despite previous efforts, studies have not led to concluding findings about underlying neurobiology involved in this disorder thus far. It remains unclear whether changes in structural connectivity are linked to genetic vulnerability or whether changes in structural connectivity are present over the lifespan.

SAD serves as an excellent model disorder for implementing innovative methodological approaches to examine neurobiological mechanisms underlying adolescent internalizing psychopathology. In this thesis, I have applied two research methods to investigate white matter microstructure in SAD. First, we used a family study design to investigate whether changes in white matter might be candidate endophenotypes of SAD. An endophenotype is a measurable, heritable trait that is found in individuals with a certain disorder and their unaffected relatives, serving as a biological marker for genetic risk [55]. Second, we conducted a mega-analysis in the largest dataset of white matter parameters in SAD to date to investigate differences between patients with SAD and healthy controls in a large age group (age 8 – 65 years) in 25 white matter tracts, covering the majority of the brain.

In the family study in **Chapter 4**, we found that increased FA in the bilateral SLF co-segregated with social anxiety symptoms, and confirmed that all studied white matter characteristics were at least moderately heritable. This suggests that alterations in the bilateral SLF could be candidate endophenotypes for SAD. Next, the mega-analysis in **Chapter 5** revealed several novel findings: individuals with SAD, particularly adults, showed lower FA in tracts like the corpus callosum and fornix; there were widespread sex-by-diagnosis and age-by-diagnosis interactions; and the pattern of these findings overlapped with those from other psychiatric disorders, suggesting transdiagnostic neurobiological features. In conclusion, we found several alterations in white matter microstructure in patients with SAD, revealing candidate endophenotypes and differences in white matter microstructure in SAD patients. I will discuss each study in more detail below.

Candidate endophenotypes of SAD

In **Chapter 4**, we explored changes in white matter microstructure in families selected based on a high genetic risk for SAD to investigate candidate endophenotypes of SAD by using data from the Leiden Family Lab on Social Anxiety Disorder (LFLSAD). An endophenotype has to be associated with the disorder (criterion 1), be state-independent (criterion 2), heritable (criterion 3) and has to co-segregate with the disorder within families of probands while already present in a preclinical state (criterion 4) [55-58]. The LFLSAD is the first comprehensive two-generation family neuroimaging study on SAD. It has been designed specifically to examine the heritability and first part of the co-segregation criteria of candidate endophenotypes of SAD [59]. Other results of this study have been published already [60-64]. In previous work, white matter characteristics were found to be moderately heritable and changes in white matter microstructure were reported in SAD [8, 65]. This combination of heritability (endophenotype criterion 3) and association with the disorder (endophenotype criterion 1) makes white matter characteristics promising candidate endophenotypes for SAD. However, the criterion of co-segregation within families of probands (criterion 4, first element) has not been examined yet. In this study, we investigated associations with social anxiety symptoms and heritability of three a priori selected

TOIs, being the UF, SLF and ILF, in a cross-sectional family study design. We expected to find a negative association between the level of social anxiety symptoms and FA in the UF, SLF and ILF. Furthermore, we expected estimates of all WM parameters to be at least moderately heritable.

Using TBSS, two analyses were conducted to investigate associatons between FA and social anxiety symptoms. First, we employed a voxelwise analysis and an analysis of the averaged values of WM parameters over the whole TOI. In addition, we performed an exploratory voxelwise analysis of the whole WM skeleton to investigate WM microstructure outside the a-priori defined regions. Finally, general heritability of white matter parameters was estimated.

Results showed that increased FA in the left and right SLF co-segregated with symptoms of social anxiety, as reflected by a positive correlation. Specifically, voxelwise analyses revealed that results were located in the SLF II. Furthermore, all investigated characteristics of white matter microstructure were at least moderately heritable.

The SLF II is the major part of the SLF and is mostly concerned with visuospatial attention and processing. Structurally it connects the caudal part of the inferior parietal lobule (IPL) and intraparietal sulcus with the posterior part of the prefrontal cortices. Functionally, the SLF II is thought to connect the parietal part of the ventral attention network with the prefrontal component of the dorsal attention network and is involved in the DMN [21, 22, 66-71]. Interestingly, our findings are in contrast with previous literature, which reported lower FA specifically in the SLF III in patients with SAD compared to healthy controls [72-74]. These conflicting results will be examined more thoroughly in the general discussion section of part 2, also incorporating the findings described in **Chapter 5**.

In sum, these findings suggest that alterations in white matter microstructure in the bilateral SLF could be candidate endophenotypes of SAD as they co-segregated within families genetically vulnerable for SAD and are heritable. However, longitudinal studies are needed to investigate state-independency. To complement this family-based approach and examine structural connectivity related to SAD in a large cohort, we next conducted a large-scale mega-analysis within the framework of the Enhancing NeuroImaging Genetics through Meta-Analysis (ENIGMA)-Anxiety Working Group.

Mega-analysis of white matter microstructure in SAD

As described in **Chapter 5**, the ENIGMA-Anxiety framework was used to explore changes in white matter microstructure in patients with SAD and healthy control participants in a mega-analysis. White matter microstructure is known to develop well into the third decade of life with a peak mostly in adolescence [75]. Previous studies on white matter differences in SAD yielded inconclusive results, probably at least partly due to a limited number of studies which used small sample sizes and were largely conducted in adults [8]. Thus, this study addressed the knowledge gap on structural connectivity in SAD over the ages by investigating changes in white matter microstructure in a large cohort spanning a wide age range (8 – 65 years). We expected to find differences in FA between patients with SAD and healthy control participants, as well as age-specific alterations in adults and adolescents.

Data from 12 research samples worldwide (n = 2104) was combined to investigate white matter microstructure in 25 regions of interest in a pre-registered mega-analysis. We compared white matter microstructure data from patients with SAD (n = 487) to healthy controls (n = 1604) and investigated interactions with sex and age. Furthermore, sensitivity analyses were applied to investigate the role of comorbidity, medication and associations with clinical symptoms. Following analyses in the full sample, separate analyses were performed in adult (> 21 years old) and adolescent samples (≤ 21 years old).

We reported several novel findings. First, patients (full sample) showed lower FA in several tracts, such as the corpus callosum and fornix, when compared to healthy controls. This pattern persisted throughout several sensitivity analyses and was replicated in the adult sample. Second, widespread sex-by-diagnosis interactions across the brain were observed across the full sample. Third, several age-by-diagnosis interactions were found in the full sample and in the adolescent sample. Fourth, the regions and direction of results correlated with those reported in previous ENIGMA DTI studies on major depressive disorder, bipolar disorder and schizophrenia [76-78]. Interestingly, our findings were not found in previous studies on white matter microstructure in SAD. These studies reported lower FA in other regions, such as the UF. Yet, as reviewed by Parsaei et al, previous studies on SAD investigated a limited number of regions in small cohorts with limited age ranges [8], which might explain why the main results were not reported previously. In contrast, the wide age range and comprehensive tract coverage in our study may have reduced sensitivity to alterations restricted to more narrowly defined populations, particularly as such effects might only survive the less stringent correction for multiple comparisons used in regionally focused analyses. Our findings suggest that some neurobiological changes in white matter tracts in individuals with SAD might be part of broader transdiagnostic neurobiological features underlying psychopathology. However, future studies are needed to confirm this hypothesis.

General reflections on part 2

In conclusion, the studies in **Chapter 4 and 5** investigated structural connectivity in SAD using two innovative designs. We have conducted the first family-study to explore whether changes in white matter microstructure could be candidate endophenotypes of SAD and performed the first mega-analysis of white matter microstructure in SAD. These studies have led to several novel findings. First, we found evidence that microstructural changes in the SLF might be promising candidate endophenotypes of SAD. Second, mega-analyses within the largest composed dataset to date showed that patients with SAD have lower FA compared to healthy controls in several white matter tracts. Moreover, lower FA in the corpus callosum and fornix might be indicative of transdiagnostic neurobiological changes, as correlations were found with patterns of white matter alterations previously demonstrated in patients with schizophrenia, major depressive disorder and bipolar disorder [76-78]. Third, we found widespread sex-by-diagnosis interactions across the brain which have been described only in selected tracts previously [79, 80].

Surprisingly, results on structural connectivity from our studies in the second part of this thesis (**Chapter 4 and 5**) did not coincide but were contradictory. It is puzzling that increased FA in the

SLF co-segregated with symptoms of social anxiety (representing a positive correlation) in the study in **Chapter 4**, whereas patients with SAD had lower FA in the SLF compared to healthy controls in the study in **Chapter 5**. It should be noted that this result did not remain significant after correction for multiple comparisons. In these paragraphs, I will discuss several differences between these studies and propose multiple hypotheses. The first difference lies in the selection of tracts and study populations. We investigated three a priori selected TOIs, being the SLF, UF and ILF, in families selected based on a high genetic risk for SAD (**Chapter 4**) and used a case-control design to explore 25 TOIs in a mega-analysis in **Chapter 5**. The family study might be more sensitive to detect subtle genetic influences or preclinical states which are not captured in a broad case-control design like the mega-analysis, although this does not account directly for contradictory findings on increased and decreased FA. The second difference is found in the way white matter microstructure was investigated. Although both studies investigated white matter microstructure using TBSS, we also investigated voxelwise changes in the family study. This revealed higher FA in a specific subregion of the SLF, being a cluster within the SLF II. As we investigated averaged FA over the bilateral SLF in **Chapter 5**, we may have missed subtle regional FA alterations within the SLF in patients with SAD. Specifically, we cautiously hypothesize that while overall FA might be reduced across the entire SLF, a localized increase in FA could exist within the SLF II. Such subtle, region-specific variations may not be discernible using methods that rely on averaged FA values across the entire (bilateral) tract. This might be particularly critical for detecting early or subtle markers of psychopathology, which potentially only become evident in genetically enriched cohorts or preclinical disease stages. Furthermore, it is plausible that a reduction in FA within regions of the SLF could be masked or "levelled out" by a compensatory increase in FA within the SLF II, leading to non-significant findings in analyses on average FA of the SLF.

Moreover, it is important to note that higher FA values do not automatically indicate improved white matter microstructure. An apparently paradoxical increase in FA can arise from several microstructural phenomena. For instance, the presence of crossing white matter fibers within a voxel can artificially elevate FA if one fiber population undergoes significant degradation or atrophy while another remains relatively preserved. Elevated FA might reflect compensatory processes, such as enhanced fiber alignment coherency, as the brain adapts to underlying pathological changes [28, 81-83].

Therefore, while the results may appear contradictory at first glance, multiple hypothesized explanations could account for these findings. Future investigations would ideally incorporate more detailed analysis methods such as tractography, a method which is able to trace anatomical connections of white matter between several brain regions, to elucidate the precise fiber architecture and directionality of the SLF. While the existing data from **Chapter 5** may present challenges for such advanced analyses, applying tractography to the data collected in **Chapter 4** would be more feasible and promising to gain deeper insights into SLF microstructure in SAD.

In conclusion, future studies are needed to replicate the findings on alterations in white matter microstructure in SAD. Further research should additionally include information about important

covariates such as age-of-onset and ethnicity in a large longitudinal dataset, as the studies in **Chapter 4 and 5** were cross-sectional, thus unable to investigate the course of structural changes over the lifespan, and did not report on these covariates. Investigating age-of-onset might aid in understanding subtypes of a disorder, providing insights in the interaction between white matter development throughout the lifespan and a mental illness. For example, differences in white matter between early and late onset psychosis have been previously reported [84], although single studies which focused on internalizing disorders did not reveal any differences in white matter microstructure between patients with adolescent- and adult-onset major depressive disorder [78]. Regarding ethnicity, differences in white matter microstructure have been reported in healthy Black, White, Asian and Hispanic participants [85, 86]. Thus, ethnicity might influence results on white matter alterations in internalizing disorders.

Taken together, our findings underscore the importance of large-scale datasets across the lifespan and innovative exploration of heritable components in SAD. Building on these insights and the limitations identified across both parts of this thesis, I now consider the broader implications of this work and several key directions that emerge for future research.

General Discussion and Suggestions for Future Research

Internalizing disorders are unique and complex, rising from numerous interactions between environmental, genetic, biological, developmental and temperamental factors that lead to a mental illness [4, 30, 51]. This thesis aimed to untangle some of these complex interactions in young people with internalizing disorders by investigating brain networks using several methods. We have explored longitudinal changes of structural and functional connectivity and investigated white matter microstructure in families with SAD and in a mega-analysis. The results add to the existing body of evidence i) that divergent structural brain connectivity is present in adolescents with internalizing disorders compared to healthy peers, and ii) that changes in white matter microstructure are present in SAD, of which some might be transdiagnostic or candidate endophenotypes. While these methods have provided additional insights into brain networks in young people with internalizing disorders, further research is necessary. Several suggestions are outlined below.

Larger, more inclusive and diverse datasets

One important direction for future work is the development of larger, more inclusive and diverse datasets. A considerable body of evidence in structural and functional neuroimaging in internalizing disorders presents us with at least some neurobiological ground that contributes to the complex interactions mentioned above, such as changes in regions of the corticolimbic network and a heritable base for SAD [4, 9, 87-90]. This evidence is largely compiled over the years by studies that have investigated a highly selected group of patients, usually without medication or comorbid disorders, in small sample sizes, using cross-sectional designs and different analysis methods. Such studies complicate reproducibility, comparability and may not adequately reflect the complexity of real-world clinical populations with internalizing disorders, where symptoms are heterogeneous, frequently comorbid and often influenced by ongoing treatment [91-94]. Although small in study size and duration, the studies presented in the

first part of this thesis used broad inclusion criteria to improve the generalizability of findings and capture the full spectrum of internalizing symptomatology. Multi-site collaborations, such as those facilitated by the ENIGMA consortium, offer a valuable infrastructure for aggregating data at scale and increasing statistical power [93, 95].

Moreover, careful consideration is needed in terms of participant selection. Inclusion strategies should aim to reduce selection bias by including a broad range of individuals, spanning various demographic backgrounds and ethnicities, treatment histories and symptom profiles if available to increase the generalizability of findings to real-world clinical populations.

Transdiagnostic and dimensional frameworks

Building on the call for more inclusive datasets, there is a pressing need to re-evaluate how study populations are defined and characterized. Rather than relying on reductionistic diagnostic categories, such as those in the DSM-5, future studies should adopt transdiagnostic approaches that reflect the symptom heterogeneity and comorbidity typical of clinical populations and move beyond traditional categorical diagnostic frameworks. Dimensional and transdiagnostic approaches, such as the Research Domain Criteria (RDoC) and the Hierarchical Taxonomy of Psychopathology (HiTOP) [96, 97], may better capture the complexity and spectrum-like nature of internalizing psychopathology.

On the other hand, next to practical and implementation challenges such as training of clinicians, dimensional and transdiagnostic frameworks used to capture internalizing psychopathology might be more susceptible to lower inter-rater reliability and differences between and within diverse populations, leading to inaccurate diagnoses and interventions. Moreover, the line between 'healthy' and 'pathological' might be blurred when using a dimensional rather than a categorical scale, risking under- or overpathologization of normal human experiences. Lastly, these frameworks could complicate communication between healthcare professionals, researchers and insurance providers.

Longitudinal and multimodal designs

Next to large, inclusive datasets and transdiagnostic frameworks, longitudinal and multimodal study designs should be prioritized in future research [98]. Including waitlist control groups, consisting of patients who do not receive any treatment, while also comparing clinical populations to normative samples could provide essential benchmarks for interpreting neurobiological deviations. As this method is not feasible for long-term investigations due to ethical reasons, other options could include naturalistic cohorts, in which patients are followed over many years. Furthermore, continued investigation of candidate endophenotypes may yield important markers for risk stratification and early intervention in individuals at risk for developing psychopathology. Finally, future research will benefit from deep phenotyping approaches that integrate clinical, cognitive, behavioral and neurobiological data. Projects such as FRENCHMINDS (https://pepr-propsy.fr/2024/09/30/frenchminds/) demonstrate the potential of these interdisciplinary, multimodal research efforts, as they aim to identify transdiagnostic profiles in patients with a wide variety of psychiatric disorders. Specifically, FRENCHMINDS aims to

include a large number of psychiatric patients and healthy controls in a study with a 14-month follow-up, collecting (neuro)biological data on two timepoints and monthly data on sleep, activity and symptoms to identify transdiagnostic profiles focused on social withdrawal and anhedonia. Such platforms can serve as valuable models to expand our understanding of internalizing disorders and tailoring interventions to the individual.

Improve methodological rigor and data richness

Lastly, from a methodological standpoint, improving rigor and depth of data is crucial. While studies using MRI-scans have yielded important insights into neurobiological alterations in internalizing disorders, our capacity to identify reliable neurobiological markers remains limited. Advancements in software, hardware, and analytical techniques, including artificial intelligence, may enhance our ability to detect clinically meaningful patterns in the future. Nevertheless, the value of MRI as a research tool is likely to increase only if it is embedded within broader, more inclusive research frameworks as described in the paragraph above.

Moreover, it is important to address platforms such as the Open Science Framework (OSF; https://osf.io/). These initiatives aim to increase transparency, collaborations and reproducibility of research. For example, it is possible to pre-register study plans (the pre-registration for the study in **Chapter 5** can be found here: https://osf.io/5ycag/). Preregistration of hypotheses and analytic plans can enhance transparency, reduce analytic flexibility and increase reproducibility. By combining technological advances with robust open science practices, researchers can work toward building a more reliable and cumulative knowledge base that ultimately benefits both scientific understanding and clinical applications in mental health research.

Explanatory reach of neuroimaging

Ultimately, neuroimaging represents just one piece of a much larger puzzle of all available research methods that can be used to untangle underlying factors in adolescent internalizing disorders. While it may eventually assist in identifying individuals at ultra-high risk, such as those exhibiting endophenotypes of social anxiety or in guiding personalized treatment strategies (e.g., tailoring interventions based on individual patterns of white matter microstructure, which are not available yet), its explanatory reach is inherently limited. Psychiatric disorders are shaped by a vast constellation of biopsychosocial factors, many of which remain poorly understood. Although we may never fully elucidate the precise mechanisms through which these disorders arise or why certain individuals fail to respond to treatment, progress remains both possible and necessary. The work presented in this thesis aims to contribute to that progress, offering insights that may ultimately improve outcomes for patients and their families.

Conclusion

This thesis aimed to advance our understanding of the neurobiological mechanisms underlying internalizing disorders in adolescence, with a particular focus on structural and functional brain connectivity. In the first section of this work we investigated longitudinal trajectories of functional and structural connectivity in adolescents with anxiety and depression. The findings revealed divergent developmental patterns in functional connectivity compared to healthy peers and changes in functional and structural connectivity which were associated with changes in symptom severity over time. These results highlight the dynamic nature of brain development in youth with internalizing disorders and suggest that changes in brain networks may contribute to internalizing psychopathology in adolescence.

The second section of this thesis focused specifically on SAD using two innovative study designs to explore changes in white matter microstructure. We examined a unique sample of families enriched for SAD and conducted a large-scale mega-analysis within the ENIGMA consortium. Our findings provided further support for white matter alterations in SAD, including changes in white matter microstructure as candidate endophenotype. In addition, we found transdiagnostic patterns in white matter microstructure with other internalizing disorders and potential sex-specific changes in white matter.

Taken together, the studies presented in this thesis offer novel insights into neural correlates of adolescent internalizing disorders. These findings are grounded in real-world clinical variation and employ innovative, multimodal methodologies, including longitudinal neuroimaging, investigation of families and large mega-analyses. Through these comprehensive, methodologically diverse approaches, this thesis aims to narrow the gap between theoretical neuroscience and clinical practice.

Ultimately, the insights gained from this thesis should support the broader goal of improving outcomes for young individuals struggling with internalizing psychopathology. By identifying neurobiological alterations that diverge from healthy peers over time, and by highlighting the need for sex-sensitive and transdiagnostic approaches, this work contributes to the road to more personalized and developmentally informed mental health care.

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A

Appendices

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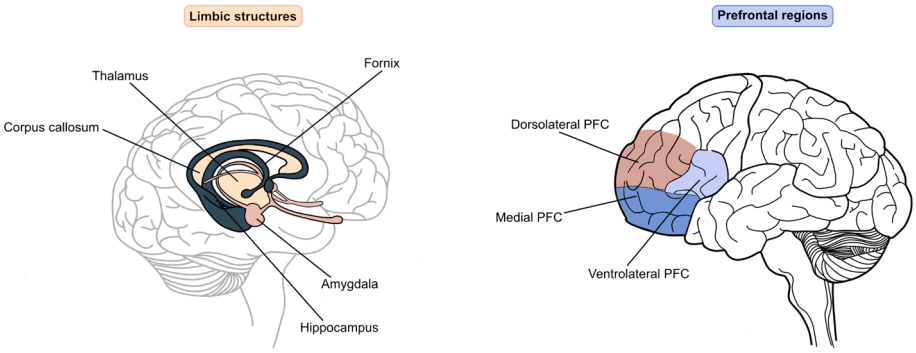
Nederlandse samenvatting

Introductie

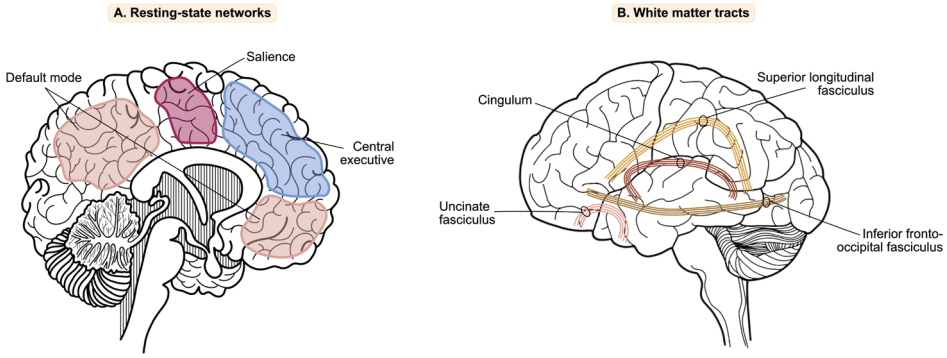
Angst- en depressieve stoornissen zijn veelvoorkomende problemen bij jongeren die vaak een grote impact hebben op het dagelijks leven, schoolprestaties en sociale relaties. We noemen deze stoornissen ook wel *internaliserende* stoornissen. Deze stoornissen beginnen vaak in de puberteit, een cruciale ontwikkelingsperiode waarin veel fysieke, mentale en sociale veranderingen plaatsvinden. Jongeren met internaliserende problemen ervaren niet alleen op dat moment veel last, maar lopen ook een verhoogd risico op blijvende problemen in hun volwassen leven, zoals verslavingen, verminderd functioneren op school of werk, en moeite met het onderhouden van vriendschappen en relaties. Belangrijk is dat angst- en depressieve stoornissen vaak samen voorkomen bij dezelfde persoon, een fenomeen dat comorbiditeit wordt genoemd. Deze overlap in symptomen is veelvoorkomend en leidt vaak tot ernstiger klachten en een slechtere prognose. Ondanks het beschikbare behandelaanbod, zoals psychotherapie en medicatie, blijven veel jongeren last houden van hun klachten. Om behandelingen te kunnen verbeteren en mogelijk zelfs te voorkomen dat jongeren deze problemen ontwikkelen, is het essentieel om beter te begrijpen wat er in hun hersenen gebeurt tijdens deze belangrijke ontwikkelingsfase.

De hersenen van adolescenten zijn volop in ontwikkeling. In deze periode verandert niet alleen de structuur van de hersenen, maar ook de manier waarop verschillende hersendelen met elkaar samenwerken. Een belangrijk aspect hierbij is het evenwicht tussen delen van de hersenen die emoties genereren (zoals de amygdala in het limbische systeem) en delen die helpen bij het reguleren van deze emoties (zoals de prefrontale cortex). Bij gezonde jongeren ontwikkelen de emotionele hersengebieden zich eerder dan de regulerende gebieden, wat kan leiden tot typisch adolescent gedrag zoals verhoogde emotionaliteit en risico-nemend gedrag. Bij gezonde jongeren ontwikkelt dit evenwicht zich geleidelijk, maar bij jongeren met angst en depressie lijkt dit proces verstoord te zijn. Dit netwerk van hersendelen dat betrokken is bij emoties wordt het corticolimbisch netwerk genoemd (zie Figuur 1).



Figuur 1 Het corticolimbisch netwerk.

Eerdere studies hebben veranderingen aangetoond in zowel de structuur als de werking van de hersenen bij jongeren met internaliserende stoornissen (zie Figuur 2), maar de meeste studies hebben slechts op één moment gemeten. Hierdoor weten we weinig over hoe deze hersenveranderingen zich ontwikkelen over tijd en hoe ze samenhangen met het verloop van de klachten. Longitudinaal onderzoek, waarbij dezelfde jongeren op meerdere momenten worden onderzocht, is daarom noodzakelijk om deze ontwikkelingsprocessen beter te begrijpen.



Figuur 2 Functionele en structurele connectiviteit in adolescenten met internaliserende problemen.

Dit proefschrift richt zich op het onderzoeken van hersennetwerken bij jongeren met angst en depressie door te kijken naar zowel de structurele als functionele connectiviteit van de hersenen. *Structurele* connectiviteit verwijst naar de fysieke verbindingen tussen hersendelen via witte stofbanen, vergelijkbaar met snelwegen die verschillende steden met elkaar verbinden. *Functionele* connectiviteit gaat over hoe deze hersendelen met elkaar communiceren en samenwerken tijdens rust, wat je zou kunnen vergelijken met het verkeer over die snelwegen. Dit proefschrift bestaat uit twee delen: het eerste deel onderzoekt hoe deze 'snelwegen' en de communicatie daarover veranderen over tijd bij jongeren met angst en depressie, terwijl het tweede deel zich richt op de 'snelwegen' bij één specifieke angststoornis, namelijk de sociale angststoornis. Sociale angststoornis is één van de meest voorkomende psychiatrische aandoeningen met een levenslange prevalentie van 4-13% en begint vaak in de kindertijd of vroege adolescentie. Patiënten met deze stoornis ervaren intense angst om door anderen beoordeeld te worden, wat leidt tot aanzienlijke beperkingen in hun dagelijks functioneren en kwaliteit van leven. In het tweede deel van dit proefschrift wordt op innovatieve manieren onderzocht óf, en zo ja, welke structurele hersenveranderingen erfelijk zijn, en of er verschillen zijn tussen gezonde mensen en mensen met een sociale angststoornis. Hiervoor wordt de grootste internationale database tot nu toe gebruikt, verkregen uit een wereldwijd internationaal consortium.

Dit proefschrift heeft twee hoofddoelen: ten eerste het onderzoeken van longitudinale veranderingen in structurele en functionele hersenconnectiviteit bij adolescenten met internaliserende stoornissen, en ten tweede het verdiepen van ons begrip van witte stof microstructuur bij sociale angststoornis door zowel te kijken naar erfelijke kwetsbaarheid als naar structurele veranderingen in het grootste internationale cohort tot nu toe.

Deel 1: veranderingen in hersennetwerken over de tijd in internaliserende stoornissen

In **Hoofdstuk 2** onderzoeken we of de structuur van de witte stof in de hersenen anders ontwikkelt bij jongeren met angst en depressie vergeleken met gezonde leeftijdsgenoten. Witte stof bestaat uit zenuwbanen die verschillende hersendelen met elkaar verbinden en is cruciaal voor effectieve communicatie binnen het brein. Deze structuren ontwikkelen zich gedurende de adolescentie en blijven zich verfijnen tot ver in de volwassenheid. Voor deze studie hebben we jongeren twee keer gescand met een MRI-scanner: aan het begin van de studie en drie maanden later. Hierbij hebben we gebruik gemaakt van een geavanceerde MRI-techniek, diffusion tensor imaging (DTI), waarmee we de microstructuur van witte stof in detail kunnen bestuderen.

Hoewel we geen verschillen vonden in hoe de witte stof zich over deze drie maanden ontwikkelde tussen jongeren met internaliserende stoornissen en gezonde jongeren, ontdekten we wel een interessante bevinding binnen de groep jongeren met problemen. Degenen die aan het begin van de studie een bepaald patroon in hun witte stof microstructuur lieten zien, met name hogere waarden van fractionele anisotropie (een maat voor de organisatie van witte stof) in specifieke hersenbanen, vertoonden drie maanden later meer verbetering in hun depressieve klachten. Dit suggereert dat de structurele kenmerken van de hersenen aan het begin van de behandeling mogelijk voorspellende waarde hebben voor het verloop van depressieve symptomen. Deze bevinding is klinisch relevant omdat het in de toekomst mogelijk zou kunnen bijdragen aan het identificeren van jongeren die meer of minder kans hebben op verbetering, wat kan helpen bij het personaliseren van behandelstrategieën. Het is belangrijk om op te merken dat de periode van drie maanden relatief kort is voor structurele hersenveranderingen, wat mogelijk verklaart waarom we geen groepsverschillen in ontwikkeling vonden. Toekomstig onderzoek met langere follow-up periodes is nodig om deze bevindingen verder te onderzoeken.

Hoofdstuk 3 gaat over hoe verschillende delen van de hersenen met elkaar samenwerken wanneer iemand in rust en wakker is, een benadering die resting-state functionele connectiviteit wordt genoemd. We hebben specifiek gekeken naar de amygdala, een hersengebied dat centraal staat in emoties en angst. De amygdala is geen homogene structuur, maar bestaat uit verschillende subkernen die elk hun eigen functie hebben. Daarom hebben we niet alleen naar de hele amygdala gekeken, maar ook specifiek naar verschillende onderdelen: de laterobasale amygdala (betrokken bij angstverwerking en emotionele leerprocessen) en de centromediale amygdala (belangrijk voor angstreacties). Daarnaast hebben we ook gekeken naar grotere netwerken in het brein, de zogenaamde resting-state netwerken, waaronder het affectieve netwerk, het default mode netwerk, het salience netwerk en het central executive netwerk.

We ontdekten dat de functionele connectiviteit tussen een specifiek deel van de amygdala, namelijk de laterobasale amygdala, en andere hersendelen zich anders ontwikkelde bij jongeren met angst en depressie dan bij gezonde jongeren over een periode van drie maanden. Deze bevinding was specifiek voor dit subgebied van de amygdala; we vonden geen vergelijkbare verschillen voor de andere amygdala-subkernen. Interessant genoeg vonden we geen significante verschillen in ontwikkeling wanneer we keken naar de connectiviteit tussen de grotere resting-state netwerken. Bij de groep jongeren met internaliserende problemen zagen we bovendien dat veranderingen in de hersencommunicatie van de basolaterale amygdala samenhingen met veranderingen in hun klinische symptomen over tijd. Jongeren bij wie de functionele connectiviteit toenam, lieten andere symptoomveranderingen zien dan jongeren bij wie deze afnam.

Deze resultaten onderstrepen het belang van het bestuderen van specifieke subregios van de amygdala in plaats van alleen naar het geheel te kijken. De verschillende onderdelen van de amygdala hebben duidelijk verschillende functies en ontwikkelingspatronen, en door deze te onderscheiden kunnen we een genuanceerder beeld krijgen van wat er ander is bij jongeren met internaliserende stoornissen. Een belangrijke kanttekening is dat het in deze studie onduidelijk blijft of de geobserveerde veranderingen in functionele connectiviteit het resultaat zijn van behandeling (alle jongeren ontvingen reguliere klinische zorg), natuurlijk herstel over tijd, of een combinatie van beide. Toekomstig onderzoek met controlegroepen die geen behandeling ontvangen zou hier meer duidelijkheid over kunnen verschaffen, hoewel dit ethisch complex is bij klinische populaties.

Deel 2: Structurele connectiviteit in sociale angststoornis

In **Hoofdstuk 4** zoomen we in op de sociale angststoornis en gebruiken we een unieke onderzoeksaanpak: het familiestudiedesign. De sociale angststoornis komt vaak voor in families, wat suggereert dat er erfelijke factoren een rol spelen. Voor dit onderzoek hebben we hele families onderzocht waarbij sociale angststoornis vaker voorkomt dan in de algemene bevolking. Door verschillende familieleden te bestuderen, waaronder mensen met een sociale angststoornis en hun familieleden die geen stoornis hebben, kunnen we onderzoeken of bepaalde hersenkenmerken erfelijk zijn en mogelijk al aanwezig zijn voordat iemand de stoornis daadwerkelijk ontwikkelt. Deze hersenkenmerken worden endofenotypes genoemd: biologische markers die in de causale keten zitten tussen genetische aanleg en de symptomen van een stoornis.

We onderzochten de witte stof microstructuur en vonden dat een specifiek patroon in een belangrijke witte stof verbinding, de superior longitudinale fasciculus (een bundel zenuwbanen die frontale en pariëtale hersengebieden met elkaar verbindt), samenhing met sociale angstklachten binnen deze families. Zowel mensen met een sociale angststoornis als hun niet-aangedane familieleden vertoonden hogere waarden van fractionele anisotropie in deze hersenbaan vergeleken met gezonde controles zonder familiegeschiedenis. Dit patroon werd gevonden onafhankelijk van of iemand daadwerkelijk de diagnose had of niet, wat suggereert dat dit hersenpatroon inderdaad een endofenotype is: een erfelijke kwetsbaarheid die wordt doorgegeven binnen families.

Bovendien konden we aantonen dat de kenmerken van witte stof microstructuur die we onderzochten inderdaad een substantiële erfelijke component hebben door gebruik te maken van heritabiliteitsanalyses binnen de familiestructuren. Dit betekent dat een deel van de individuele verschillen in deze hersenkenmerken kan worden toegeschreven aan genetische factoren. Deze bevindingen zijn belangrijk omdat ze laten zien dat structurele hersenafwijkingen al aanwezig kunnen zijn voordat iemand daadwerkelijk een sociale angststoornis ontwikkelt, wat erop wijst dat deze veranderingen mogelijk deel uitmaken van de kwetsbaarheid voor de aandoening in plaats van enkel een gevolg ervan te zijn. In de toekomst zou dit hersenpatroon mogelijk kunnen dienen als een vroege marker voor het risico op sociale angststoornis, wat zou kunnen helpen bij het identificeren van mensen die baat zouden kunnen hebben bij preventieve interventies. Wel is meer onderzoek nodig met grotere steekproeven en longitudinale designs om te bevestigen of deze marker daadwerkelijk voorspellende waarde heeft voor het ontwikkelen van de stoornis.

Hoofdstuk 5 beschrijft een grote internationale samenwerking binnen het ENIGMA consortium (Enhancing NeuroImaging Genetics through Meta-Analysis), waarbij we gegevens van twaalf onderzoeksgroepen wereldwijd hebben samengevoegd om witte stofbanen bij mensen met een sociale angststoornis te onderzoeken. Dit is de grootste studie ooit gedaan naar de structurele hersenconnectiviteit bij sociale angststoornis, met in totaal 495 mensen met een sociale angststoornis en 1612 gezonde controles, variërend in leeftijd van 8 tot 65 jaar. Door deze grote aantallen te bereiken via internationale samenwerking, konden we veel betrouwbaardere en robuuste bevindingen verkrijgen dan in eerdere studies met kleinere steekproeven mogelijk was.

In deze mega-analyse hebben we gebruik gemaakt van gestandaardiseerde analysemethoden die in alle deelnemende centra op dezelfde manier werden toegepast, wat de vergelijkbaarheid en betrouwbaarheid van de resultaten vergroot. We vonden dat mensen met een sociale angststoornis lagere waarden van fractionele anisotropie lieten zien in belangrijke witte stof verbindingen vergeleken met gezonde controles. Deze verschillen werden met name gevonden in het corpus callosum (de grote verbinding tussen de linker- en rechterhersenhelft die betrokken is bij interhemisferische communicatie) en de fornix (een belangrijke verbinding binnen het limbische systeem die de hippocampus verbindt met andere hersenstructuren en betrokken is bij geheugen en emotieregulatie).

Opvallend was dat we ook substantiële verschillen zagen tussen mannen en vrouwen in onze analyses. We vonden dat vooral vrouwen met een sociale angststoornis afwijkende patronen in witte stof microstructuur vertoonden vergeleken met gezonde vrouwen, terwijl deze effecten minder uitgesproken waren bij mannen. Dit suggereert dat er mogelijk sekse-specifieke neurobiologische mechanismen zijn bij sociale angststoornis, een bevinding die belangrijke implicaties kan hebben voor onze conceptualisering van de stoornis en mogelijk ook voor behandeling. Toekomstig onderzoek zou zich meer moeten richten op deze sekseverschillen om te begrijpen waarom vrouwen mogelijk kwetsbaarder zijn voor structurele hersenveranderingen bij sociale angststoornis.

Interessant genoeg vertoonden de hersenveranderingen die we vonden bij mensen met een sociale angststoornis sterke overeenkomsten met patronen die eerder zijn gerapporteerd bij andere psychiatrische aandoeningen, zoals schizofrenie en depressie, in vergelijkbare ENIGMA studies. Dit suggereert dat sommige structurele hersenveranderingen misschien niet uniek of specifiek zijn voor één enkele stoornis, maar mogelijk gemeenschappelijke neurobiologische kenmerken representeren die bij meerdere psychiatrische aandoeningen voorkomen. Deze bevinding past binnen een groeiende wetenschappelijke trend die psychiatrische stoornissen niet ziet als volledig afzonderlijke entiteiten, maar eerder als aandoeningen die op een spectrum liggen met gedeelde onderliggende mechanismen. Dit heeft belangrijke implicaties voor hoe we psychiatrische classificatiesystemen conceptualiseren en hoe we behandelingen kunnen ontwikkelen die gericht zijn op deze gedeelde mechanismen in plaats van op symptomen van individuele stoornissen.

Discussie

De studies in het eerste deel van dit proefschrift hebben laten zien dat jongeren met angst en depressie een andere ontwikkeling doormaken in hoe verschillende hersendelen met elkaar communiceren (*functionele* connectiviteit), specifiek in de verbindingen van de basolaterale amygdala met andere hersengebieden. Deze veranderingen in functionele communicatie hingen samen met veranderingen in symptomen. Daarentegen vonden we over een periode van drie maanden geen groepsverschillen in de ontwikkeling van fysieke verbindingen tussen hersendelen (*structurele* connectiviteit).

Dit verschil tussen functionele en structurele bevindingen is begrijpelijk vanuit een neurobiologisch perspectief: veranderingen in hoe hersendelen met elkaar communiceren (zoals gemeten met resting-state fMRI) kunnen relatief snel optreden, binnen weken tot maanden, terwijl veranderingen in de fysieke structuur van witte stofbanen (zoals gemeten met DTI) een veel langzamer proces zijn dat maanden tot jaren in beslag neemt. Onze follow-up periode van drie maanden was waarschijnlijk te kort om structurele veranderingen te detecteren, maar wel voldoende om functionele veranderingen waar te nemen. Dit onderstreept het belang van het kiezen van geschikte tijdsintervallen voor verschillende typen neurobiologische metingen en suggereert dat toekomstig onderzoek met langere follow-up periodes nodig is om structurele ontwikkeling beter in kaart te brengen.

De studies over sociale angststoornis in het tweede deel van dit proefschrift hebben op twee manieren bijgedragen aan ons begrip van structurele hersenveranderingen bij deze stoornis. Ten eerste identificeerden we in de familiestudie specifieke hersenkenmerken in de superior longitudinale fasciculus die mogelijk erfelijk zijn en deel uitmaken van de genetische kwetsbaarheid voor sociale angststoornis. Dit biedt een interessant aanknopingspunt voor verder onderzoek naar de biologische basis van de aandoening en opent mogelijk wegen voor vroege identificatie van risicopersonen. Ten tweede vonden we in de internationale mega-analyse structurele veranderingen in het corpus callosum en de fornix bij patiënten met sociale angststoornis, waarbij we ook belangrijke sekseverschillen ontdekten die erop wijzen dat vrouwen mogelijk anders worden getroffen dan mannen.

Een opvallende bevinding die nader verklaard moet worden, is dat we in hoofdstuk 4 (de familiestudie) verhoogde waarden van fractionele anisotropie vonden in de superior longitudinale fasciculus bij families met sociale angststoornis, terwijl we in hoofdstuk 5 (de mega-analyse) juist verlaagde waarden van fractionele anisotropie zagen bij patiënten in andere witte stofbanen. Dit lijkt op het eerste gezicht tegenstrijdig, maar kan worden verklaard door verschillende factoren. Ten eerste keken we in elk hoofdstuk naar verschillende witte stofbanen (superior longitudinale fasciculus versus corpus callosum en fornix). Ten tweede gebruikten we verschillende onderzoekspopulaties en analysemethoden: de familiestudie keek specifiek naar genetisch verrijkte families en gebruikte een endofenotype-benadering, terwijl de mega-analyse een breed patiëntencohort onderzocht met gestandaardiseerde methoden. Ten derde is het mogelijk dat erfelijke kwetsbaarheid (zoals gemeten in families) zich anders manifesteert in hersenstructuur dan de daadwerkelijke aandoening bij patiënten. De verhoogde waarden bij families zouden kunnen wijzen op een compensatiemechanisme of een kwetsbaarheidsfactor, terwijl de verlaagde waarden bij patiënten mogelijk meer het effect van de chronische stoornis zelf weerspiegelen.

Er zijn een aantal belangrijke beperkingen van het huidige onderzoek. Ten eerste was de follow-up periode in de longitudinale studies relatief kort (drie maanden), wat betekent dat we vooral vroege veranderingen in hersennetwerken hebben kunnen observeren. Langere follow-up studies zijn nodig om te begrijpen hoe hersennetwerken zich ontwikkelen over meerdere jaren en hoe deze ontwikkeling samenhangt met het beloop van internaliserende stoornissen op de lange termijn. Ten tweede waren alle jongeren in onze longitudinale studies in behandeling, waardoor we niet konden onderscheiden welke hersenveranderingen het gevolg waren van behandeling en welke van natuurlijk herstel. Toekomstige studies met onbehandelde controlegroepen (waar ethisch verantwoord) of studies die specifieke behandelinterventies vergelijken, zouden hier meer inzicht in kunnen geven.

Tot slot is het belangrijk om concrete aanbevelingen voor toekomstig onderzoek te bekijken. Deze omvatten onder meer: het gebruik van grotere en meer diverse steekproeven om de generaliseerbaarheid van bevindingen te vergroten; longitudinaal onderzoek over langere periodes om zowel structurele als functionele hersenveranderingen beter in kaart te brengen; meer aandacht voor sekseverschillen in neurobiologisch onderzoek naar internaliserende stoornissen; het combineren van verschillende neuroimaging technieken om een completer beeld te krijgen van hersenveranderingen; en het voortzetten van internationale samenwerkingen zoals binnen het ENIGMA consortium om grotere en meer betrouwbare datasets te creëren. Uiteindelijk is het doel van dit onderzoek om bij te dragen aan betere diagnostische methoden en effectievere, meer gepersonaliseerde behandelingen voor jongeren met angst en depressie, waarbij we hun unieke hersenkenmerken en ontwikkelingstrajecten in acht nemen. De bevindingen uit dit proefschrift vormen een belangrijke stap in die richting, maar maken ook duidelijk dat er nog veel werk te verzetten valt voordat we neurobiologische markers daadwerkelijk kunnen implementeren in de klinische praktijk.

Curriculum Vitae

Eline Roelofs was born in 1992 in 's-Hertogenbosch and raised in Berlicum, the Netherlands. In 2010, she completed her secondary education (gymnasium) at Gymnasium Beekvliet in Sint-Michielsgestel. Following graduation, she spent six months in Kelowna, BC, Canada, attending Kelowna Secondary School as part of an international exchange program.

In 2011, Eline commenced her medical studies at Leiden University Medical Center (LUMC). During her undergraduate years, she gained valuable research experience working as a student assistant at the Department of Clinical Genetics at LUMC from 2012 to 2016, where she contributed to database development for investigating the p16-Leiden mutation. Her growing interest in neurobiology led her to join the Department of Psychiatry at LUMC as a research assistant for the NESDA study from 2015 to 2017, where she served as a scan buddy for neuroimaging data collection.

Seeking to expand her research expertise internationally, Eline undertook a seven-month research internship in 2017 with the PsychoNeuroEndocrinology Group at the Centre for Psychiatry, Imperial College London. During this placement, she investigated the influence of nutritional state on inhibitory motor control, presenting her findings at the British Feeding and Drinking Group Annual Meeting in Lyon, France, in 2018.

After completing her medical degree in 2018, Eline began working as a junior doctor at the Department of Child and Adolescent Psychiatry at LUMC, gaining experience across various in- and outpatient clinics. Her continued fascination with the neurobiological underpinnings of psychiatric disorders motivated her to pursue doctoral studies, and in 2019, she commenced her PhD under the supervision of professor Robert Vermeiren and professor Nic van der Wee.

In 2021, Eline embarked on her specialist training as a Child and Adolescent Psychiatry resident at LUMC while simultaneously continuing her doctoral research, successfully combining clinical practice with academic investigation. She completed a EMDR course for children and adolescents in 2023. Her multidisciplinary approach to understanding the mind extended beyond traditional medical training when she completed a 200-hour vinyasa yoga teacher training in 2024, reflecting her holistic perspective on mental health and well-being.

Eline is currently in the final stages of completing her residency in Child and Adolescent Psychiatry at Youz, The Hague. She lives in Leiden with her partner Tom and cat Zeus.

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Portfolio

PhD trajectory overview

My PhD trajectory commenced in 2019 at Leiden University Medical Center (LUMC) under the primary supervision of Prof. Dr. R.R.J.M. Vermeiren and Prof. Dr. N.J.A. van der Wee, with Dr. J.M. Bas-Hoogendam serving as co-promotor. My doctoral research is embedded within the research program "New methods for child psychiatric diagnosis and treatment outcome evaluation" and is summarized within the thesis "Untangling the adolescent brain – investigating neural networks in youth with anxious and depressive symptoms."

In the foundational year of 2019, I established my methodological and regulatory framework. I completed mandatory training in "Basic Methods and Reasoning in Biostatistics" (42 hours) and "Responsible Research" (42 hours), with a particular focus on laws and regulations governing clinical research under relevant Dutch Acts (WMO, GDPR/AVG). I acquired advanced neuroimaging skills through the FSL course (42 hours) and initiated my teaching portfolio, contributing to courses for (bio)medical students on depression and translational neuroscience.

In 2020, I developed my scientific communication capabilities through specialized training (40 hours) and presented online at two major international conferences: the annual meeting of the Society of Biological Psychiatry (SOBP) and the annual congress of the European College of Neuropsychopharmacology (ECNP). I also published my first peer-reviewed publication as first author, in which I investigated white matter microstructure as candidate endophenotypes of social anxiety disorder (SAD) in the Leiden Family Lab study on SAD, advancing my understanding of familial anxiety mechanisms. I co-authored a book chapter on the pathogenesis of SAD and expanded my teaching responsibilities to over 60 hours, supervising medical and biomedical students.

The year 2021 demonstrated continued research productivity with a second first-author publication exploring adolescent anxiety and depression trajectories in relation to white matter microstructure. A significant milestone occurred in March 2021 when I commenced my Residency in Psychiatry at the LUMC, successfully combining clinical training with research activities. I maintained teaching commitments (10 hours) alongside these dual responsibilities.

During 2022-2023, I sustained engagement with the national research community through attendance at the NVVP spring conference ("voorjaarscongres") while continuing student supervision and balancing research, clinical training, and educational duties.

The period spanning 2023 to 2025 featured a significant organizational achievement: I undertook a major leadership role coordinating and conducting a mega-analysis within the international ENIGMA Anxiety working group. This large-scale logistic operation involved collecting and managing international multi-site data, coordinating cross-cultural teams, and implementing quality control procedures to investigate

brain structural correlates of social anxiety at unprecedented scale. This project demonstrates my capacity to manage complex collaborative projects and my dedication to advance open science practices.

During 2024-2025, I presented at the EPA and SOBP congresses in Budapest and Toronto, respectively, and presented my work at the ENIGMA Developmental Group Leadership Summit in Amsterdam. Furthermore, I visited the ECNP congress in Amsterdam and completed the "Workshop Scientific Conduct for PhDs," strengthening commitment to research ethics. Moreover, I received the Young Neurolab NL travel grant, facilitating international collaboration.

Regarding societal outreach, I participated in science communication at Corpus Kids Academy, an interactive event introducing brain research to over 200 children. This initiative exemplifies my dedication to making neuroscience accessible, fostering scientific curiosity in youth, and bridging academic research with public understanding of mental health.

Throughout my PhD trajectory, I accumulated over 400 hours of formal training across methodological coursework, scientific communication, teaching, and conferences. My work bridges clinical child and adolescent psychiatry with advanced neuroimaging, investigating white matter microstructure and neural networks underlying adolescent anxiety and depression. The trajectory showcases substantial development of scientific insight, from foundational analyses to leadership of an international collaborative project. The integration of extensive teaching and supervision, pioneering team science initiatives, clinical training, ethical research leadership, and public outreach positions me as an emerging researcher in developmental neuropsychiatry, committed to translating neuroscientific findings into improved approaches for youth mental health while engaging diverse audiences and maintaining rigorous research standards.

CRediT statement

CRediT table for the thesis of Eline Roelofs

Ch.	Type*	Short Title	Conceptualization	Data Curation	Formal Analysis	Funding Acquisition	Investigation	Methodology	Project Administration	Resources	Software	Supervision	Validation	Visualization	Writing – Original Draft	Writing – Review & Editing	Preregistered	Preprinted	Published with Peer Review
1	Introduction	Introduction	■												■	■	☐	☐	☐
2	PhD project chapter	Second DTI project		■	■				■		■			■	■	■	☐	☐	☑
3	PhD project chapter	First resting-state project		■	■			■	■		■			■	■	■	☐	☐	☑
4	PhD project chapter	First DTI project		■	■			■	■		■				■	■	☐	☐	☑
5	PhD project chapter	Organizing a mega-analysis	■	■	■		■	■	■	■	■			■	■	■	☑	☐	☐
6	Discussion	Discussion	■												■	■	☐	☐	☐

**PhD project chapters are the direct result of the PhD project of the PhD candidate.*
Some theses also include Collaboration Chapters, to which the PhD candidate has contributed but fall outside the PhD project.

Dissemination table

1.	**Roelofs EF, Nestor LJ, Ali SN, Lingford-Hughes AR, Nutt DJ, Goldstone AP Active nutritional state and Body Mass Index influence Anterior Cingulate Cortex activation during motor response inhibition**	
	Chapter in this thesis	Not in this thesis
	Conference contributions of the PhD candidate	British Feeding and Drinking Group annual meeting 2018. DOI: 10.1016/j.appet.2018.05.223
2.	**Bas-Hoogendam JM, Roelofs EF, Westenberg PM, van der Wee NJA Pathogenesis of social anxiety disorder**	
	Chapter in this thesis	Not in this thesis
	Other forms of dissemination	In: Simon N, Hollander E, Rothbaum BO, Stein DJ, editors. The American Psychiatric Association Publishing Textbook of Anxiety, Trauma, and OCD-Related Disorders, Third Edition. Washington, DC: American Psychiatric Association Publishing; 2020.
3.	**Roelofs EF, Bas-Hoogendam JM, van Ewijk H, Ganjgahi H, van der Werff SJA, Barendse MEA, et al. Investigating microstructure of white matter tracts as candidate endophenotypes of Social Anxiety Disorder - Findings from the Leiden Family Lab study on Social Anxiety Disorder (LFLSAD)**	
	Chapter in this thesis	4
	Conference contributions of the PhD candidate	ECNP 2020. DOI: 10.1016/j.euroneuro.2019.12.070 SOBP 2020. DOI: 10.1016/j.biopsych.2020.02.641
	Publication in peer reviewed journal	DOI: 10.1016/j.nicl.2020.102493
4.	**Roelofs EF, Bas-Hoogendam JM, van der Werff SJA, Valstar SD, van der Wee NJA, Vermeiren RRJM Exploring the course of adolescent anxiety and depression: associations with white matter tract microstructure.**	
	Chapter in this thesis	2
	Publication in peer reviewed journal	DOI: 10.1007/s00406-021-01347-8
5.	**Roelofs EF, Bas-Hoogendam JM, Winkler AM, van der Wee NJA, Vermeiren RRJM Longitudinal development of resting-state functional connectivity in adolescents with and without internalizing disorders**	
	Chapter in this thesis	3
	Publication in peer reviewed journal	DOI: 10.1016/j.nsa.2024.104090
6.	**Roelofs EF, Groenewold NA, Farkas K, Zhu AH, Gao S, Borgers T, et al. White matter microstructure alterations in social anxiety disorder – a mega-analysis across 12 cohorts in the ENIGMA-Anxiety Working Group**	
	Chapter in this thesis	5
	Pre-registration	https://osf.io/fkswz
	Conference contributions of the PhD candidate	SOBP 2025. DOI: 10.1016/j.biopsych.2025.02.282
	Publication in peer reviewed journal	Submitted

Overview of completed courses and other training

Mandatory activities		
Month/Year	**Title**	**Hours**
03/2019	Leiden University Onboarding Programme Inform & Connect (2 activities)	10
09/2019	Basic Methods and Reasoning in Biostatistics	42
11/2019	Responsible Research (2 activities)	42
05/2025	Workshop Scientific Conduct for PhDs	5

Scientific courses, workshops and other training activities		
Month/Year	**Title and description**	**Hours**
03/2019	FSL-course. Five-day theoretical and practical course on using the FMRIB Software Library (FSL) for structural, functional and diffusion image analysis	42
2019	Basic course on Regulations and Organization of Clinical Trials ("BROK"). Mandatory course focused on laws and regulations when conducting clinical research that falls under the scope of several Dutch laws	20
01/2020	Corsendonk course. Five-day course for researchers in psychiatry	40
06/2020	Communication in Science for PhD's. Seven sessions focused on scientific presenting and writing	40

Transferable skill courses, workshops and other training activities		
Month/Year	**Title and description**	**Hours**
2019	Teaching of FOS-course "Depression and other stress-related disorders", including supervision of master students writing their research proposal	14
2019	Teaching two classes of the half minor "Translational Neuroscience"	5
2020	Teaching (bio)medical students, including the same courses as those in 2019 and supervision of two medical students for their scientific research project for 4 to 6 months each	63
2021	Teaching medical students "Vraagstukken psychisch functioneren"	6
2023	Teaching FOS-course "Depression and other stress-related disorders"	5
2024	Corpus Kids Academy. One day to introduce brain research to children	8

Dissemination, acknowledgement, esteem and other relevant scientific activities

Other scientific activities related to this thesis		
Month/year	**Description**	**Linked to chapter(s)**
2020	ECNP. Poster presentation at the annual meeting of the European College of Neuropsychopharmacology, online due to the COVID-19 pandemic	4
2020	SOBP. Poster presentation at the annual meeting of the Society of Biological Psychiatry, online due to the COVID-19 pandemic	4
2022	Suffugium grant appplication for innovative projects in child- and adolescent psychiatry. Not awarded	5
2024	EPA. Oral presentation at the annual meeting of the European Psychiatry Association in Budapest, Hungary	2
2025	SOBP. Poster presentation at the annual meeting of the Society of Biological Psychiatry in Toronto, Canada	5
2025	Young Neurolab NL travelgrant, awarded	5

Dankwoord

Een promotietraject is lang en intens, zeker als deze gecombineerd wordt met een opleiding tot medisch specialist. Ik wil graag iedereen bedanken die hieraan heeft bijgedragen. Bij een aantal mensen wil ik graag in het bijzonder stilstaan.

Robert, dankzij jouw vastberadenheid kon ik beginnen aan dit onderzoeksproject en ik wil je dan ook graag bedanken voor je brede blik, vertrouwen en niet-aflatende steun. Jouw bevlogenheid voor (het onderzoek binnen) de kinder- en jeugdpsychiatrie is aanstekelijk.

Nic, sinds ik in 2016 als geneeskundestudent aanklopte voor een wetenschapsstage in het buitenland heb je me weten te enthousiasmeren voor de psychiatrie en het neurobiologisch onderzoek. Met je kritische, nuchtere houding en het vermogen om de grote lijn vast te houden, heb je me gevormd als onderzoeker.

Janna Marie, als co-promotor heb je me ontelbare keren geholpen met je indrukwekkende kennis, niet-aflatende precisie en enthousiasme en passie voor het onderzoek. Dank je wel voor alle momenten dat ik even kon bellen, appen, koffiedrinken of langslopen.

I'd like to thank these researchers for their expertise and relevant contributions to studies in this thesis: **Kinga Farkas** from Semmelweis University; **Habib Ganjgahi** from the University of Oxford; **Nynke Groenewold** from Stellenbosch University; **Daniel Pine** from NIMH; **Steven van der Werff** and **Michiel Westenberg** from Universiteit Leiden; **Anderson Winkler** from Texas University.

Collega's van de kantoortuin in het LUMC, dank je wel voor alle gezellige momenten en enthousiasme in de afgelopen jaren. Ondanks dat ik vaak in- en uitvloog was het altijd een warm welkom en was er altijd ruimte voor koffie en een moment om bij te praten.

Natuurlijk wil ik ook **alle AIOS en ANIOS-collega's van het LUMC, Curium en Youz** bedanken. Jullie humor, steun en aandacht heeft me er altijd weer doorheen gesleept. Vooral mijn jaargenoot **Sarah**, dank je wel voor de leuke en minder leuke tijden die we samen hebben beleefd.

Lieve Plukkies: Adina, Anne, Camille, Iris, Marieke, Micaela, Nina en Ymke, dank je wel voor alle gekke, chaotische en hilarische avonden, weekenden en vakanties. **Maaike**, tegelijk aan een promotietraject beginnen en proberen vol te houden in COVID-tijd is zwaar. Ik ben heel blij dat we elkaar hebben mogen ondersteunen tijdens de talloze wandelingen, wijnavonden en koffie momenten.

Fanny, there are no words to describe your authenticity and lovingness. What started at yoga class grew into an amazing friendship during my PhD journey. You had an exceptional sense of when I needed grounding, space, or simply a reminder to breathe. Thank you for holding the space, especially when my work–life balance became wobbly, and for your steady encouragement, hugs and laughs. They helped me stay connected to myself throughout this process.

Lotje, Karin en Nicole, we zijn ooit begonnen in oranje overalls als dispuutsgenoten in Leiden en inmiddels zijn we uitgewaaierd over het land. Ondanks de afstand zijn jullie een constante factor gebleven tijdens mijn promotietraject. Dank jullie wel voor jullie betrokkenheid en jullie talent om van alles een feest te maken. Jullie herinnerden mij eraan dat er naast deadlines en manuscripten ook altijd ruimte is voor plezier en ontspanning.

Sevgi, onze lange reis door Zuid-Afrika met jou heeft een enorme band gecreëerd. Dank je wel voor het inchecken als ik weer eens onder een steen zat in drukke onderzoeks- en klinische periodes. Je steun in lastige en mooie tijden heeft me ontzettend geholpen tijdens dit traject. Ik ben heel blij dat je naast me staat als paranimf.

Anne, sinds dag 1 van onze studie geneeskunde zijn we vriendinnen. Wat ben ik dankbaar dat we elkaar niet zijn kwijtgeraakt ondanks alle life-events. Dank je wel dat ik mezelf mag zijn, in welke staat dan ook. Jouw luisterend oor, wijze advies en onvoorwaardelijke steun zijn onbetaalbaar geweest tijdens dit promotietraject.

Mijn schoonfamilie, jullie enthousiasme voor iedere stap in de opleiding en onderzoek is aanstekelijk. Dank je wel voor alle leuke momenten en weekenden. **Merel**, dank je wel voor de illustraties van de cover!

Saskia, ik ben zo blij dat wij steeds hechter worden als zussen, waar we ook zijn in de wereld. Dank je wel voor alle voice messages, memes en fijne momenten samen. **Mijn ouders**, dank je wel voor jullie liefde en steun.

Liefste **Tom**, jij zet me altijd weer op de grond als ik aan het fladderen ben. Jouw kracht om door te zetten als het nodig is en te ontspannen wanneer het kan helpt me om in balans te blijven. Je inspireert me om uit mijn comfort zone te stappen en alles uit het leven te halen. Samen kunnen we de wereld aan.