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

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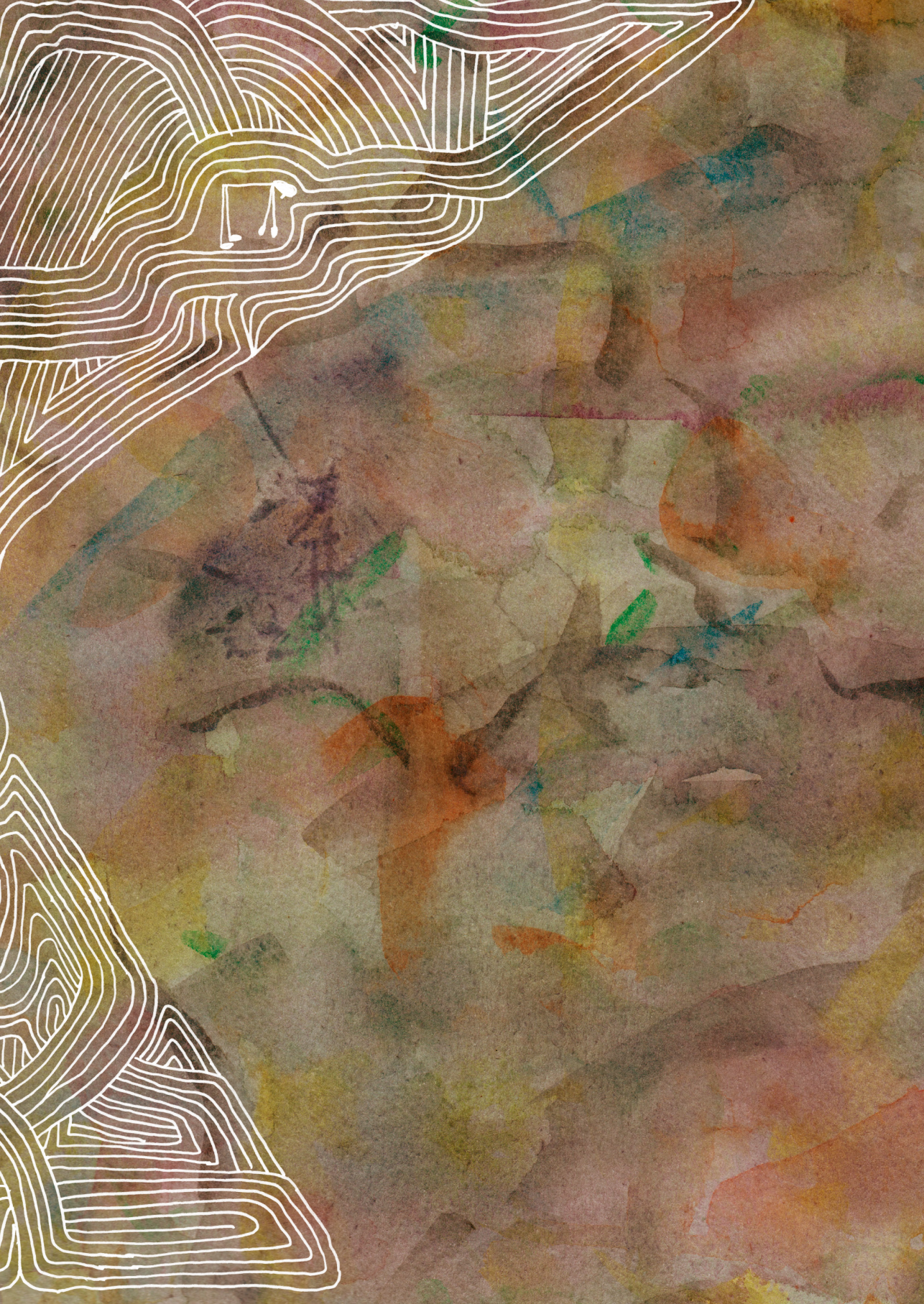


Discussion
&
Appendices

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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