GENETIC, EPIGENETIC AND NEUROIMAGING MARKERS ASSOCIATED WITH CONDUCT DISORDER AND CALLOUS-UNEMOTIONAL TRAITS IN FEMALES

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A thesis submitted to the University of Birmingham for the degree of Doctor of Philosophy

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January 2022

ABSTRACT

The aim of this thesis was to better understand the pathways from (epi)genetic variation to brain structure and response in youth with CD and varying levels of CU traits. Epigenetic and genetic data from female youth from a mix-sex sample were integrated with structural and functional neuroimaging data to explore whether there was evidence for sex-specific associations between these variables. DNA methylation, grey matter volume (GMV), brain response to emotional faces and *OXTR* genotype were investigated using either linear regression analyses or structural equation modelling (SEM). In chapter 3, salivary DNA data from female youth were analysed and I identified a region on chromosome 1 (incorporating the *SLC25A24* gene), which showed differential methylation according to the CD x CU interaction effect. Specifically, I observed an inverse pattern of correlation between CU traits and methylation in females with CD (positive) as compared to TD females (negative). Across the whole cohort, level of methylation of this region was also negatively associated with GMV in several brain regions including the superior frontal gyrus, dorsolateral prefrontal cortex, supramarginal gyrus, secondary visual cortex and ventral posterior cingulate cortex. Chapter 4 examined the association between this *SLC25A24* gene methylation and brain response to emotional faces. A positive association between *SLC25A24* methylation and brain response to faces (i.e., angry, fearful and neutral) was observed in regions across the whole brain. Across all faces, the significant regions were within the right hemisphere (ventral caudate, para-hippocampal region, superior temporal cortex and mid-temporal region). Response to angry or fearful, as compared to neutral, faces was also positively associated with *SLC25A24* methylation in areas across both hemispheres, including the inferior frontal gyrus, angular gyrus, pre-central gyrus, occipital lobe, ventral posterior cingulate and dorsal anterior cingulate cortex (ACC). In chapter 5, I tested whether a structural equation model including *OXTR* genotype data, age, IQ, site, CU-trait score and brain response data from the amygdala (during emotional face processing) could explain a significant amount of the variance in CD symptoms in males-only, females-only and mixed-sex youth. Neither direct associations between *OXTR* genotype and variation in CD symptoms, nor indirect associations via other factors in the model explained a significant amount of overall CD symptom variation. In males only, there was an association between left amygdala response to angry faces and *OXTR* genotype (as represented by a composite risk score including data from 34 SNPs). Overall, the findings presented here demonstrate that both sex and level of CU traits are crucial factors to consider

when investigating the biological mechanisms and neural correlates associated with CD. The findings reported in chapter 3 were published in the Translational Psychiatry journal in September 2021:

Farrow, E., Chiocchetti, A. G., Rogers, J., Pauli, R., Gonzalez-Madruga, K., Smaragdi, A., ... & De Brito, S. (2021). *SLC25A24* gene methylation and gray matter volume in females with and without conduct disorder: an exploratory epigenetic neuroimaging study. *Translational Psychiatry*.
https://doi.org/10.1038/s41398-021-01609-y

ACKNOWLEDGEMENTS

I am really grateful to my supervisors, Dr Stephane De Brito and Dr Magdalena Chechlacz, for being constant sources of support and encouragement since I began this PhD. They have both provided invaluable contributions to my work through their advice on methodological issues and analysis techniques and by taking the time to provide detailed feedback and suggestions to improve each section as it was written. I am particularly grateful to Stephane for the regular zoom contact during the lockdown periods and for maintaining an optimistic, positive attitude through the disruptions caused by the COVID-19.

I am also incredibly grateful to all members of the FemNAT-CD consortium who have contributed to this work, especially those who worked hard to collect and organise this large dataset that I have had the opportunity to work with. Especially, I am indebted to the team in Frankfurt for hosting me for a placement in 2019 and teaching me how to analyse epigenetic data and to Dr Andreas Geburtig-Chiocchetti for providing on-going support and expertise on both the epigenetic and genetic aspects of this project. I would also particularly like to thank Dr Ruth Pauli for her friendship, help and reassurance over the past 4 years. I feel very privileged to have had the chance to work with so many kind, knowledgeable and inspiring researchers.

Finally, I would also like to thank my friends (Amy, Becky, Jyoti, Mel and Peter) for always being there to chat, run or cycle with me when I needed a break from work, and my parents, for 29 years-worth of love and prayers, which have kept me going through the tougher times.

Funding

My PhD studentship has been funded by the British Biosciences and Biotechnology Research Council (BBSRC) through the Midland Integrative Biosciences Training Partnership (MIBTP) Doctoral training scheme. The placement in Frankfurt was funded via a Short-term Research Grant from the Deutscher Akademischer Austauschdienst (DAAD), funding ID no. 57442045.

The FemNAT-CD project was funded by the European Commission under the 7th Framework Health Programme with Grant Agreement no. 602407.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

APPENDIX A: SUPPLEMENTARY MATERIALS FOR CHAPTER 3

APPENDIX B: SUPPLEMENTARY MATERIALS FOR CHAPTER 4

APPENDIX C: SUPPLEMENTARY MATERIALS FOR CHAPTER 5

KEY ABBREVIATIONS

ACC	Anterior Cingulate Cortex
AI	Anterior Insula
ADHD	Attention-Deficit Hyperactivity Disorder
ASD	Autism Spectrum Disorder
ASPD	Antisocial Personality Disorder
CD	Conduct Disorder
CU	Callous-unemotional
DSM	Diagnostic and Statistical Manual of Mental Health Disorders
EWAS	Epigenome-wide Association Study
GAD	Generalized Anxiety Disorder
GMV	Grey Matter Volume
GWAS	Genome-wide Association Study
ICU	Inventory of Callous-unemotional Traits
K-SADS	Schedule for Affective Disorders and Schizophrenia in School-aged Children
LPE	Limited Prosocial Emotions
ODD	Oppositional Defiant Disorder
OXTR	Oxytocin Receptor Gene
PFC	Prefrontal Cortex
PDS	Pubertal Development Scale
SEM	Structural Equation Model/Modelling
SES	Socio-economic Status
SLC25A24	Solute Carrier family 25-member 24 gene
SNP	Single Nucleotide Polymorphism
TD	Typical development/typically developing
WAIS	Wechsler Intelligence Scale
WISC	Wechsler Intelligence Scale
YPI	Youth Psychopathic Inventory

CHAPTER 1: INTRODUCTION TO CONDUCT DISORDER AND CALLOUS UNEMOTIONAL TRAITS

1.1 General Introduction and Research Questions

Conduct Disorder (CD) is a psychiatric diagnosis given to youth under the age of eighteen who demonstrate repetitive patterns of anti-social behaviours. It is a highly heterogeneous diagnosis, with over 32,000 combinations of symptoms which can lead to a CD diagnosis (Nock, Kazdin, Hiripi, & Kessler, 2006), thus youth with CD may exhibit a wide range of antisocial behaviours with differing affective characteristics (DSM-5; American Psychiatric Association, 2013). The clinical presentation of CD varies, both according to an individual's sex and level of callous-unemotional (CU) traits (i.e. a lack of empathy, absence of guilt after wrongdoing, shallow emotionality) (Fairchild et al., 2019). Inter-individual variations in CD symptom patterns may be better understood by considering the genetic predispositions and environmental risk factors which contribute to an individual being at increased risk of developing CD (Wesseldijk et al., 2017). These environmental and genetic risk factors also vary depending on the individual's level of CU traits and sex (e.g. (Jacobson, Prescott, & Kendler, 2002;Van Hulle, Waldman, & Lahey, 2018). Additionally, research indicates that the associations between CD symptoms and brain structure and function also differ according to level of CU traits and sex (Blair, Veroude, & Buitelaar, 2018).

Chapter 1 of this thesis provides an overview of CD and introduces the relevant neuroimaging, genetic and epigenetic literature which has informed the research questions of the thesis. Each

section begins with CD in general, and then looks at the disorder according to variations in levels of CU traits and sex differences. I then conclude this chapter with an outline of the three experimental studies reported in the main body of this thesis.

1.2 Conduct Disorder

1.2.1 Definition and Prevalence

CD is a psychiatric disorder that onsets in childhood or adolescence and is characterized by a repetitive and persistent pattern of antisocial behaviour in which the basic rights of others and societal norms are violated (American Psychiatric Association, 2013). CD is one of the most common reasons for referral to Child and Adolescent Mental Health Services and has a highly negative impact on the affected individual as well as their families, teachers, and society (Scott, Knapp, Henderson, & Maughan, 2001). Globally, among school-aged children the estimated prevalence of CD is around 3% (Coghill, 2013; Kazdin, 2003), although prevalence estimates are higher in boys (3-4%) than girls (1-2%) (Polanczyk, Salum, Sugaya, Caye, & Rohde, 2015). In 2015, the estimated prevalence of CD in 5-16-year olds in England was 5.6% (Public Health England, 2019). Anti-social behavioural issues are among the leading reasons for school dropout, which is a major concern to the EU (European Commission Education Committee, 2018) and affects approximately 15% of all adolescents in Europe. In addition to poor educational attainment, childhood behavioural disorders are associated with greater rates of future reliance on welfare sources (Erskine et al., 2014) and individuals diagnosed with behavioural issues in childhood also show increased risk of engaging in substance abuse, criminal acts and domestic abuse in later life (Kessler et al., 1996). In the United Kingdom, the financial impact of criminal acts committed by those aged 10-21 years old is estimated at around £23,000,000 per annum

(Prince's Trust, 2010) and there are also significant costs associated with providing therapeutic interventions and social support to children with CD and their families (Romeo, Knapp, & Scott, 2006). Despite this, CD remains one of the least studied psychiatric disorders (Fairchild et al., 2019).

1.2.2 Heterogeneity

Due to the variety of behaviours included as possible criteria for CD, the diagnosis may be applied to individuals presenting with entirely different patterns of symptoms (American Psychiatric Association, 2013). A diagnosis of CD is given to youth displaying 3 or more symptoms from a list of 15 in the 5th edition of the Diagnostic and Statistical Manual of Mental Health Disorders ((DSM-V; APA, 2013) – see Figure 1).

As a result, that there are over 32,000 different combinations of symptoms that may qualify for this diagnosis (Nock et al., 2006). There are four main clusters of symptoms, covering behaviours in the areas of aggression, destruction, deceitfulness, and rule-violation (American Psychiatric Association, 2013). The diagnostic criteria also contains subtype specifiers, based on symptom severity, age of CD symptom onset (during childhood [before 10 years] or adolescence) and whether or not the individual also displays limited prosocial emotions (LPE). Some have suggested that youth with CD and a comorbid diagnosis of Attention Deficit Hyperactive Disorder (ADHD) symptoms should also be considered a separate subgroup (Faraone, Biederman, Jetton, & Tsuang, 1997), but this subtyping approach has not been incorporated in current psychiatric nosologies and has lost traction in the last decade.

As may be expected, this heterogeneity impacts treatment outcomes and certain interventions may only prove effective for specific subgroups of CD (Hogstrom, Enebrink, & Ghaderi, 2013).

DSM-5 criteria for conduct disorder

A. A repetitive and persistent pattern of behaviour in which the basic rights of others or major age-appropriate societal norms or rules are violated, as manifested by the presence of at least three of the following 15 criteria in the past 12 months from any of the categories below, with at least one criterion present in the past 6 months:

Aggression to People and Animals

- Often bullies, threatens, or intimidates others.
- Often initiates physical fights.
- Has used a weapon that can cause serious physical harm to others (e.g., a bat, brick, broken bottle, knife, gun).
- Has been physically cruel to people.
- Has been physically cruel to animals.
- Has stolen while confronting a victim (e.g., mugging, purse snatching, extortion, armed robbery).
- Has forced someone into sexual activity.

Destruction of property

- Has deliberately engaged in fire setting with the intention of causing serious damage.
- Has deliberately destroyed others' property (other than by fire setting).

Deceitfulness or theft

- Has broken into someone else's house, building, or car.
- Often lies to obtain goods or favors or to avoid obligations (i.e., "cons" others).
- Has stolen items of nontrivial value without confronting a victim (e.g., shoplifting, but without breaking and entering; forgery).

Serious violations of rules

- Often stays out at night despite parental prohibitions, beginning before age 13 years.
- Has run away from home overnight at least twice while living in the parental or parental surrogate home, or once without returning for a lengthy period.
- Is often truant from school, beginning before age 13 years.

B. The disturbance in behaviour causes clinically significant impairment in social, academic, or occupational functioning.
C. If the individual is age 18 years or older, criteria are not met for antisocial personality disorder.

DSM-5 criteria for conduct disorder (cont.)

Age at onset subtype
Childhood-Onset Type: at least one criterion characteristic of CD is present prior to age 10
Adolescent-Onset Type: Absence of any criteria characteristic of CD prior to age 10
Unspecified Onset: when the age at onset of CD is unknown or insufficient information is available to determine this.

Severity
*Mild***:** Few if any conduct problems in excess of those required to make the diagnosis are present, and conduct problems cause relatively minor harm to others (e.g., lying, truancy, staying out after dark without permission, other rule breaking).
*Moderate***:** The number of conduct problems and the effect on others are intermediate between those specified in "mild" and those in "severe" (e.g., stealing without confronting a victim, vandalism).
*Severe***:** Many conduct problems in excess of those required to make the diagnosis are present, or conduct problems cause considerable harm to others (e.g., forced sex, physical cruelty, use of a weapon, stealing while confronting a victim, breaking and entering).

Figure 1. DSM-5 Criteria for Conduct Disorder with Age-of-Onset and Severity Specifiers.

The variation in symptom presentation is so significant that it has generated debates in the field as to the plausibility of considering CD as one single disorder (Richers & Cicchetti, 1993). I will now discuss the different subgroups within CD in more detail and introduce the most clearly articulated model of the underlying neurobiology of this heterogeneity – the James Blair model of neural dysfunction in individuals with psychopathic traits (Blair, 2013).

1.2.2.1 DSM-5 Subtypes

The severity specifier in the criteria for CD allows clinicians to distinguish between youth with mild, moderate or severe symptoms. See Figure 1 for more details. The age of onset specifier differentiates between individuals who display at least one symptom of CD before the age of 10 (childhood-onset) from those who show no CD symptoms before age 10 (adolescent-onset), but also covers cases where there is insufficient information to determine when symptoms began

(unspecified onset) (American Psychiatric Association, 2013). To be given the diagnosis of CD with the LPE specifier (Figure 2), there needs to be evidence that an individual has exhibited at least 2 of the following symptoms in multiple relationships and settings within the last year; (i) displays little or no remorse or guilt for their wrongdoing,(ii) is callous/uncaring (i.e. lacking in empathy or disregarding the feelings of others), (iii) fails to accept responsibility for poor performances at school or work and/or, (iv) shows little care over these poor performances and reduced emotionality (i.e. emotions 'switched off') (APA, 2013).

Limited Pro-social Emotions (LPE) Specifier for CD

To qualify for this specifier, an individual must have displayed at least two of the following characteristics persistently over at least 12 months and in multiple relationships and settings.

1. **Lack of remorse or guilt**: Does not feel bad or guilty when he or she does something wrong (exclude remorse when expressed only when caught and/or facing punishment). The individual shows a general lack of concern about the negative consequences of his or her actions. For example, the individual is not remorseful after hurting someone or does not care about the consequences of breaking rules.
2. **Callous—lack of empathy**: Disregards and is unconcerned about the feelings of others. The individual is described as cold and uncaring. The person appears more concerned about the effects of his or her actions on himself or herself, rather than their effects on others, even when they result in substantial harm to others.
3. **Unconcerned about performance**: Does not show concern about poor/problematic performance at school, at work, or in other important activities. The individual does not put forth the effort necessary to perform well, even when expectations are clear, and typically blames others for his or her poor performance.
4. **Shallow or deficient affect**: Does not express feelings or show emotions to others, except in ways that seem shallow, insincere, or superficial (e.g., actions contradict the emotion displayed; can turn emotions "on" or "off" quickly) or when emotional expressions are used for gain (e.g., emotions displayed to manipulate or intimidate others).

Figure 2. Limited Prosocial Emotions Specifier from DSM-V Conduct Disorder Criteria (American Psychiatric Association, 2013)

The LPE specifier (Figure 2) represents the most extensively researched method of subgrouping youth with CD - according to their levels of CU traits (Fairchild et al., 2019). For example, many studies separate those with CD and high vs. low levels of CU traits (e.g. (Frick & Ellis, 1999)).

This approach has gained support due to its effectiveness in identifying differing developmental trajectories of CD (Frick & Viding, 2009) and providing a reliable indicator of the severity and persistence of conduct problems (Frick, Stickle, Dandreaux, Farrell, & Kimonis, 2005). In addition to this, subtyping youth with CD according their level of CU traits has allowed researchers to identify sub-groups with different environmental, genetic and neurobiological vulnerabilities with differing levels of treatment responsivity (Fairchild et al., 2019).

1.2.2.2 CU Traits (group based on clinical features)

CU traits show considerable overlap with the core affective and interpersonal characteristics of the adult symptoms of psychopathy (Viding, Fontaine, & McCrory, 2012), and are thought to identify of a subgroup of antisocial youth who might be at risk of developing the syndrome of psychopathy in adulthood (De Brito et al., 2021). As mentioned previously, these traits include flat affects, a lack of empathy, minimal guilt or remorse after wrong-doing, and little concern over how the individual is perceived by others (Frick & White, 2008). Although varying levels of CU traits are present in several psychiatric disorders (Herpers, Rommelse, Bons, Buitelaar, & Scheepers, 2012), as well as in typically developing (TD) youth (Fanti, Demetriou, & Kimonis, 2013), there is a particularly high prevalence of these traits in individuals with antisocial personality disorders (ASPDs). Around 25-40% of those with a diagnosis of ASPD also have levels of CU traits high enough to meet the diagnostic criteria for psychopathy (Hare, 2003; Hildebrand & de Ruiter, 2004). In comparison, in the community the estimated prevalence of psychopathy is only around 1% (Coid et al., 2009; Neumann & Hare, 2008).

Proactive aggression, manipulation and causing harm without provocation are more commonly observed in the subgroup of youth with CD and high levels of CU traits (Frick, Cornell, Barry, Bodin, & Dane, 2003), while those with CD and low levels of CU are reported to have higher involvement in crimes motivated by reactive aggression (Fanti, Frick, & Georgiou, 2009). Generally, the group of youth with CD and high levels of CU traits also display the most severe conduct problems (Fairchild et al., 2019) and are more at risk of displaying serious and enduring patterns of antisocial behaviour (Viding, Fontaine, et al., 2012), which may be linked to the low treatment responsivity in this group (Euler et al., 2015; Pardini, Frick, & Moffitt, 2010). Individuals with CD and high CU traits also show the greatest impairments in their capacity for effective interpersonal functioning and socialization (Frick, Ray, Thornton, & Kahn, 2014; Viding & McCrory, 2019), which is suggested to relate to deficits in key socio-affective skills, such as their capacity for empathy and emotional recognition (Blair, Leibenluft, & Pine, 2014). Furthermore, CU traits in youth are a predictor of future offending behaviour (Kahn et al., 2013) and youth diagnosed with CD and high levels of CU traits are also at a significantly higher risk of developing psychopathic personality disorder in adulthood compared to those with CD and lower levels of CU traits (Blair et al., 2014; Frick et al., 2014). Initially, research reported that CU traits were stable during the course of development (Frick et al., 2014), but recent studies have reported that there are also some genetic and environmental effects which may contribute to developmental changes in CU traits (Takahashi, Pease, Pingault, & Viding, 2021). The fact that there are specific, problematic behavioural patterns and a poorer long-term prognosis for the subgroup of individuals with CD and high CU traits makes this an important area of research within the field of behavioural disorders (Squillaci & Benoit, 2021).

In order to assess CU traits in a research context, either self-reporting measures, parent/caregiver reporting, or both may be used (De Brito et al., 2021). This information is usually gathered via questionnaires, of which the most widely used are the Inventory of Callous-Unemotional Traits (ICU; (Essau, Sasagawa, & Frick, 2006)) and the Youth Psychopathic Traits Inventory (YPI; (van Baardewijk et al., 2008)). For studies using a person-centred approach when assessing CU traits, a median split is typically used to determine whether an individual's score is classed as a 'high' or 'low' level of CU traits (Docherty et al., 2017). However, as a continuous spectrum of scores for CU traits are usually observed within samples, it appears that CU traits are dimensional rather than defining discrete groups (Kliem, Krieg, Klatt, & Baier, 2021; Murrie et al., 2007). Thus, it is important that research studies investigating these traits are designed accordingly. The CAPE interview (Centifanti et al., 2019) is a recently developed semi-structured interview that may be used to capture the dimensional nature of CU traits more accurately than has previously been possible with traditional questionnaire designs.

1.2.2.3 Neuro-cognitive Model of Emotions

The most widely recognised model used to explain the neurobiology of CU traits in youth with CD, and the individual differences in levels of reactive vs. proactive aggression, was developed by Blair (Blair, 2013). In this model, high levels of CU traits in youth with CD are associated with dysfunction in particular brain regions, namely; the amygdala, caudate, orbitofrontal cortex, ventromedial prefrontal cortex (PFC) and anterior insula (AI) (Blair, 2013). In these youths, atypical levels of response to negative affective cues in these brain regions is linked to diminished emotional experience and lack of empathic responding to other's distress, which can result in misinterpretation of social cues and inappropriate (or anti-social) responding.

Specifically, it is suggested that lower activation of the amygdala in response to negative emotional stimuli, and of the striatum in response to positive stimuli, reduce the individual's capacity for stimulus-reinforcement learning in social situations. Thus, for youth with CD and high CU traits the salience of social cues may be poorly understood, leading to a distorted representation of the situation in the orbitofrontal cortex. This misrepresentation then contributes to impaired decision-making, observed as altered functionality in the ventromedial PFC. The low amygdala response to emotional distress cues from negative facial stimuli seen in these individuals has also been linked to deficient empathic responses to distress and suggested to contribute to an increased risk for proactive aggression in this group (Lozier, Cardinale, VanMeter, & Marsh, 2014).

Blair's model also posits that dysfunction of the AI in this group contributes to atypical emotional responding and the ensuing behaviours (Blair, 2001, 2013). This brain region is vital for interoceptive awareness (Ernst et al., 2014; Wang et al., 2019) and disruptions to this process relating to atypical AI activation are implicated in other psychiatric disorders characterised by deficient emotional responding, such as autism spectrum disorders (Barrett & Simmons, 2015; Quattrocki & Friston, 2014) and substance misuse disorder (Sönmez, Kahyacı Kılıç, Ateş Çöl, Görgülü, & Köse Çınar, 2017). However, it is important to note that there appear to be distinct neurocognitive impairments and behavioural problems between CD and these disorders. For example, CD with high CU traits is characterized by impaired affective, but not cognitive empathy and high levels of instrumental aggression, while autism spectrum disorders are characterized by impaired cognitive empathy, but do not exhibit the elevated levels of instrumental aggression seen in CD (Blair, 2008; O'Nions et al, 2014; Schwenck et al., 2012).

Nonetheless, there may be some overlap in the neural correlates of these disorder, as reduced AI volume is reported in youth with CD (Rogers & De Brito, 2016) and has been specifically associated with affective introspection in individuals with this diagnosis (Sethi, O'Nions, McCrory, Bird, & Viding, 2018). (But see (Klapwijk et al., 2016) which showed distinct patterns of brain response between CD with high CU traits and autism spectrum disorders.)

In Blair's model (Blair, 2013), the reactive aggressive behaviours observed in youth with CD and both high and low CU traits, but more common to the group with low CU traits, are associated with dysregulation of the basic neural threat circuitry. Whereas the proactive aggression and shallow emotionality observed in youth with CD and higher levels of CU traits is linked to dysfunction in a distinct network of regions associated with stimulus reinforcement learning (see Figure 3 below).

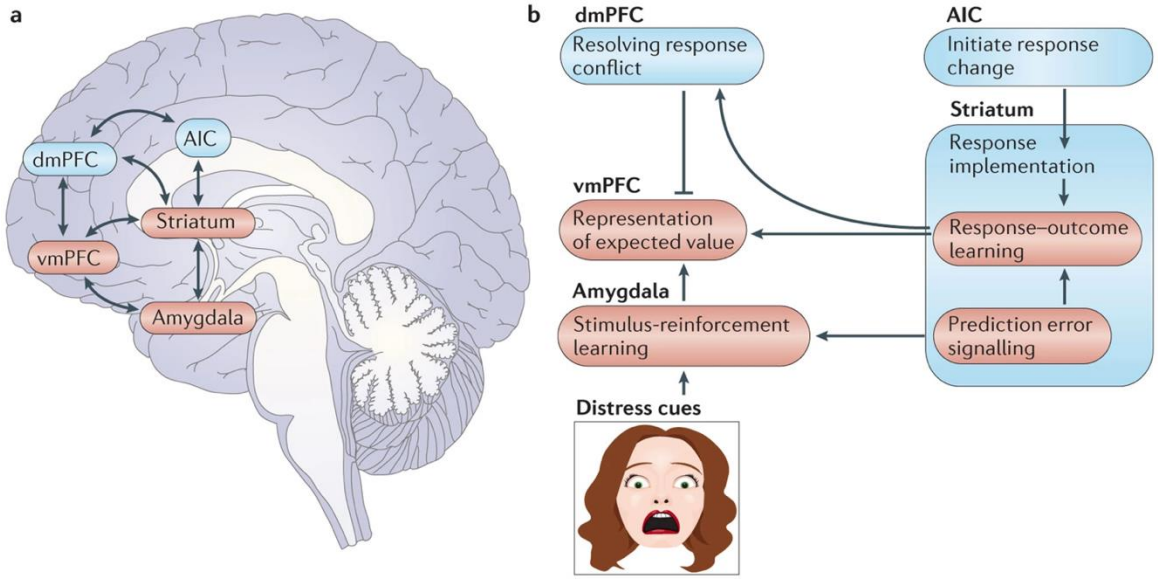


Figure 3. Blair's 2013 model of neural dysfunction in individuals with psychopathic traits (Blair, 2013). a) Regions implicated in psychopathy b) Functional Impairments associated with each region.

Hyper-responsivity of the basic neural threat circuitry is implicated as the neural basis of reactive aggression. This circuitry includes a network of brain regions including the amygdala and hypothalamus (Lickley & Sebastian, 2018). Blair posits that the reactive aggressive behaviours particularly observed in youth with CD and low levels of CU traits are linked to an increased amygdala responsiveness, specifically to threat cues. This heightened amygdala response may lead to an increased threat sensitivity in this group and thereby increase the likelihood that when a threatening trigger is encountered it will activate reactive aggressive behaviour (Blair, 2013). Early-life environmental influences (e.g. childhood maltreatment/abuse) have been linked to hyper-responsivity in this system (Hein and Monk., 2017) and may represent a latent vulnerability in youth with CD (McCrory, Gerin, & Viding, 2017), this may also be accompanied by impaired regulation of these limbic regions by the orbito-frontal and anterior cingulate cortices (Blair, 2013; Blair, Budhani, Colledge, & Scott, 2005). This may lead to hyper-vigilance to threat stimuli and heightened response behaviours, including reactive aggression. Over-stimulation of this system may also contribute to difficulty in recognising expressions of anger in youth with CD (Blair et al., 2005). Figure 3 above shows some of the core regions (a) and cognitive functions (b) implicated in relation to psychopathic traits in youth according to Blair's model.

In summary, Blair's model provides a framework for understanding the link between behaviours observed in youth with CD (in relation to their level of CU traits) and aberrant function of emotional brain networks. However, this model is incomplete as it does not discuss the influence of sex or consider that distinct patterns of neuro-cognitive dysfunction may be present in males and females with CD. This highlights the need for an updated model which also includes the influence of sex on patterns of neurocognitive dysfunction in CD.

1.2.2.4 Sex-Specific Characteristics

Sex appears to be another important factor contributing to the heterogeneity of CD. In addition to the differences in prevalence rates of CD between male and female youths (Moffitt & Caspi, 2001), several differences in common symptom presentation have also been identified. Studies consistently report a greater frequency of overt aggressive behaviours and vandalism in males with CD, compared to females (Moffitt, Caspi, Rutter, & Sylva, 2002), but higher incidences of covert behaviours (such as truancy, absconding and lying) in females with CD compared to males with the disorder (e.g. (Hipwell et al., 2002)). Females incarcerated in juvenile detention centres also show greater rates of relational aggression, but lower rates of physical aggression, compared to their male counterparts (Marsee & Frick, 2007).

One issue specifically associated with CD in females is the increased rates of teenage pregnancies observed in this group (Pedersen et al., 2011). This problem can have a wide-reaching societal impact, as it further increases the female's risk of physical and mental health issues and difficulty in integrating into full-time work. Also, the children of females with CD are themselves at greater risk for developing CD symptoms (Pedersen & Mastekaasa, 2011). In addition to this, CD in female youth is also particularly strongly associated with increased rates of teenage prostitution, chronic health issues and petty crime (Bardone et al., 1998).

There are also sex-differences in the co-morbidity of CD with other psychiatric diagnoses (Konrad et al., 2021). For example, females with CD show higher incidences of co-occurring symptoms of borderline personality disorder, post-traumatic stress disorder and depressive disorders, while rates of co-morbid ADHD are higher in males with CD (Konrad et al., 2021). However, findings for sex-specific prevalence of CD with substance use disorders and anxiety

disorders are mixed (Freitag et al., 2018; Konrad et al., 2021). Overall, lifetime rates of co-morbid psychiatric symptoms are higher in girls with CD and more severe symptoms of these comorbid psychiatric disorders are observed in females (Konrad et al., 2021). These findings are in-line with the 'gender paradox' effect observed in other psychiatric disorders, where the lesser affected sex displays more serious symptoms, impairments and co-morbidities (Eme, 1992).

In the following sections I will discuss the environmental and genetic risk factors for CD, and how these vary according to level of CU traits and sex. I will then move on to discuss the most recent structural, functional, and imaging genetics research findings in CD, and again consider how these vary in relation to level of CU traits and sex.

1.2.3 Environmental Risk Factors for CD

1.2.3.1 CD in General

Prenatal, perinatal and postnatal environmental influences have all been identified as playing a role in the development of CD symptomatology (Fairchild et al., 2019). Findings from twin-study research indicates that ~50% of the variance in CD symptoms is indeed due to environmental effects (Jaffee & Price, 2012; Latimer et al., 2012). Pre-natal environmental factors relate to the environment that the developing foetus is exposed to in utero. Prenatal factors shown to be associated with an increased risk of developing CD include maternal smoking (Gaysina et al., 2013; Silberg et al., 2003), maternal depression (Barker, Copeland, Maughan, Jaffee, & Uher, 2012), poor maternal nutrition (Liu, 2011; Raine, 2002), alcohol or substance abuse (Popova et al., 2016; Hyun Ruisch, Dietrich, Glennon, Buitelaar, & Hoekstra, 2018), pregnancy complications (Sandman et al., 2018) and exposure to situations causing severe

stress which alters the biological state of the mother (MacKinnon, Kingsbury, Mahedy, Evans, & Colman, 2018). Stress during pregnancy in particular has been linked to childhood-onset CD behaviours (Barker & Maughan, 2009). Birth complications are also potential risk factors for later development of CD (Hodgins, Kratzer, & McNeil, 2001; Lukkari et al., 2012) as they may contribute to impaired brain development and function (Kim et al., 2015).

The importance of family structure and adequate parenting have also been recognised as contributing to individual risk for developing CD. Negative parenting practises, such as inadequate supervision (Loeber & Stouthamer-Loeber, 1998), low levels of parent-child engagement (Gardner, Hutchings, Bywater, & Whitaker, 2010), harsh punishment (Baker-Henningham & Francis, 2018) and coercive practices (Granic & Patterson, 2006) are associated with higher rates of CD and sub-clinical conduct problems (Waller, Gardner, & Hyde, 2013). Childhood maltreatment (Kim-Cohen et al., 2003; Norman et al., 2012) also appears to have a significant impact on the risk of developing subsequent conduct problems. Other family factors such as having a parent in jail, living with a parent who has been divorced and witnessing domestic violence (Yockey, King, & Vidourek, 2021) have also been shown to increase risk for CD.

Sociological theories of criminal behaviour postulate that unstable communities (specifically those with high residential turnover and isolated residents) provide an optimal environment for antisocial behaviours and crime to thrive (Herrenkohl & Russo, 2001). In-line with this, several social factors such as witnessing community violence (Kersten et al., 2017), living in a neighbourhood with high levels of poverty (Piotrowska, Stride, Croft, & Rowe, 2015) and

involvement in a delinquent peer group are commonly observed in individuals with CD (Burt & Klump, 2013; Kendler, Jacobson, Myers, & Eaves, 2008). Lower socio-economic status (SES) has been consistently associated with higher rates of antisocial behaviour (e.g. (Costello, Compton, Keeler, & Angold, 2003)) although the strength of this correlation varies according to other factors, such as level of CU traits and national income inequality (Piotrowska et al., 2015). There is also some evidence that SES moderates the effects of other genetic and environmental CD risk factors (Hendriks et al., 2020).

1.2.3.2 CU

Several environmental factors have been specifically associated with an increased risk of developing CU traits, either directly or via interactions with other characteristics of the child. A recent twin-study demonstrated that 21.7% of children's baseline level of CU traits was due to non-shared environmental factors (Takahashi et al., 2021) and that the association between these environmental factors and level of CU traits varied with age (Takahashi et al., 2021). Additionally, they observed that non-shared environmental factors accounted for 43.2% of the variation in the developmental course of CU traits in 7-16 year olds (Takahashi et al., 2021). There is also evidence that there are different developmental pathways (genetic vs. environmental) leading to the development of CU traits and in particular there appear to be distinct environmental vulnerabilities for groups of individuals with high levels of CU traits with or without or co-occurring internalizing problems (Craig, Goulter & Moretti, 2021). In youth with low levels of internalising problems, a higher level of methylation of the Oxytocin receptor (*OXTR)* gene at birth has been linked to both higher CU traits at age 13 and fewer experiences of victimization by age 7 (Cecil et al., 2014). In contrast, in youth with high levels of internalising

symptoms, there was no association between level of CU traits and *OXTR* methylation, however there was a positive association between CU traits and prenatal exposure to domestic violence and conflict in the home (Cecil et al., 2014). Maternal psychopathology or criminality and maternal substance use were also reported as specific prenatal environmental risks for greater *OXTR* methylation at birth across all participants in this study (Cecil et al., 2014).

Exposure to multiple traumas or prolonged maltreatment in childhood has been suggested to play a role in the development of CU traits (Kerig, Becker, & Egan, 2010) and the shallow affect/blunted emotionality observed in this group may stem from an over-modulation of emotions, representing an adaptive response attempting to avoid post-traumatic hyper-arousal in response to traumatic situations (Mozley, Modrowski, & Kerig, 2018). Twin-studies have also reported links between parenting styles and CU traits, specifically, harsh/negative parenting has been positively associated with level of CU traits, and parental warmth negatively associated with level of CU traits (Waller, Hyde, Klump, & Burt, 2018). However, the association between negative parenting and higher CU is not reported in all twin-studies (Viding, Fontaine, Oliver, & Plomin, 2009) and so further longitudinal research is needed to fully determine the nature of the relationship between these variables.

1.2.3.3 Sex

While sex-differences in environmental risk factors are reported for a number of psychiatric disorders, including generalized anxiety disorder (GAD) (Remes et al., 2017) and depression (Chen & Yu, 2015), research indicates that the environmental influences on development of CD behaviours appear to be largely the same for males and females (Burt, Halperin, & Oldehinkel, 2018; Van Hulle et al., 2018). However, social learning of aggressive behaviours during childhood is more prevalent in males (Tremblay & Côté, 2019), indicating that observing

displays of aggression (either directly from peer groups/family, or indirectly via exposure to media violence) may be a greater risk factor for subsequently developing these behaviours for boys. While there is limited evidence of sex-specific risk factors in youth with CD, some sex-specific factors have been identified which may be protective against CD development. For example, both positive socialization and supportive interpersonal relationships appear to act as a stronger deterrent against developing CD in females than males (Ehrensaft, 2005). However, generally, the lack of studies on females with CD means that environmental factors that increase females' susceptibility to developing CD are currently still poorly understood (Freitag et al., 2018).

1.2.4 Genetic Risk Factors

1.2.4.1 CD in General

In addition to investigating environmental risk factors, in recent years an increasing number of studies have endeavoured to identify genetic influences on childhood behavioural disorders. Twin studies have played an important part in distinguishing between environmental and genetic risk factors for CD and enabling researchers to estimate the overall heritability of CD symptomatology (Burt, 2015). A recent review of CD research in twins reported that approximately half of the variance in rates of CD could be attributed to genetic factors (Polderman et al., 2015). Studies also report that the influence of genetic factors increases with age (i.e. the heritability estimates of adolescent-onset CD are higher than those for child-onset CD), due to the changing roles of genes during development, and the increased expression of certain genes during puberty (Jacobson et al., 2002).

There are various approaches for investigating the molecular genetic mechanisms associated with a specific psychiatric phenotype. For example, researchers may either investigate genetic variation across the entire genome, or regarding a single gene of interest. The methodologies which have been used for investigating genetic risks associated with CD have included both hypothesis-free and hypothesis-driven approaches (Salvatore & Dick, 2018). Generally, hypothesis-driven approaches select a specific genetic variant and investigate whether it is more common in psychiatric groups as compared to control groups (Jorgensen et al., 2009), whereas hypothesis-free approaches explore the entire genome of individuals to determine whether there are significant differences in genotype between individuals with and without psychiatric symptoms (Collins & Sullivan, 2013). The most common type of genetic variation comes from single-nucleotide polymorphisms (SNPs), which are variations in a single nucleotide from a base in the DNA sequence. However, genome-wide searches for an association between anti-social behaviour (Anney et al., 2008; Dick et al., 2011; Tielbeek et al., 2012) or aggression (Pappa et al., 2016) and specific SNPs have not yielded consistent findings.

Hypothesis-driven studies that have been used to clarify the association between CD and the variation of individual genes have been mostly directed at genes implicated in biological systems associated with socio-affective functioning (Fairchild et al., 2019). In individuals with CD, single 'candidate-gene' studies have predominantly focussed on genes associated with the function of the serotonergic, vasopressin, oxytocin and dopaminergic systems. In many cases, this has produced non-replicable or conflicting findings (e.g. with *GABRA2* (Dick et al., 2006; Sakai et al., 2010), but there are some genes for which there is substantial evidence of an association with CD symptomatology (Salvatore & D. M. Dick, 2018). These include the monoamine oxidase type A (*MAOA)* gene (Ficks & Waldman, 2014; Prom-Wormley et al.,

2009), *SLC6A4* gene (Gunter, Vaughn, & Philibert, 2010), *AVPR1A*, which has been associated specifically with aggression (Pappa et al., 2016) and *OXTR* gene (Andreou, Comasco, Aslund, Nilsson, & Hodgins, 2018; Kraaijenvanger et al., 2019; Smearman, Winiarski, Brennan, Najman, & Johnson, 2015).

To explain the biological relevance of these genes to CD (and CU traits), I will now provide an overview of the functioning of the systems of three of these neuro-hormones; dopamine, serotonin and oxytocin.

The Dopaminergic System

Dopamine is one neuro-hormone that has been widely research in association with psychopathic traits and antisocial behaviours (Ferguson, 2010; Ferguson & Beaver, 2009). Dopamine is produced in the brain in the substantia niagra, ventral tegmental area (VTA) and hypothalamus, and dopaminergic neuro-transmission plays a key role in reward processing and the development of addictions (Juarez Olguin, Calderon Guzman, Hernandez Garcia, & Barragan Mejia, 2016). Three particular genes in the dopaminergic system have been extensively studied in research in psychiatric populations. These are the gene encoding the dopamine transporter (*DAT1*; (Kim, Kim, & Cho, 2006)) and gene encoding the dopamine receptors D4 (*DRD4*; (Wu et al., 2013)) and D5 (*DRD5*; (Payton et al., 2001)). There is considerable evidence for dysregulated function of the reward systems in youth with CD. For example, atypical responses to reward and punishment have been repeatedly linked to the development of CD behaviours, specifically in youth with high levels of CU traits (Byrd, Kahn, & Pardini, 2013; Frick et al., 2003; Hyde et al., 2013). A diagnosis of CD in youth is also a strong predictor of substance abuse (e.g. (Khoddam

& Leventhal, 2016)), which is associated with altered dopaminergic/reward system function in brain networks (M. Ernst & Luciana, 2015).

The Serotonergic System

Serotonin is another neuro-hormone suggested to play an important role in the neurobiology of aggression and antisocial behaviour (Ficks & Waldman, 2014; van Goozen, Fairchild, Snoek, & Harold, 2007). Serotonin (also known as 5-hydroxytryptamine (5HT)) is a neurotransmitter synthesized from the amino acid tryptophan (Azmitia, 2007). Key genes governing the function of the serotonergic system include the *TPH1* gene, which codes for the enzyme that synthesizes serotonin from tryptophan and the serotonin receptor genes (e.g. *HTR1B, 5HT2A*) which encode genes that regulate the amount of serotonin circulating in the brain (Muller & Cunningham, 2020). The *MAOA* gene is another important gene in determining the functioning of this system, as it controls the rate at which serotonin degradation occurs following its reuptake from the synaptic cleft (Berger, Gray, & Roth, 2009). The genotypic variant which confers lower expression of the *MAOA* gene has been identified as a risk factor for antisocial behaviour in males (Byrd & Manuck, 2014; Ficks & Waldman, 2014) and this gene has also been implicated in the aetiology of CU traits (Moore et al., 2019). More generally, research has demonstrated a negative relationship between serotonin levels in cerebrospinal fluid and antisocial/aggressive behaviours in male criminal populations (Moore, Scarpa, & Raine, 2002; Virkkunen, Goldman, Nielsen, & Linnoila, 1995) and there is evidence that the serotonin system is linked to the some of the cognitive (e.g. impaired stimulus-reinforcement learning) and emotional (e.g. poor recognition of emotional distress/fear) deficits (Moul, Dobson-Stone, Brennan, Hawes, & Dadds, 2013) observed in individuals with high levels of CU traits.

The Oxytocinergic System

In humans, the neuro-hormone oxytocin is strongly implicated in social and emotional behaviour (Puglia, Lillard, Morris, & Connelly, 2015) thus making it relevant to both CD and CU traits (Cecil et al., 2014; Dadds, Allen, et al., 2014; Dadds & Rhodes, 2008). Oxytocin is a neuro-hypophysial peptide with a 9 amino-acid structure, synthesized by neurons in the hypothalamus (Russell, 2018). The oxytocin system is governed by three main genes: the structural gene (*OXT*), the *OXTR* gene and the central oxytocin secretion gene (*CD38).* Oxytocin's biological functioning is particularly strongly linked to the levels of expression of *OXTR* (Nikolova & Hariri, 2015).

Variations in the levels of oxytocin have been shown to influence key socio-affective behaviours, including emotional recognition, emotional responding and emotional learning (Bartz, Zaki, Bolger, & Ochsner, 2011; Kirsch, 2015). Experimental manipulation of oxytocin levels (achieved using intranasal sprays) also attenuates brain responses to social cues from emotional faces (Domes et al., 2010) and imbalances in the oxytocinergic system have been linked to atypical amygdala activation during socio-affective processing (Puglia et al., 2015). Polymorphic variations of *OXTR* have been specifically linked to severe conduct problems and high CU traits in youth (Beitchman et al., 2012; Dadds, Moul, et al., 2014) and reduced expression of the *OXTR* gene due to epigenetic modifications has also been associated with high CU traits(Cecil et al., 2014; Dadds, Moul, et al., 2014).

Genome-Wide Association Studies (GWAS) are a powerful method for investigating the genetic basis of complex diseases. Researchers identify DNA sequence variations that occur more

commonly in a particular group, and this allows them to make predictions about the biological basis of diseases, and potentially identify individuals at greater risk of developing a disease (Bush & Moore, 2012). However, GWAS on antisocial phenotypes and CD symptomatology have not yet consistently identified genetic variants linked to antisocial behaviour or CU traits. Most studies do not report any loci which show genome-wide significant associations with measures of antisocial behaviour (Derringer et al., 2015; Salvatore et al., 2015; Tielbeek et al., 2012) or psychopathic tendencies (Viding et al., 2010). One exception, however, is a study of 3963 American participants (including 872 CD cases and 3091 controls) that reported four genome-wide significant loci, including two located in the region of the *C1QTNF7* (C1q and tumour necrosis factor-related protein 7) gene (Dick et al., 2011).

Table 1 below contains the summary information from six GWAS studies that investigated genetic variation associated with antisocial behaviour or CD symptomatology. The work by Dick et al, 2011 (described above) is the only study to report positive findings at a genome-wide level of significance (Dick et al., 2011). Other studies report nominally significant associations between antisocial behaviours and specific variants (e.g. Anney et al., 2008; Salvatore et al., 2015), but in general these studies do not provide evidence for the existence of genetic variants which are significantly associated with CD symptomatology.

Table 1: Scoreboard of GWAS studies on antisocial behaviour/CD symptomatology

Title	Authors	Year	Sample Size	Participant characteristics	Genotyping Method	Main Findings
Genome-wide association study of conduct disorder symptomatology	Dick et al.	2011	n=3963	Data from individuals in the Study of Addiction: Genes and Environment (SAGE).	Illumina Human 1M BeadChip	Four genome-wide significant loci identified, including two located in the region of the gene *C1QTNF7* (C1q and tumour necrosis factor-related protein 7) gene.
Conduct disorder and ADHD: Evaluation of conduct problems as a categorical and quantitative trait in the international multicentre ADHD genetics study	Anney et al.	2008	n=958	ADHD diagnosis	Perlegen Array (~600,000 loci tagged)	Nine genes showed nominal associations with clinical and sub-clinical CD symptoms (p<.00001); *A2BP1*, *c12orf28*, *FLJ39061*, *KIRREL3*, *LOC729257*, *PAWR*, *PKD1L2*, *PKD1L3*, and *RGL1*
Genome-Wide Association Studies of a Broad Spectrum of Antisocial Behavior	Tielbeek et al.	2017	n=25781	Multiple population cohorts included	Various from different studies	A calculated Polygenic Risk Score of a range of ASBs predicted some of the variation in ASPD in a sub-group of criminal offenders, but overall, no individual genetic variants exceeded minimum significance threshold for links to anti-social behaviour.

Genome-wide association data suggest ABCB1 and immune-related gene sets may be involved in adult antisocial behavior.	Salvatore et al.	2015	n=1379	Adult participants from the Collaborative Study on the Genetics of Alcoholism (COGA)	Illumina Human 1M BeadChip	No single SNP met the strict genome-wide significance threshold ($P \leqslant 5 \times 10{-}8$). Most highly associated SNP (rs4728702, $P=5.77 \times 10{-}7$) located on the protein-coding adenosine triphosphate-binding cassette, sub-family B, member 1 (*ABCB1*)
Genome-Wide Association Study of Behavioral Disinhibition in a Selected Adolescent Sample. Behavior Genetics	Derringer et al.	2015	n=1901	Adolescents from the CADD projects, half of the participants were from high-risk populations (i.e. undergoing substance abuse treatment or involvement with the criminal justice system)	Affymetrix 6.0 GeneChip microarray	No single SNP was significantly associated with behavioural disinhibition (risky/impulsive behaviour)
In search of genes associated with risk for psychopathic tendencies in children: a two-stage genome-wide association study of pooled DNA. Journal of Child Psychology and Psychiatry, and Allied Disciplines.	Viding et al.	2011	n=1186	General population sample came from the Twins Early Development Study (TEDS)	Affymetrix 6.0 GeneChip microarray	None of the SNPs reached genome-wide statistical significance.

Candidate Gene Studies in CD

In addition to the genome-wide research approaches, a number of studies have investigated individual genes in relation to CD (Veroude et al., 2016). In particular, candidate gene studies have focused on genetic variants associated with the function of the serotonergic and catecholaminergic systems (Gunter et al., 2010). For example, a 2014 meta-analysis reported a significant association between the low activity *MAOA* allele and aggressive or antisocial behaviour (Ficks & Waldman, 2014), and between the short allele of *5-HTTLPR* and aggressive or antisocial behaviour. Other single gene studies have linked CD symptomology to genetic variations in the vasopressin V1a receptor (*AVPR1A*; (Veroude et al., 2016)), *RBFOX1* (a gene involved in regulating neuro-developmental processes) (Fernàndez-Castillo et al., 2020), and *OXTR* genes (Andreou et al., 2018; Zhang et al., 2018). However, small sample sizes and a failure to reproduce the findings from candidate gene studies has led to criticism of these hypothesis-driven approaches for being over-simplistic and unsuitable for studying genetically complex phenotypes, such as CD (Dick et al., 2015; Duncan & Keller, 2011).

1.2.4.2 CU Traits

Genetic epidemiological studies have demonstrated that some clinical aspects of conduct problems have high rates of heritability, including aggression (Rhee & Waldman, 2002) and CU traits (Viding, Blair, Moffitt, & Plomin, 2005). Research estimates for the contribution of genetic factors to the level of CU traits across the population range from 40-78% (Viding & McCrory, 2012). Findings from the Twins Early Development Study (TEDS; Viding et al, 2005) demonstrated that in 7-year-olds, the group differences in CU-trait scores between children with very high levels of CU traits (those in the 90th percentile) and those with normative levels of CU traits is mainly due to genetic variation, with an estimated heritability rate of 0.68 (Viding et al.,

2005). In this group of children with extremely high levels of CU traits, antisocial behaviour also appears to have stronger genetic constituents (0.81 heritability rates of antisocial behaviour in children with elevated CU traits, compared to 0.3 in children with low CU traits) (Viding, Jones, Frick, Moffitt, & Plomin, 2008). This large-scale study also found that the rates of heritability for CU traits are similar between children with high CU traits and antisocial behaviours and children with high CU traits only (Larsson, Viding, & Plomin, 2008). Studies on adopted children demonstrate that the effects of genetic risk for CU traits and antisocial behaviours can be reduced, but not completely removed, by exposure to positive early-life environmental stimuli, such as affirmative parenting, warmth, and positive reinforcement (Hyde et al., 2016).

A recent review of studies investigating the genetic underpinnings of CU traits (Moore et al., 2019) concluded that, at present, the most significant findings from single "candidate" gene studies are for genes involved in governing the function of the serotonin and oxytocin systems. These are two neurohormones that have been repeatedly linked to variation in socio-affective functioning (Di Simplicio & Harmer, 2016; Kirsch, 2015; Muller, Anacker, & Veenstra-VanderWeele, 2016), however there are conflicting findings in the literature as to the relevance of specific genotypes. For example, a study investigating adolescent participants' levels of psychopathic traits and emotional dysfunction (i.e. scores from the affective component of the Hare Psychopathy Checklist-Youth Version (PCL-YV) (Forth et al., 2003)) reported that those who were homozygous for the short allele for the *5-HTTLPR* promoter polymorphism of the *SLC6A4* gene (which impacts the function of the serotonin transporter) had significantly higher scores on these measures than individuals who possessed at least one copy of the *5-HTTLPR* long allele (Fowler et al., 2009). In contrast, a 2011 review that examined the findings

for *SLC6A4* genotype in relation to neuropsychological, psychophysiological and brain imaging data concluded that individuals homozygous for the long allele of the *5-HTTLPR* gene may be at increased risk of higher levels of psychopathic traits (Glenn et al., 2011), while the short allele conferred a greater risk for several other mental health- related conditions (e.g. depression, anxiety, alcoholism). Research on this gene in child participants has also identified a positive association between participants' levels of CU traits and the number of *5-HTTLPR long* alleles (Brammer, Jezior, & Lee, 2016), and a separate study determined that youth who had the long/long *5-HTTLPR* genotype had higher CU traits only if they also reported of low SES (Sadeh et al., 2010).

Similarly, for genes in the oxytocin system, such as the *OXTR* gene, there are mixed findings on how genotype of individual SNPs is related to levels of callousness and antisocial behaviours. In some studies, variation in the *OXTR* gene is reported to characterize children with high levels of CU traits and conduct problems (Dadds, Moul, et al., 2014). For example, the AA genotype at SNP rs237885 has been positively associated with CU traits (Beitchman et al., 2012) and for rs1042778, homozygous status for the minor allele (i.e. genotype TT) is positively associated with CU traits (Beitchman et al., 2012). However, other studies have reported no association between *OXTR* genotype and level of CU traits in children with severe aggressive behaviours (Malik, Zai, Abu, Nowrouzi, & Beitchman, 2012).

These mixed findings illustrate the limitations of investigating single SNPs in relation to complex psychiatric phenotypes, such as levels of CU traits. Research designs which investigate the combined effect of multiple SNPs of a single gene or create a polygenic risk score (e.g.

(Ruisch et al., 2020)), may be more suitable for research of this nature, and understanding the interactions of genetics with environmental factors is also crucial for understanding the aetiology of these traits (Viding & McCrory, 2018).

1.2.4.3 Sex

Some studies have also reported sex differences in the genetic architecture of CD (Meier, Slutske, Heath, & Martin, 2011; Rose, Dick, Viken, Pulkkinen, & Kaprio, 2004; Tielbeek et al., 2017), but these appear to be exceptions to the norm (Burt et al., 2019). In general, twin studies report absent or minor sex-differences in the genetic origins of antisocial behaviour (e.g. (Jacobson et al., 2002; Van Hulle, Rodgers, D'Onofrio, Waldman, & Lahey, 2007), for example a recent longitudinal twin study conducted over a period of 27 years found no sex-effects on the proportion of variance in CD symptoms explained by genetic factors (Wesseldijk et al., 2018). While twin studies are a widely used method for disentangling genetic and environmental influences, it has been suggested that they may inflate the likelihood of false negative findings and are thus ill-suited to identifying sex differences in behaviour at an etiologic level (Burt et al., 2019). Nevertheless, the current evidence does not support distinct genetic underpinnings of antisocial behaviour between males and females (Burt et al., 2019).

It is also possible that while the underlying genetic factors in the aetiology of CD are not sex-specific, the magnitude with which they contribute to risk for CD is (Burt et al., 2019). However, the research on quantitative sex differences in antisocial behaviours presents conflicting findings. Bartels et al (2003) reported that a higher percentage of the variance in aggressive and rule-breaking behaviours is explained by genetic factors in males than in females at age 12 (Bartels et al., 2003), but in contrast, Rose et al (2004) report that at age 14, the genetic influences on

antisocial behaviour are greater for females (Rose et al., 2004). Other studies have found no clear evidence of sex-specific quantitative differences in the genetic aetiology of antisocial behaviour (Burt, Krueger, McGue, & Iacono, 2001; Taylor, Iacono, & McGue, 2000). Thus, the exact nature and extent of genetic effects on CD risk susceptibility is still largely unknown. One reason for these inconsistent findings may be that genetic factors also interact with other factors (e.g. environmental factors), which have not been adequately controlled for in research thus far.

1.2.5 Epigenetics (Environmental x Gene Interplay)

1.2.5.1 CD in General

As discussed previously, both genetic and environmental factors are implicated in the development of CD and in relation to the different CU-trait phenotypes. In order to understand the aetiology of CD and CU traits, it is also important to understand the interplay between genetics and the environment (Fairchild et al., 2019). Epigenetic modulation of gene expression is one possible mechanism resulting from this interaction. Epigenetic modifications are changes to the layer of biological material which sits above the core nucleotide sequence of DNA (El-Sayed, Koenen, & Galea, 2013) and recent research has demonstrated that the specific epigenetic modification of DNA methylation is associated with CD symptomatology (Gescher et al., 2018; Moul, Dobson-Stone, Brennan, Hawes, & Dadds, 2015; Provencal et al., 2014).

The process of DNA methylation involves addition of a methyl group to a specific site on the DNA molecule, which then influences the expression of the gene associated with that particular site (Allis & Jenuwein, 2016). Depending on the precise location of the methylation, there may be an increase or decrease in gene expression. For example, DNA methylation at the promoter site of a gene is particularly linked to gene silencing (Nikolova & Hariri, 2015; Suzuki & Bird,

2008). The suggested mechanism for this is that the addition methyl group(s) reduce the accessibility of the gene's transcriptional machinery, thus decreasing gene expression (Allis & Jenuwein, 2016). Methylation is subject to both genetic and environmental influences. Many DNA methylation signatures are stable and heritable and therefore may be inter-generationally transmitted (Pacht et al., 2021). For example this has been shown to be the case for methylation changes associated with the experiences of holocaust survivors (Yehuda and Lehrner, 2018). However, DNA methylation is also known to be influenced by exposures to environmental factors including dietary nutrients, chemical exposure, and lived experiences of stress or trauma(e.g.; Cao et al.,2013)

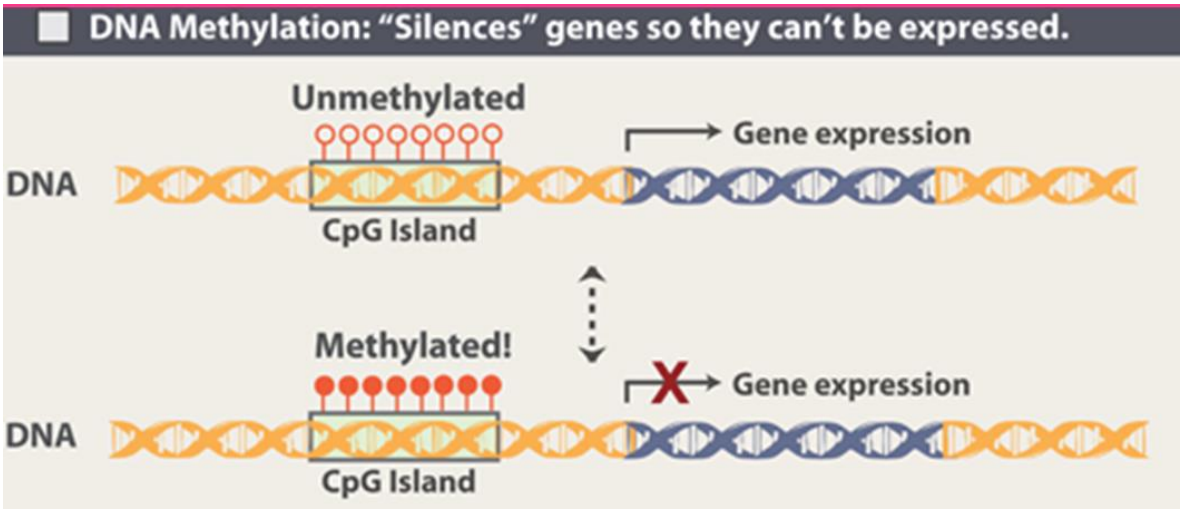


Figure 4. Diagram of DNA Methylation at CpG Island Leading to Reduced Gene Expression (Dozmorov, 2016)

There are few studies investigating the variation in longitudinal, intra-individual changes in DNA methylation, however preliminary evidence indicates that an individual's

DNA methylation profile is dynamic across both short-term (hours to days) and long-term (months to years) timescales (Ciccarone et al., 2018; Gruzieva et al., 2019).

There is also evidence that differential DNA methylation is associated with variations in both brain structure (Fagiolini, Jensen, & Champagne, 2009) and function (Wheater et al., 2020). In-line with this, atypical patterns of DNA methylation have been reported in several psychiatric disorders, such as Schizophrenia, depression, PTSD and Autism Spectrum Disorder (ASD) (Liu, Jiao, Wang, & Yuan, 2018), which are also associated with abnormal brain structure or function (e.g. (de Mendonça Filho, Alves, & Silveira, 2021; Opel et al., 2020). Recently, a small epigenome-wide association study (EWAS) showed that early-onset conduct problems are associated with elevated methylation at various loci, including sub-significant associations with genes previously implicated in CD such as the *MAOA* gene and the brain derived neurotrophic factor (*BDNF)* gene (Cecil et al., 2018). More generally, childhood aggression has been linked to greater methylation at the promoter region of the *SLC6A4* gene (Booij et al., 2010; Wang et al., 2012), which encodes a protein involved in the transfer of serotonin from synapses to presynaptic neurons (Ramamoorthy et al., 1993), and also with elevated methylation at a number of loci associated with regulating cytokines, which in turn regulate the immune system (Provencal et al., 2014). However, these findings were not replicated in a recent EWAS (Pappa et al., 2016).

1.2.5.2 CU Traits

Differential DNA methylation has also been associated with variation in CU traits (Cecil et al., 2014; Dadds, Moul, et al., 2014; Moul et al., 2015). In males with antisocial behaviours, higher levels of methylation of the serotonin receptor gene (*HTR1B*) has been linked to increased CU

traits (Moul et al., 2015), but only in a particular genotypic group (G/T heterozygous at SNP rs1156881). A recent study on males with CD reported that methylation of the *OXTR* gene interacts with level of CU traits to predict brain responses during face processing (Aghajani et al., 2018). Specifically, they observed that overall neural activity strength (during an emotional face processing task) was differentially associated with *OXTR* methylation level at low, moderate, and high CU levels, in CD versus healthy control participants (Aghajani et al., 2018) (see Figure 5). Finally, in a longitudinal study of mixed-sex youth, *OXTR* methylation at birth was positively correlated with level of CU traits at age 13, but only in individuals with lower levels of co-occurring internalising problems (Cecil et al., 2014).

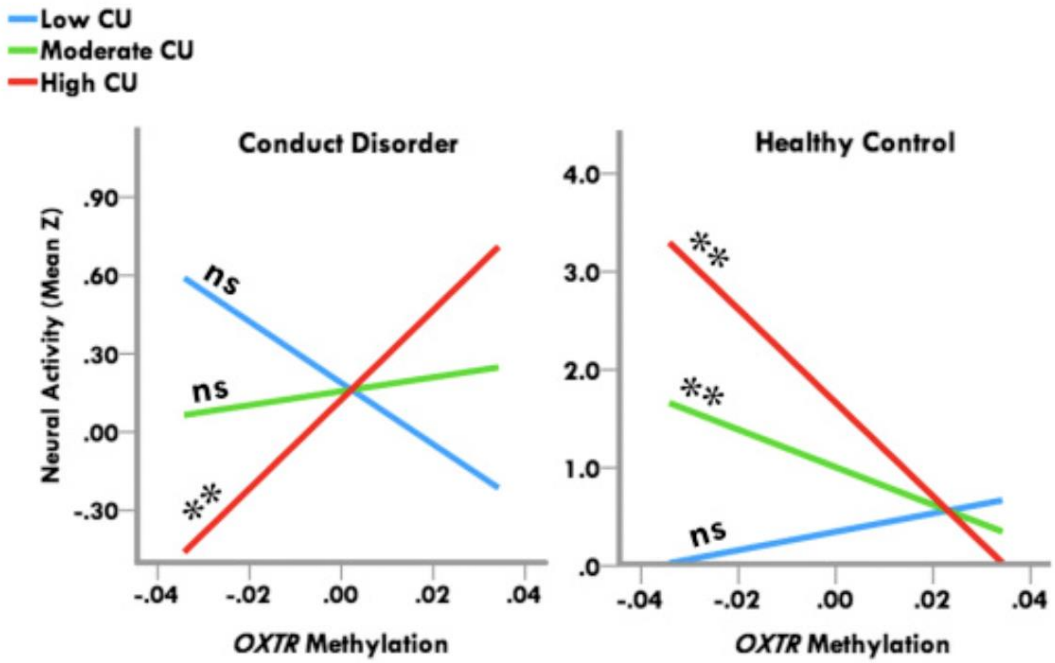


Figure 5. Differing directions of association between brain activity and DNA methylation of the *OXTR* gene according to CD diagnostic status and level of CU traits (low, moderate, high) in adolescent males (Aghajani et al., 2018)

1.2.5.3 Sex

Some researchers have suggested that the existing research methodologies are not sufficient to differentiation sex-specific epigenetic modifications that are specific to one psychiatric disorder (Xia et al, 2019). Accordingly, there is not a single published study examining sex-differences in epigenetic markers associated with CD. However, differential markers of epigenetic variation are known to play a key role in mediating sex differences in brain and behaviour (Qureshi & Mehler, 2010). In particular, DNA methylation of genes involved in synaptic processes has been implicated in sexually differential human brain characteristics (McCarthy et al., 2014; Xu et al., 2014). For example, in post-mortem brain tissue samples, sex differences were observed in the levels of methylation of families of genes involved in coding for synapse-related pathways and signaling neuronal pathways (Xia et al., 2014). Several genes potentially involved in psychiatric disorders have also shown different methylation levels between males and females (Xia et al., 2021). In particular, DNA methylation at the same locations on genetic variants are reported to have different effects on males and females in genes associated with schizophrenia and ASD (Xia et al., 2021). Additionally, genes reported to be upregulated in ASD have been shown to be hypermethylated in females, which may indicate that the relative amount of gene alteration required for females to qualify for an ASD diagnosis is greater than for males (Xia, Chen, Jiang, Liu, & Chen, 2019). This has been suggested as an explanation for the difference in ASD prevalence between males and females (Xia et al., 2019). As there is also considerable disparity in prevalence rates of CD according to sex, sex-specific epigenetic modifications may be an important area for future research to clarify the nature of the sex differences in the prevalence of this disorder and its symptomatology.

1.2.6 Functional Neuro-imaging Evidence

1.2.6.1 CD in General

Neuro-imaging studies examining brain response in CD have used a wide range of tasks, including response inhibition (e.g. (Rubia et al., 2008; Sun et al., 2018; Zhu et al., 2014)), reward-processing (e.g. (Stuart F White et al., 2014)) working memory (e.g. (Fridberg, Gerst, & Finn, 2013)) and theory of mind (e.g. (Arango-Tobón et al., 2020)). However, the majority have focussed on emotional processes (Fairchild et al., 2019), for example, using tasks in which participants view affective stimuli, such as a picture of another person in distress/pain or images of faces expressing emotion. Meta-analytical evidence has demonstrated that, compared to TD youth, those with CD display reduced response in the amygdala (Noordermeer, Luman, & Oosterlaan, 2016), ACC (Alegria, Radua, & Rubia, 2016) and parts of the basal ganglia (Noordermeer et al., 2016) during emotional processing tasks. Another recent study reported that youth with CD showed lower response in the AI than TD youth when viewing emotional faces (Menks et al., 2021), however this effect disappeared when the researchers corrected the results for the fact that the CD group spent less time focussing on the eye-regions of the face.

1.2.6.2 CU Traits

Functional neuro-imaging research in participants with CD with varying levels CU traits has also largely focussed on emotional processing and these studies have generally reported reduced responses in individuals with CD and higher levels of CU/psychopathic traits (Blair & Zhang, 2020). For example, reduced amygdala response to fearful faces has been repeatedly observed in participants with CD and high CU traits (Jones, Laurens, Herba, Barker, & Viding, 2009; Lozier, Cardinale, VanMeter, & Marsh, 2014; Marsh et al., 2008), whereas amygdala hyper-

responsiveness to fearful faces is more commonly observed in participants with CD and low levels of CU traits (Sebastian et al., 2014; Viding, Sebastian, et al., 2012; White et al., 2016). However, it is not clear whether these associations extend to non-clinical populations, as in youth with varying levels of conduct problems below the threshold for CD diagnoses, brain responses to fearful faces show no association with level of CU traits (Dotterer, Hyde, Swartz, Hariri, & Williamson, 2017).

In a 2016 meta-analysis, task-related lowered response in the hypothalamus, thalamus, ventral striatum and ventromedial PFC were all reported in youth with conduct problems and psychopathic traits as compared to control youth (Alegria et al., 2016). This meta-analysis also reported greater response for this group in the dorsolateral PFC and the right caudate (Alegria et al., 2016). Another recent meta-analysis in adults (Poeppl et al., 2019) concluded that decreased activity in the right amygdala, dlPFC and frontal cortices is also consistently observed in relation to psychopathy, along with elevated levels of activity in the fronto-insular cortices bilaterally (Poeppl et al., 2019). Psychopathy has also been linked to dysfunction in the default mode network of the brain (Johanson, Vaurio, Tiihonen, & Lahteenvuo, 2019). This network includes regions such as the dorsomedial PFC, ventral PFC, PCC, precuneus and lateral parietal cortex (Raichle, 2015) and is implicated in self-referential processing (Kjaer, Nowak, & Lou, 2002), moral judgement (Greene & Haidt, 2002; Harrison et al., 2008), and emotional reflection (Gusnard, Akbudak, Shulman, & Raichle, 2001). Atypical activation in this system is observed in both adult males (Sheng, Gheytanchi, & Aziz-Zadeh, 2010) and females (e.g. (Lindner et al., 2018)) with psychopathic traits, and has also been reported in relation to CU traits in mix-sex youth sample with a history of being arrested (Cohn et al., 2015).

1.2.6.3 Sex

As previously mentioned, research in the field of CD is dominated by studies on male participants (Freitag et al., 2018) and thus there are relatively few MRI studies directly examining sex differences in brain response in youth with CD. There are also few studies that have investigated the neural correlates of emotional processing specifically in females with CD. The first study to examine brain response to emotional faces in females with CD (Fairchild et al., 2014) observed that, compared to TD females, the CD group had lower activation of the medial orbitofrontal cortex and greater activation of the AI on viewing emotional faces (across facial stimuli depicting angry, sad and neutral expressions). Another recent study investigating emotional regulation in females with CD reported atypical left angular gyrus and dorsolateral PFC (dlPFC) activity during effortful emotional regulation (Raschle et al., 2019), compared to TD females. This appears to be in-line with findings of reduced activity in these regions in males with CD during tasks with an emotional component (Blair et al., 2018). However, sex-specific findings have been reported for brain regions such as the ACC (Alegria et al., 2016; Cao, Sun, Dong, Yao, & Huang, 2018), superior temporal gyrus (Cao et al., 2018), mid-frontal and mid temporal gyri (Cao et al., 2018). A 2016 meta-analysis found a greater magnitude in reduction of activity of the ACC is reported in males as compared to females with CD during emotional processing (Alegria et al., 2016). In addition, in a recent rs-fMRI study, Cao et al (2018) observed that, compared to females with CD, males with CD displayed greater levels of spontaneous brain activity in the superior temporal gyrus, but lowered levels of activity in the left ACC, mid-frontal and mid-temporal gyri (Cao et al., 2018).

In TD participants, sex-differences are reported in fMRI studies involving emotional conflict processing (Cservenka & Ray, 2017) and in the brain mechanisms mediating reward and

addiction (Becker & Chartoff, 2019). As atypical neural responses associated with these processes are also reported in youth with CD (Blair et al., 2018), there is a precedent to suggest that sex-specific abnormalities in brain response may exist, but further research in mixed-sex participant samples is needed to confirm this. Importantly, research into other psychiatric disorders, such as ASD, has highlighted the importance of using a life-span approach to address this question (Walsh, Wallace, Gallegos, & Braden, 2021) as this allows researchers to capture variations in sex-differences at particular neuro-developmental periods.

1.2.7 Structural Neuro-imaging Evidence

1.2.7.1 CD in General

Meta-analyses of structural MRI research in youth with CD symptomatology report reductions in grey matter volume (GMV) in multiple cortical and sub-cortical brain regions (Noordermeer et al., 2016; Raschle et al., 2015; Rogers & De Brito, 2016). Another recent study, which included participants with CD-only (i.e. no other current comorbid psychiatric disorders), demonstrated that compared to TD males, boys with CD had decreased GMV in the right pre-postcentral cortex, supramarginal gyrus and right putamen, but increased GMV in the orbitofrontal cortex and superior temporal gyrus (Gao et al., 2020).

Differences in brain surface structure are also associated with CD and can be assessed using surface-based morphometry (SBM) methods. SBM techniques allow researchers to measure the cortical thickness, surface area, or local gyrification of the brain, which have been shown to follow different developmental trajectories and be under different genetic influences (Raznahan et al., 2011). As the volume of a brain region is a product of the brain's surface area and cortical

thickness, in VBM studies it can be difficult to determine which of these inter-related metrics are driving observed differences in GMV (Hutton et al., 2009). Thus, SBM methods give researchers the opportunity to investigate precisely which of these individual components are contributing to volumetric differences in CD (Fairchild et al., 2015). Specifically, youths with CD have been reported to have reduced cortical thickness in the superior temporal gyrus (Fairchild et al., 2015) and cortical thinning in relation to CD symptomatology has also been reported in the ventromedial PFC, superior temporal cortex, fusiform gyrus, precentral gyrus and precuneus (Fairchild et al., 2019). Additionally, within CD youth lower levels of gyrification are reported in the ACC, orbitofrontal cortex and ventromedial PFC (Hyatt, Haney-Caron, & Stevens, 2012; Jiang et al., 2015; Wallace et al., 2014) and insula (Fairchild et al., 2015) alongside reduced orbitofrontal cortex surface area (Fairchild et al., 2015). As cortical thickness, surface area, and folding metrics each provide unique information (Hutton et al., 2009; Raznahan et al., 2011), these findings demonstrate the importance of SBM techniques to fully understand the nature of the structural brain correlates associated with CD.

1.2.7.2 CU Traits

A number of studies have examined the neural correlates of CU traits and showed that they are related to variations in GMV in several areas of the brain. For example, reductions in GMV in association with CU traits have been reported in multiple regions across both the paralimbic cortex and limbic system (Caldwell et al., 2019). Additionally, negative associations between psychopathic traits (a measure which has considerable overlap with CU traits) and GMV have been reported in the orbitofrontal cortex, bilateral temporal poles, and posterior cingulate cortex (PCC) (Ermer, Cope, Nyalakanti, Calhoun, & Kiehl, 2013), as well as the right mid-frontal

cortex and left parietal lobe (Yang et al., 2015), and the right ACC (Sebastian et al., 2016). Some other studies have reported a positive association between level of CU traits and GMV within the paralimbic brain regions (Fairchild et al., 2019; Wallace et al., 2014). For example, compared to TD male youth, GMV in male youth with CD and high CU traits is increased in posterior regions of the orbitofrontal cortex, the ACC and the anterior temporal lobe (De Brito et al., 2009). Finally, in TD male youth, CU traits were found to be positively correlated with GMV in the bilateral AI (Raschle et al., 2017).

Several Diffusion Tensor Imaging (DTI) studies have also reported a reduction in the structural connectivity in white matter tracts in adolescents with CD and high CU traits (Gonzalez-Madruga et al., 2020; Rogers et al., 2019; Sethi et al., 2018). Lower connectivity is reported both between limbic areas of the brain (e.g. in the uncinate fasciculus and the retrosplenial cingulum tracts, (González-Madruga et al., 2020)), and across a wider network of regions, including the dorsal 'default-mode' network and anterior thalamic pathways (Rogers et al., 2019; Sethi et al., 2018). Authors of a recent review of neuro-imaging research in relation to CD and CU traits (Blair & Zhang, 2020) have suggested that these findings of whole-brain impairments in connectivity in individuals with psychopathy/CU traits provide evidence for extensive cognitive problems in this population.

1.2.7.3 Sex

Finally, there is preliminary evidence indicating that the GMV correlates in youth with CD are moderated by sex (Fairchild et al., 2013), and that these sex-effects on brain structure may also interact with the individual's level of CU traits. For example, Fairchild et al (2013) demonstrated a sex-by-diagnosis interaction effect on GMV in the AI, whereby females with CD had reduced

GMV in this region compared to TD female adolescents, but in males an opposite pattern was observed (Fairchild et al., 2013). They also observed a positive correlation between level of CU traits and right orbitofrontal cortex volume uniquely in females (across both CD and TD participants) (Fairchild et al., 2013). In TD males, high levels of CU traits have been linked to increased GMV in the bilateral AI, whereas no association was observed in females (Raschle et al., 2018).

Smaragdi et al (2017) used SBM analysis to determine that, for both sexes, CD was associated with cortical thinning and greater levels of gyrification in the ventromedial PFC (Smaragdi et al., 2017), but they also observed some sex-specific findings. Specifically, they reported a sex-by-diagnosis interaction effect on cortical thickness of the supramarginal gyrus (in males cortical thinning was associated with CD compared to TD males, while the reverse pattern was present in females) (Smaragdi et al., 2017). They also reported a sex specific association between CD and cortical surface area in the superior frontal gyrus (SFG). Relative to controls, males with CD had larger surface area and greater levels of gyrification in this region, whereas in females with CD surface area and gyrification of the SFG was lower than in TD females (Smaragdi et al., 2017). Given the sex-specific functional neuro-imaging findings in CD youth (Alegria et al., 2016; Cao et al., 2018), and as organizational differences in the brain are linked to functional differences (Batista-García-Ramó & Fernández-Verdecia, 2018), sex differences in the structural neural correlates of CD and CU traits may be anticipated.

Findings in TD participants also set a precedent to expect sex-specific human brain morphology in CD, as, in addition to overall brain volume being larger in males than females (Ruigrok et al., 2014), higher tissues densities are observed in males in brain regions implicated in CD, including

the amygdala, hippocampus, precuneus, temporal poles and posterior cingulate gyri (Ruigrok et al., 2014). Additionally, research in TD populations indicates sex differences in patterns of lateralization (e.g. (Chiarello et al., 2009)) and there is also evidence to support lateralized dysfunction associated with antisocial behaviours (Leshem, 2020).

1.2.8 Imaging Genetics/Epigenetic Neuroimaging research

1.2.8.2 CD in General

Imaging genetics research explores genetic variation alongside neuroimaging data on brain structure, function, or connectivity to investigate the neural mechanisms linking genetic and molecular factors to cognition and behaviour (Bogdan, Hyde, & Hariri, 2013). While a number of imaging genetics studies have been published on psychiatric conditions such as ADHD (Park et al., 2015), major depressive disorder (MDD; Pereira et al., 2018), PTSD (Nisar et al., 2020) and schizophrenia (Hass et al., 2015; Walton et al., 2014), there are few imaging genetics publications on CD – only two imaging genetics and one epigenetic neuroimaging studies at present. The studies that have linked genetic and neuroimaging markers in CD have generally, like in other disorders, focussed on a single gene, rather than adopting a genome-wide approach (e.g. Jiang et al, 2019; Sun et al, 2018). For example, during an inhibitory control task, individuals with CD and the high-expression allele of *MAOA* (*MAOA*-H) were demonstrated to have greater deactivation in the precuneus, supplementary motor areas and dorsal ACC than those with the low-expression allele (*MAOA*-L) (Sun et al., 2018). One recent study in male juvenile offenders with CD also demonstrated that methylation (an epigenetic marker linked to gene expression) of the *OXTR* gene interacted with participants level of CU traits to predict both activity in frontoparietal brain regions and also disconnection between the amygdala and frontal

regions in response to facial expressions (Aghajani et al., 2018). Overall, these findings provide preliminary evidence to suggest that both genetic and epigenetic variations may play a part in the underlying pathophysiology of CD.

Research that combines both brain imaging and molecular genetics has been identified as the crucial next step in the field of antisocial behavioural research (Raine, 2008) and collaborative work on understanding the relationship of 'genes to brain to antisocial behaviour' is currently underway through large-scale consortiums (e.g. ENIGMA; Bearden et al, 2017, IMAGEN; Quinlan et al., 2017, PGC; Sullivan et al., 2018). These larger scale studies also provide an opportunity for researchers to investigate the association between genetic variation and neuro-imaging data in CD in relation to CU traits and sex, which is currently an unexplored field.

1.3 Summary

In summary, evidence suggests that the prevalence, clinical presentation, long-term prognosis and both genetic and environmental risk factors for CD differ according to sex and level of CU traits. Neuroimaging research has also demonstrated that there are sex-differences in both structural and functional brain measures in CD participants, and that these differences are influenced by variation in level of CU traits. Both genome-wide and candidate gene research methodologies have been used to study the genetic and epigenetic basis of CD and CU traits, and this research has identified variations in genes linked to biological systems including the dopaminergic, serotonin and oxytocinergic systems. These biological systems are implicated in cognitive and affective processes known to be impaired in individuals with CD, and particularly

in the subgroup with high CU traits. Imaging genetics, which combines genetic and neuroimaging data, provides an opportunity for a greater understanding of the mechanisms that link genetic variation, epigenetic modifications and neuro-imaging markers.

1.4 Thesis Outline

The primary aim of this thesis is to integrate epigenetic and genetic data with structural and functional neuroimaging data to better understand the pathways from (epi)genetic variation to brain structure and response in youth with CD and varying levels of CU traits. Chapter 2 describes the relevant methodologies used in the three studies reported in the thesis and also gives more detailed information on the FemNAT-CD project (from which the data used in these studies was taken). Chapters 3 and 4 outline two studies that investigated the link between epigenetic variation and structural/functional brain markers in females in relation to CD and CU traits. In Chapter 3, I report an EWAS investigating DNA methylation in relation to CD diagnostic status and CU traits, and then examine whether these finding in turn relate to whole-brain GMV in adolescent females. In Chapter 4, I analyse the findings from the EWAS study in the previous chapter, alongside functional MRI data from a face processing task, to probe the link between this epigenetic modification and brain response to emotional faces in females. In Chapter 5 I use a SEM approach to investigate the associations between genotype of the *OXTR* gene, CU traits, CD symptom score and bilateral amygdala activation during the aforementioned emotional face processing task. This modelling is used in both a mixed-sex sample and independently in male-only and female-only participant groups to clarify how these factors contribute to overall CD symptom score according to sex.

*Please note that the findings from chapter 3 have been published in the Translational Psychiatry journal:

Farrow, E., Chiocchetti, A. G., Rogers, J., Pauli, R., Gonzalez-Madruga, K., Smaragdi, A., ... & De Brito, S. (2021). *SLC25A24* gene methylation and gray matter volume in females with and without conduct disorder: an exploratory epigenetic neuroimaging study. *Translational Psychiatry*.
https://doi.org/10.1038/s41398-021-01609-y

CHAPTER 2: METHODOLOGIES AND THE FEMNAT-CD PROJECT

2.1 Overview

The aim of this thesis is to integrate epigenetic and genetic data with neuroimaging data to better understand the biological and brain mechanisms at work in female youth with CD and varying levels of CU traits. This chapter introduces the different data collection approaches and analytical strategies used to investigate the research questions of this thesis. The four main methodological areas covered in this chapter are (i) Structural and function MRI neuroimaging, (ii) Epigenetic analysis (with specific focus on DNA methylation analysis), (iii) Genotyping and structural equation modelling (of the *OXTR* gene), and (iv) clinical/behavioural measures. Clinical, behavioural, and demographic data were drawn from questionnaires administered as part of the FemNAT-CD project (Freitag et al., 2018). For each different methodological area, I provide a brief overview of the topic, a theoretical description of how experimental methods may be used to obtain this information, and then finally the details of the practicalities and specific data methodologies employed in the studies reported in this thesis.

2.2 MRI Methods

2.2.1 Overview

Magnetic Resonance Imaging (MRI) is a widely used method for obtaining anatomically detailed images of the human body, using the principles illustrated in Maxwell's electromagnetic equations (Collins, 2016). It is especially valuable because of its capacity to produce three-dimensional images without exposing the person to harmful radiation or invasive medical procedures (Symms, Jäger, Schmierer, & Yousry, 2004).

2.2.2 MRI Physics

Maxwell's equations explain that the movement of charged particles may induce electric and magnetic fields. They also describe how the behaviour of these electromagnetic fields vary when they interact with different types of matter (Griffiths, 2005). For example, the rate of decay of an electromagnetic signal is different for different tissues in the human body, as is the degree of absorption of electromagnetic waves of different frequencies.

$$\nabla \cdot \mathbf{E} = \frac{\rho_v}{\varepsilon} \qquad \text{(Gauss' Law)}$$

$$\nabla \cdot \mathbf{H} = 0 \qquad \text{(Gauss' Law for Magnetism)}$$

$$\nabla \times \mathbf{E} = -\mu \frac{\partial \mathbf{H}}{\partial t} \qquad \text{(Faraday's Law)}$$

$$\nabla \times \mathbf{H} = \mathbf{J} + \varepsilon \frac{\partial \mathbf{E}}{\partial t} \qquad \text{(Ampere's Law)}$$

Figure 6. Maxwell's Equations of Electricity and Magnetism

The human body is composed of many different types of particles but contains a particularly high percentage of hydrogen molecules (approximately 62% of atoms in the human body are hydrogen). Hydrogen nuclei have an important physical property which MRI imaging makes use of (Berger, 2002) - this is the fact that they have an intrinsic 'spin' of magnitude s= ½. Nuclei with spin of magnitude 1/2 can be in one of two possible states in relation to an external magnetic field: parallel or anti-parallel (Westbrook & Talbot, 2018). These nuclei also have angular momentum which results from their intrinsic spin, and when the nuclei are positioned in an external magnetic field, a torque (twisting force) is generated which acts perpendicular to the direction of the angular momentum and perpendicular to the external field. This torque deflects the nuclei onto a circular path and this circular motion is known as precession (Plewes &

Kucharczyk, 2012). The specific speed/frequency of the precession is influenced by the strength of the magnetic field and varies according to the particles size, mass and spin.

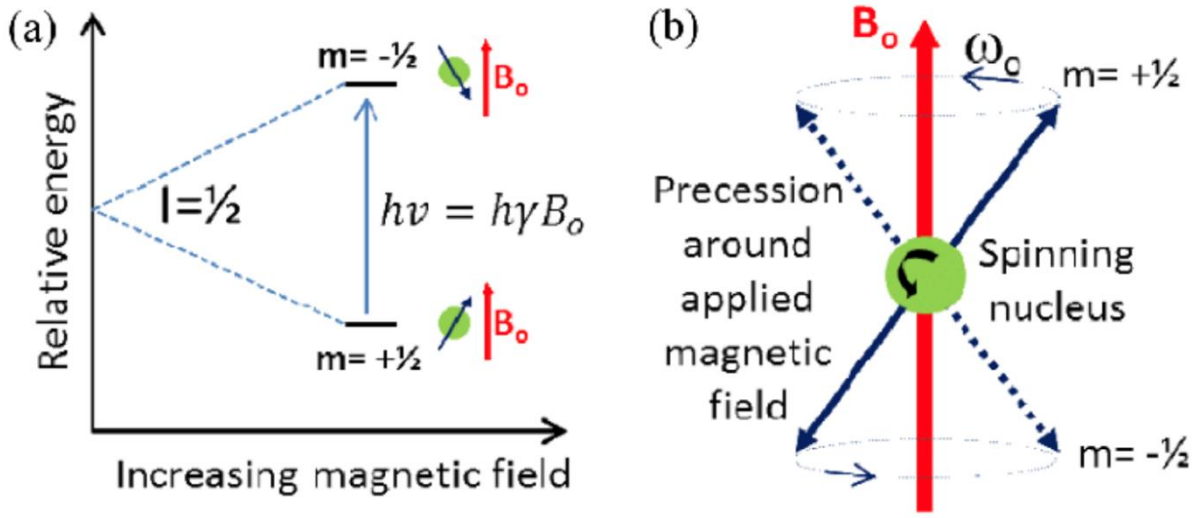


Figure 7. The splitting of nucleus spin states (m=+½ and −½) under the influence of an external magnetic field (Kim, Lee, & Kafle, 2013)

For each state there is an associated energy. The difference in energy between the parallel and anti-parallel states can be calculated using the equation for magnetic potential energy:

$$\boldsymbol{U = \mu.B}$$

(i.e. The potential energy, U, is equal to the scalar product of the magnetic moment of a particular particle and the external magnetic field).

In order for a particle to move from one state to another, it must absorb or emit the amount of energy equal to the energy difference between the two states (Plewes & Kucharczyk, 2012). An

unequal ratio of particles in the two states will cause a hydrogenous material to have an overall net magnetisation.

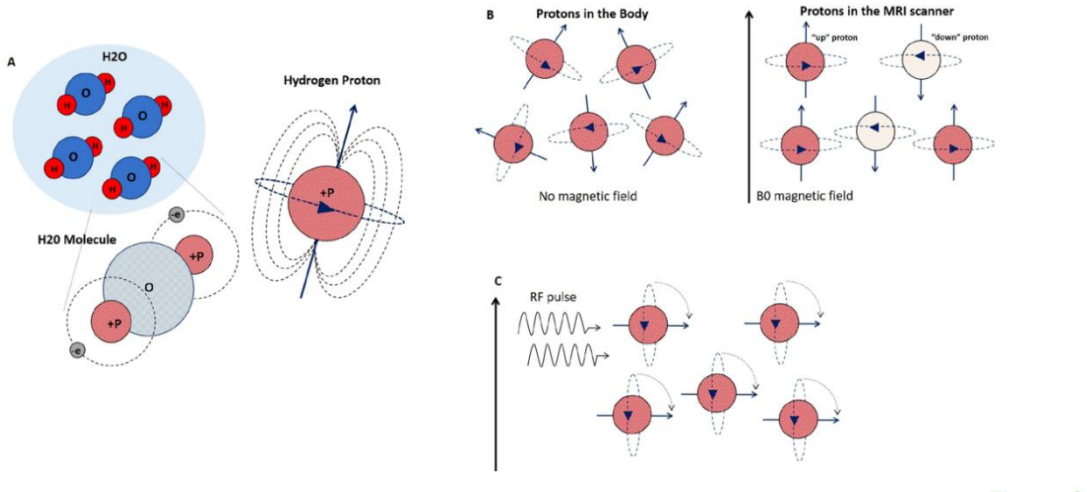


Figure 8. Hydrogen nucleus (i.e. proton) behaviour in a magnetic field (Broadhouse, 2019). A) shows the hydrogen protons within a water (H_20) molecule and the direction of precession about the spin axis on a single proton. B) shows the disordered configuration of proton spins in the absence of a magnetic field compared to proton spins aligned parallel or anti-parallel to the magnetic field in an MRI scanner. C) shows all spins aligned in one direction after a RF pulse is applied.

An MRI scanner provides the magnetic and energetic conditions needed to generate images of biological tissues according to the different ratios of hydrogen nuclei in each of the two states within that particular tissue (and other tissues in its immediate surrounding) (Westbrook & Talbot, 2018). Powerful magnets within the scanner generate a strong external magnetic field. These magnets move and create a changing magnetic field.

Hydrogen nuclei re-align to the changing field so that the system is in its lowest energy state, which is the most stable state for any system. As a hydrogen nucleus drops down from the high

energy to lower energy state it emits energy in the form of an electromagnetic wave (Plewes & Kucharczyk, 2012).

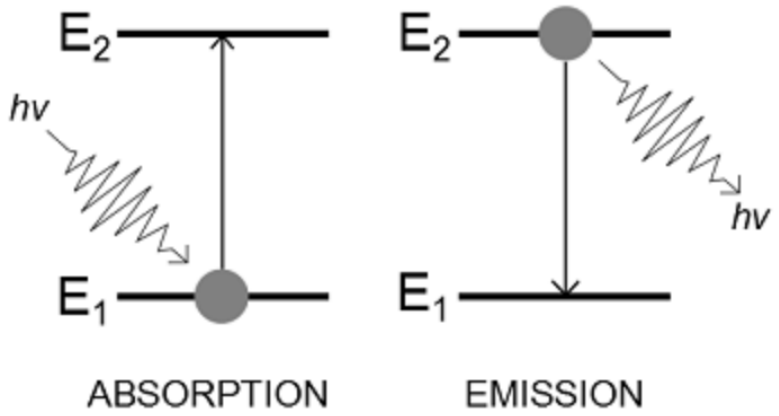


Figure 9. Transitions between energy states E1 and E2 occurring due to absorption or emission of energy E=hv (Plewes and Kucharczyk, 2012)

The scanner also produces pulses of energy (in the form of radio-frequency (RF) pulses) which can 'excite' hydrogen nuclei, causing them to move from the lower energy state to the higher energy state when they absorb this energy (Westbrook & Talbot, 2018).

The time taken for hydrogen nuclei to move from the high to low energy states in response to the change of the external magnetic field varies according to the immediate environment of the nucleus (i.e., the particles in the tissues around it and their properties). This relaxation time has two components; the Longitudinal relaxation time (T_1) and the Transverse relaxation time (T_2) (Westbrook & Talbot, 2018). The values of these two quantities, and the ratio between them is specific to the tissue in which the hydrogen nuclei are situated. MRI imaging uses this fact to create images where the intensity of given tissues corresponds to values of these quantities (T_1 and T_2). This difference in intensity according to tissue-type allows us to distinguish between different types of structures in the body (Grover et al., 2015). For example, MRI imaging can

differentiate between grey matter, white matter and cerebrospinal fluid in the brain because of the differing densities of hydrogen nuclei in each tissue. These different densities result in a unique relaxation time for each tissue in its specific location and the different tissues are thus displayed on the image as different intensities (colours).

For more detailed information on the Physics of MRI please see (Elster, 2018).

2.2.3 Imaging Brain Response (fMRI)

Functional MRI uses the same basic principles are structural MRI, but in this case, the differing magnetic properties of the molecules oxy-haemoglobin and deoxy-haemoglobin are used to create an image (Ogawa, Lee, Kay, & Tank, 1990; Ogawa et al., 1992). Haemoglobin is a molecule within the blood that is used to transport oxygen around the body. The oxygen plus haemoglobin complex (Oxy-haemoglobin) has zero magnetic moment (i.e. it is magnetically 'neutral'), but when it loses the oxygen molecule to become deoxyhaemoglobin it becomes paramagnetic (this means it is weakly attracted by an external magnetic field). This change results in a subtle increase in the local magnetic signal, which is measured by fMRI and known as the Blood Oxygen Level Dependent (BOLD) signal (Ogawa et al., 1990; Ogawa et al., 1992). When neural activity increases in one area of the brain, oxygenated blood is required to flow to that region to sustain the activity. This means that the concentration of oxyhaemoglobin in this region increases, and so measuring the value of the BOLD signal allows us to indirectly measure variations in brain response (Buxton, 2009; Huettel, Song, & McCarthy, 2004).

For more detailed information on the Physics of fMRI please see (Buxton, 2009).

2.2.4 Voxel-Based Morphometry and Image Pre-processing

Voxel-based morphometry (VBM) is mass-univariate statistical technique that is commonly used to analyse MRI images of the brain (May & Gaser, 2006). Indeed, VBM analysis involves splitting the image up into small 3-dimensional sections (known as voxels) and comparing the signal intensity from each voxel, hence the reference to mass-univariate statistics. During this procedure different tissues-types (e.g., grey and white matter) can be extracted separately for analysis, meaning this technique is particularly effective for investigating local regional differences in brain structures (John Ashburner & Friston, 2000). A particular advantage of the VBM approach is that it is suitable for whole-brain analysis, and so can be used without the need for a priori hypotheses about particular regions of interest (Ridgway et al., 2008). VBM has been used extensively to analyse brain structure in psychiatric populations (Scarpazza & De Simone, 2016) and is as one of the most statistically valid methods for comparing grey matter volumes between clinical and healthy groups (John Ashburner & Friston, 2000).

Classical VBM methods use T1- weighted MRI images, which undergo extensive pre-processing before statistical analysis occurs. The main steps of this pre-processing are: segmentation, normalization, modulation and smoothing (Mechelli, Price, Friston, & Ashburner, 2005). Segmentation uses the voxel intensity values to segment the images according to the main tissue-type in a particular region - the three main tissue components of the brain are grey matter, white matter and cerebrospinal fluid (CSF). Normalization involves transforming images so that they are standardised within one stereotactic space, meaning that different size/shaped brains can be compared directly. The modulation stage is used to correct for volumetric changing during the spatial normalization process. This involves scaling the image intensities according to the amount of contraction that has occurred during the previous spatial normalization step to ensure

that the total amount of grey matter is the same as in the original image (Whitwell, 2009). Finally, in the smoothing stage the intensity of each voxel is replaced by the weighted average of a number of its surrounding voxels. The precise number of voxels averaged at a point depends on the size of the smoothing kernel used. The intensity corrections during smoothing help to minimise inter-participant variability and ensure that the image data are normally distributed (Ashburner & Friston, 2000; Salmond et al., 2002). This means that, after smoothing, these normally distributed data conform more closely to a Gaussian field model, which is an important assumption of VBM, and thus increases the validity of parametric tests (Ashburner & Friston, 2000).

To account for the fact that each pre-processing step has the potential to introduce bias, corrections for multiple comparisons are made. For research designs involving group comparisons, the family-wise error (FWE) correction is proven to be reliable and particularly effective for reducing the rate of false positives (Scarpazza, Tognin, Frisciata, Sartori, & Mechelli, 2015). Chapter 3 provides further details of how each of these pre-processing stages were implemented for this study.

2.2.5 sMRI Data Analysis in this Thesis

The VBM MRI analysis for this thesis was conducted using Statistical Parametric Mapping version 12 (SPM12) software, in-line with standard recommendations (Kurth, Gaser, & Luders, 2015). This involved using the Computational Anatomy Toolbox (CAT12: (Gaser & Dahnke, 2016) and the Template O' Matic (TOM) toolbox (Wilke, Scott K Holland, Mekibib Altaye, & Christian Gaser, 2008) during pre-processing. For the normalization step, the TOM toolbox was used to create a customised tissue probability map based on the age and sex of the participants,

as recommended for VBM analysis in paediatric participant groups (Gaser & Dahnke, 2016; Wilke et al., 2008). Quality control was done using a combination of visual inspection of the scan images, and by using the CAT12 quality rating B- as a minimum cut-off criterion, so that scan images with a lower quality rating were excluded. The CAT12 image quality ratings are estimated using values from the tissue segmentation and are scaled using standardised parameters from the analysis of both synthetic and real MRI data, such as the Brain Web Phantom (BWP; Collins et al., 1998). Smoothing was completed using a 6mm full width at half maximum gaussian kernel and the images were normalised to Montreal Neurological Institute (MNI) space using a customised Diffeomorphic Anatomical Registration Through Exponentiated Lie Algebra (DARTEL (Ashburner, 2007)) template. The General Linear Modelling (GLM) function in SPM was then used to investigate the association between GMV and other variables of interest.

2.2.6 fMRI Pre-processing and Data Analysis in this Thesis

This thesis includes fMRI data from an emotional face processing task, similar to that previously used in experiments with both males (Passamonti et al., 2012) and females (Fairchild et al., 2014) with CD. While in the MRI scanner, participants were shown grey-scale images of human faces showing angry, sad or neutral expressions. These images included photographs of both male and female facial expressions and participants are asked to indicate the gender of the face by pressing a button. A face trial was comprised of a 1,000-millisecond presentation of the face, followed by presentation of a 750-millisecond null stimulus (a fixation cross). In a null trial the fixation cross was shown for 1,750 milliseconds. Each block included 5 images of faces and 5 fixation crosses. In total, a block lasted 17.5 seconds. The images were presented in 12 epochs of

each emotional expression (i.e. 60 angry, 60 sad and 60 neutral faces), thus the total duration of the task was 10 minutes 30 seconds. Figure 10 below shows a visual representation of this task.

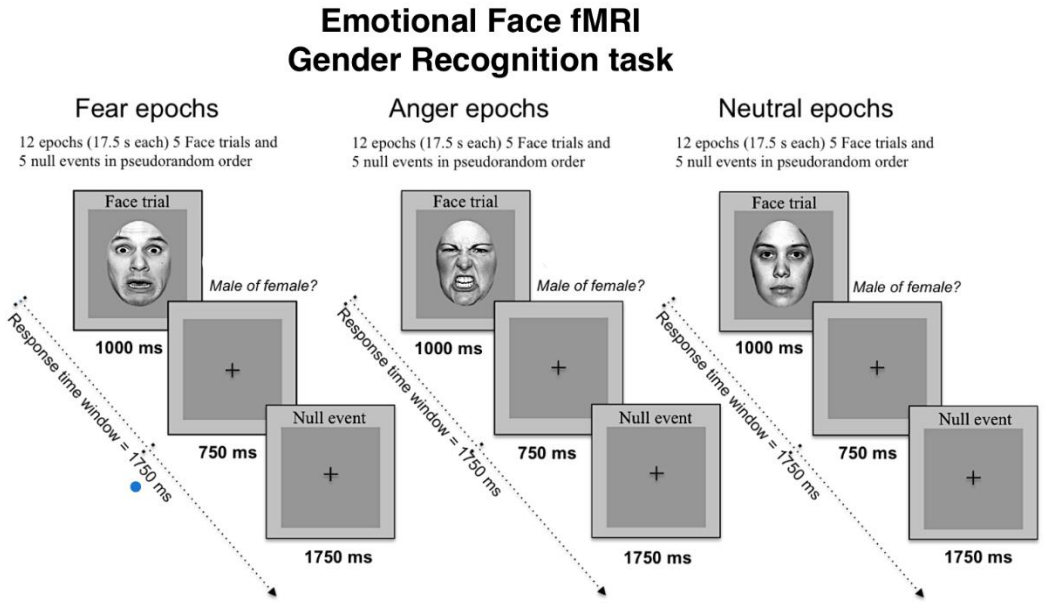


Figure 10. Emotional Face Processing Task based on Passamonti et al 2012 (Passamonti et al., 2012)

The fMRI data pre-processing for this thesis was done using the SPM12 (University college London, UK; http://www.fil.ion.ucl.ac.uk/spm/doc/manual.pdf) and ART (Neuroimaging Tools and Resources Collaboratory; https://www.nitric.org/projects/artificat_detect) packages. The main steps of the pre-processing were: (i) realignment (and unwarping) of the functional data with reference to the participants first scan image, (ii) co-registration of all scans to the participants T1- weighted 'reference' structural scan by rigid body transformations, (iii) image normalisation to standard space using a DARTEL template (Ashburner, 2007), generated with the TOM8 toolbox (Wilke et al., 2008), (iv) data smoothing using a 6mm Gaussian kernel, and, (v) motion correction using the ART toolbox. The ART toolbox includes a facility to identify individual regressors associated with motion and signal intensity, which can then be included in the design matrix for first-level analyses.

After data pre-processing, first-level (individual subject) analysis was conducted. This involved modelling the intensity at each voxel (using GLM), based on the elements included in the design matrix (Price & Friston, 2002). The design matrix was used to define a contrast vector between 2 different trial conditions from the task (i.e., emotional face vs. fixation or emotional face vs. different emotional face). The second-level analysis then involved GLM of voxel intensity for a given contrast across all participants.

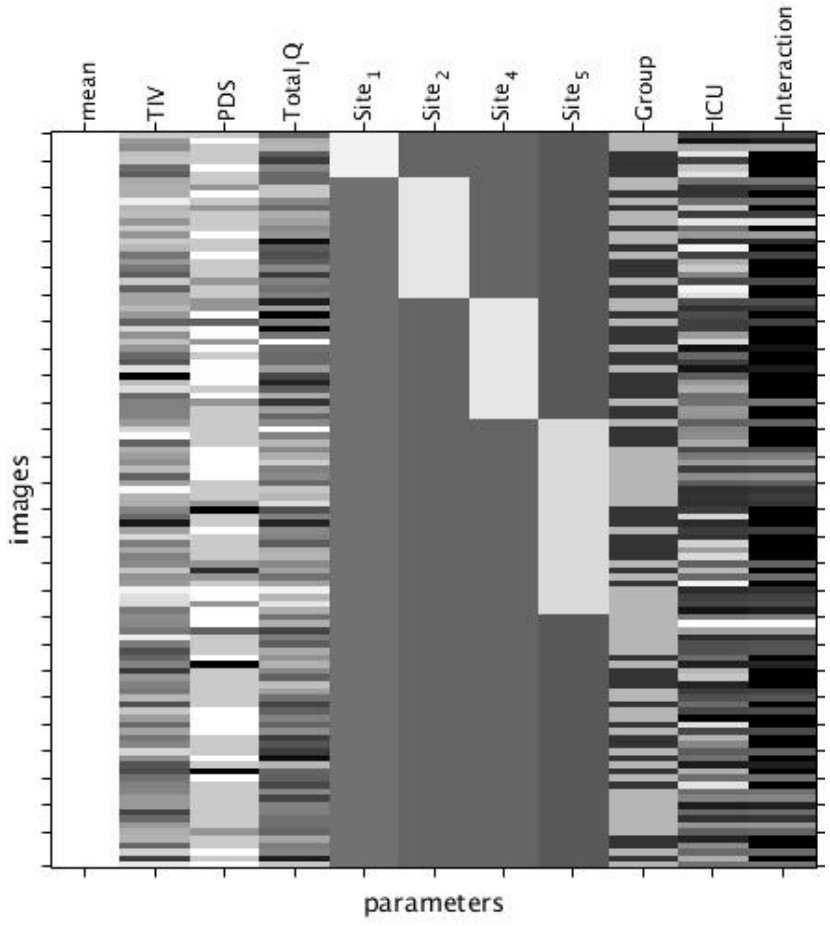


Figure 11. Design matrix for multivariate modelling for fMRI analysis in SPM. Mean BOLD signal difference between two contrasts is based on a linear model including covariates of total intracranial volume (TIV), PDS, Site (dummy coded). Variables of interest are (i) CD, (ii) CU, (iii) CDxCU interaction

2.3 Epigenetic Processing and Analysis

2.3.1. Overview

As mentioned in chapter 1, epigenetic modifications play an active role in regulating gene expression. As these epigenetic changes can occur in response to environmental stimuli, they represent a mechanism by which environmental factors may influence biological processes. One of the principle epigenetic modifications is DNA methylation. Genome-wide detection of methylation signatures via micro-array techniques and bisulphite sequencing allow researchers to map the methylome at a single-base level of resolution (Parle-Mcdermott & Harrison, 2011) and thus identify the detailed epigenetic profile of a DNA sample.

2.3.2 Experimental Approaches for Detecting DNA Methylation

The two main approaches for detecting methylation signals are via methylation array and sequencing methods (Barros-Silva, Marques, Henrique, & Jerónimo, 2018). Treating DNA with the chemical sodium bisulphite is the most commonly used method for distinguishing between methylated and unmethylated cytosine bases. The addition of sodium bisulphite converts the unmethylated cytosine to uracil, while methylated cytosine remains unchanged (Adusumalli, Mohd Omar, Soong, & Benoukraf, 2015; Barros-Silva et al., 2018). After this conversion, the uracil is converted to thymine via a polymerase chain reaction (PCR) process.

The Illumina Infinium Methylation Assays were used to extract the DNA methylation data reported in this thesis. These devices use bilsufite-converted genomic DNA to determine the cytosine methylation at specific cytosine-dense regions of the DNA molecule, known as CpG islands. The assay distinguishes the methylated from un-methylated loci using two site-specific probes (one designed for the methylated locus, the other for the unmethylated locus). These

probes are located on tiny silica beads across the Illumina chip. The overall level of methylation at a particular site can be computed from the ratio of signals from methylated (M): unmethylated (U) probes. The level of methylation can then be summarised by the beta value where:

$$\beta = \frac{\mathrm{Max}(M,0)}{\mathrm{Max}(M,0)+\mathrm{Max}(U,0)+100}$$

Figure 12. Beta Value Equation

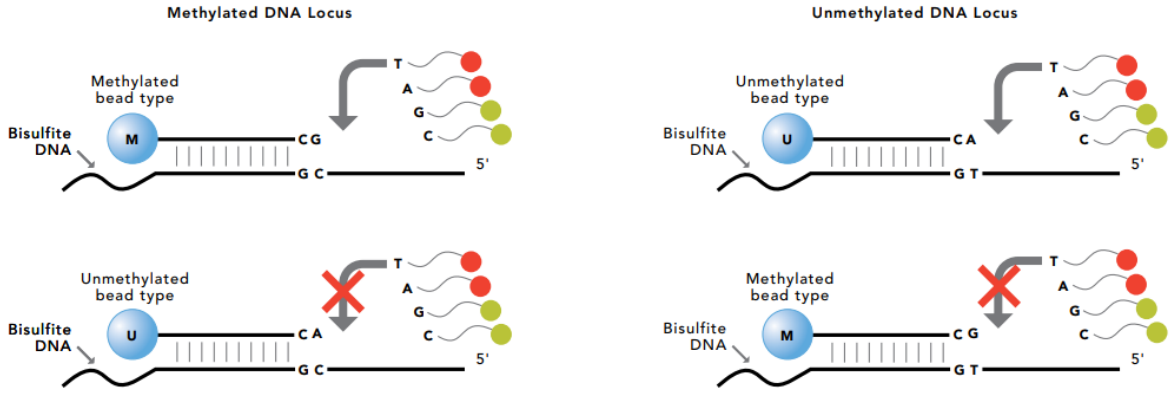


Figure 13. R/G Illumina BeadArray Methylation measurement (Illumina, 2009)

This can be performed at genome-wide level, or applied to a specific sub-region of DNA. Thus, the key strengths of this DNA methylation analysis approach are; (i) that it allows researchers to either target specific epigenetic regions of interest or profile methylation patterns across the whole genome at single-base resolution, and, (ii) it can be performed with low amounts of input DNA. However, a major limitation of this approach is that it provides poor coverage of regions of the genome which have low CpG density (Laird, 2010).

The two most commonly used Illumina Methylation arrays are the Infinium HumanMethylation450karray and the EPIC array. The EPIC array allows the analysis of over 850, 000 DNA methylation sites. Each site is associated with two measurements: a methylated measurement and an unmethylated measurement, in two different colour channels (red and green). Many different software packages have been developed for DNA methylation analysis, however the most widely and comprehensively tested pipelines for pre-processing and analysing methylation data use packages in the *Bioconductor* (Gentleman et al., 2004) suite (Xu et al., 2021), thus this software was used in the analysis in this thesis (see secion 2.3.3 for full details).

2.3.3. Epigenetic Analysis and Pre-processing in minfi

For the studies reported in this thesis, the *minfi* (Fortin, Triche, & Hansen, 2017) package from the *Bioconductor (*Gentleman et al., 2004*)* suite in R software (*version 3.6.0*) was used to analyse the methylation data from the Illumina EPIC array. A standard pre-processing pipeline was used, as shown in Figure 14 (Wilhelm-Benartzi et al., 2013).

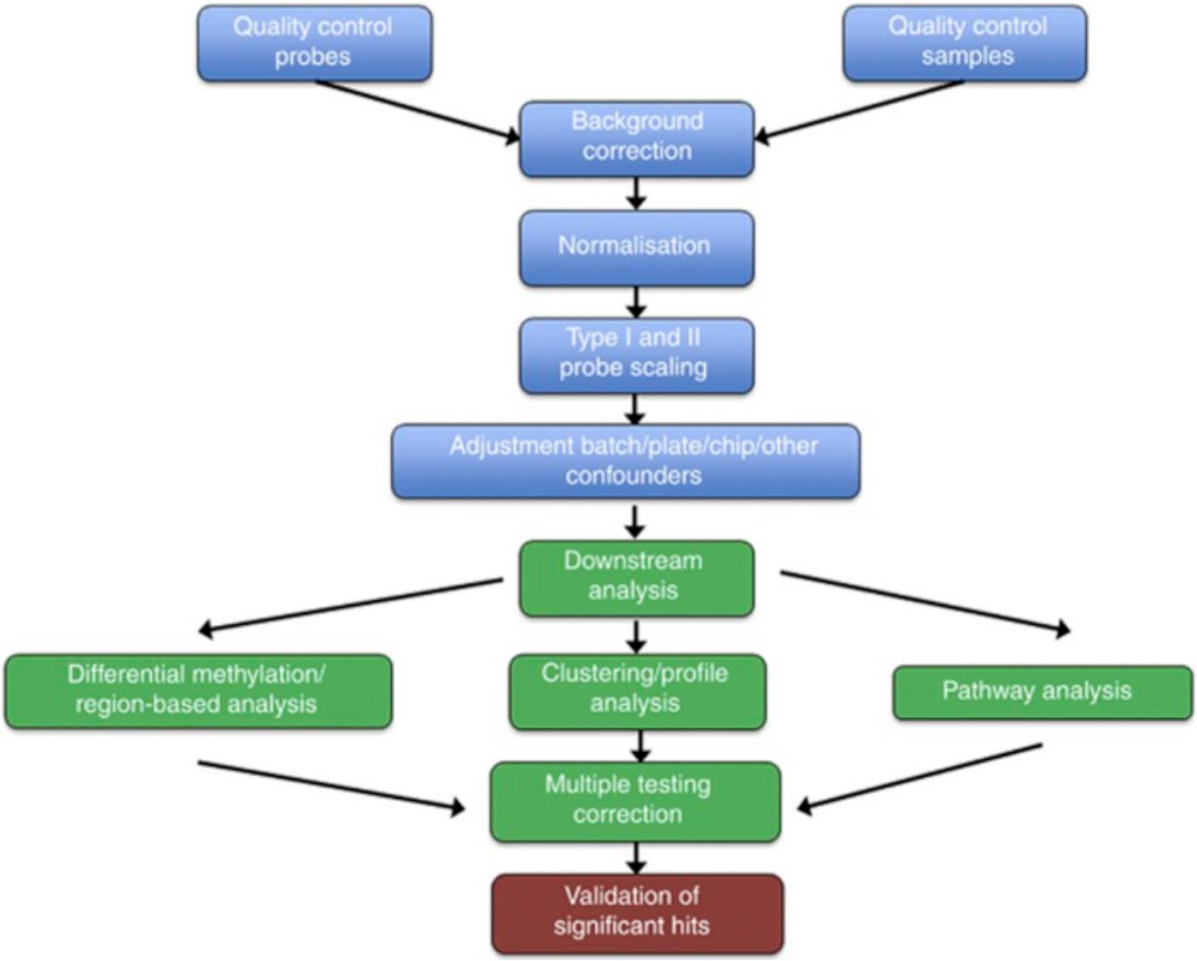


Figure 14. 8 Standard pipeline for pre-processing methylation data (Wilhelm-Benartzi et al, 2013)

This pre-processing involved reading in raw input data in the form of IDAT files and then several quality control steps to remove unreliable probes. This included the removal of failed/noisy probes (as recommended by (McCall & Almudevar, 2012)), removing probes in regions coinciding with the location of known SNPs with a data-base annotated minor allele frequency (MAF) > 10% and removing cross-reactive probes. Between-array normalisation was then performed to remove batch effects and probes were mapped to their genomic location using

the human reference genome hg19. Linear regression modelling was then used to model methylation in terms of; (i) CD case status, (ii) total ICU score and, (iii) the interaction effect between these factors (CD x CU). I computed both Beta values and M values for each locus, but as M values are considered more statistically suitable for methylation analysis (Du et al., 2010), I used only these values in the subsequent stages of analysis. The *Bumphunter* Algorithm was then used to compare methylation levels across multiple 'candidate regions' across the entire genome, to determine whether methylation differed according to (i) CD case status, (ii) total ICU score and, (iii) the CD x CU interaction.

The main functions used in the analysis are shown in Table 2 below, alongside a basic description of their use. The full R code for this analysis is included in Appendix A1 of this thesis.

Table 2: R functions used in methylation data pre-processing

FUNCTION	DESCRIPTION
READ.METHARRAY.SHEET()	Reads .idat files into R
DETECTIONP()	Identifies failed probes
PREPROCESSFUNNORM()	Performs functional normalisation
ADDSNPINFO()	Intersects probes with dbSNP

DROPLOCIWITHSNPS()	Removes probes in range of common SNPs
DROPXREACTIVELOCI()	Removes cross-reactive probes
GETSEX()	Estimate sex from each sample
GETM()	Compute M-values
GETANNNOTATION()	Returns requested annotation of each methylation loci
PDATA()	Extracts descriptive information for the samples
NUM.SV()	Calculates number of surrogate variables for model
LMFIT()	Fits a linear model to the data
EBAYES()	Computes statistics by empirical Bayes moderation
BUMPHUNTER()	Identifies 'Bumps' (DMRs) in genomic data

2.4 *OXTR* Genotyping (and SEM/path analysis)

2.4.1 Overview

This section provides a brief summary of the experimental steps involved in genotyping SNPs, an introduction to SEM, and how these two methods were combined in chapter 5 to investigate

genotype of the *OXTR* gene in relation to CD symptoms. Identifying genetic loci associated with increased risk of psychiatric disorders has the potential to shed light on the biological mechanisms underlying these disorders (Geschwind & Flint, 2015) and investigating SNPs of a gene is one of the most common methods for measuring genetic variation (Müller-Myhsok, 2005). SNP micro-arrays use known nucleotide sequences as probes to hybridize with DNA test samples, and they then measure the intensity of the output signals to provide information on the SNPs present in the test sample (LaFramboise, 2009). This genetic information can be incorporated into genomic SEM models to determine the degree of overlap between the SNP variation and symptoms of a particular psychopathology or phenotypic trait (Grotzinger et al., 2019).

2.4.2 *OXTR* Genotyping

In the past two decades, there have been rapid developments in the technologies which enable researchers to investigate genetic sequence variations (Durmaz et al., 2015). SNPs account for a high proportion of genetic variation and it is estimated that there are > 10 million SNPs across the human genome (Kruglyak & Nickerson, 2001; Lappalainen, Scott, Brandt, & Hall, 2019). Consequently, the creation of efficient, affordable methods for identifying SNPs has been an important focus of the field of molecular genetics over the past 20 years. Currently, SNP microarray platforms have the capacity to genotype hundreds of SNPs from thousands of individuals in a single assay (Array, 2007; Illumina, 2009).

The Illumina BeadArray consists of tiny silica beads coated with multiple copies of an oligonucleotide probe, assembled in microwells on a small planar chip (Illumina, 2009). The two-stage allele detection process consists firstly of hybridizing fragments of the test DNA to the

probes on the array (where each probe corresponds to a specific genomic location) and then synthetic (enzymatic) single-base extension, using a specific labelled nucleotide for assay readout. Figure 15 shows a diagram of the steps involved in genotyping using the Illumina BeadArray and Figure 16 shows how the stages are spread across 3 days in this process.

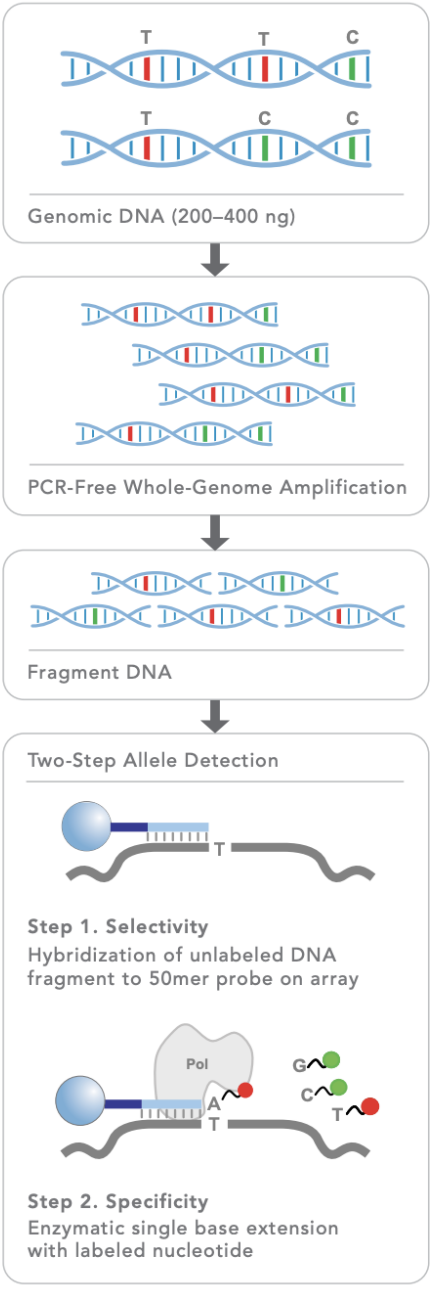


Figure 15. Illumina Infinium Assay Chemistry (Illumina, 2009)

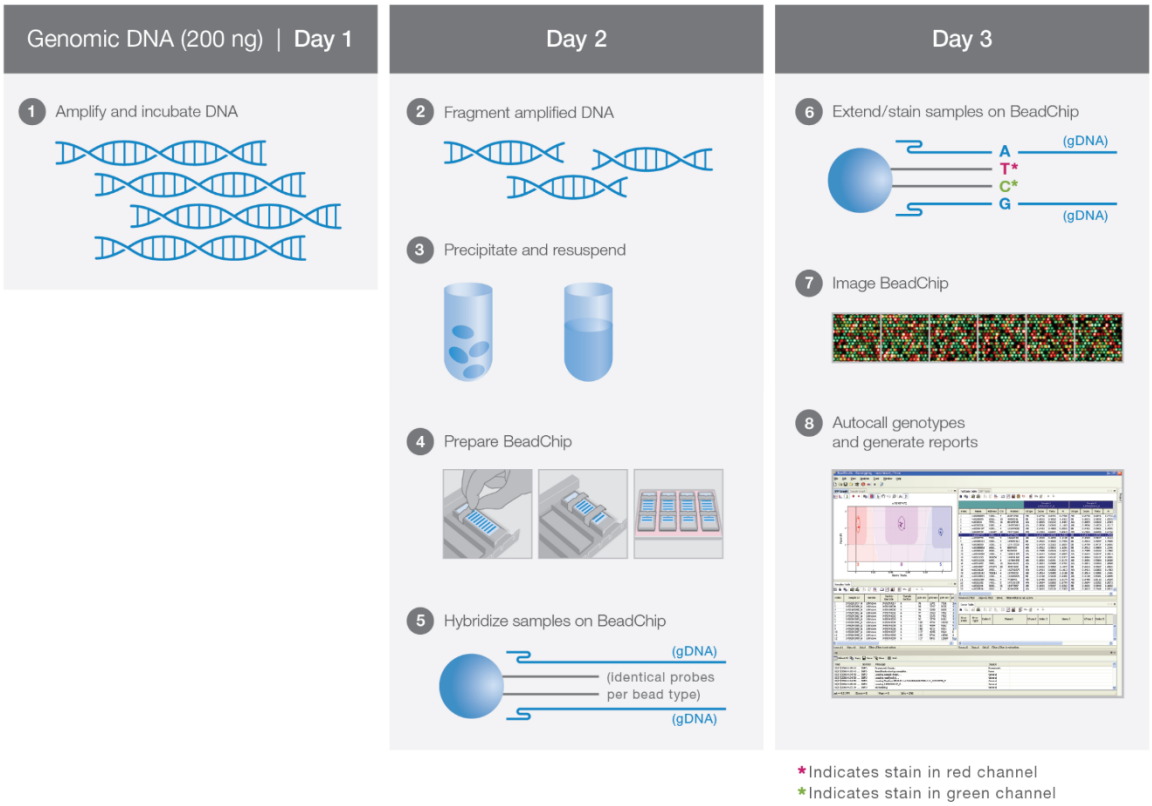


Figure 16. Illumina Infinium Workflow (Illumina, 2009)

2.4.3 SEM in Psychology/Psychiatry

SEM involves using mathematical equations to describe a series of cause-effect relationships between multiple variables in a composite model. The performance of this model can then be tested against a baseline-model to determine the goodness of fit of the proposed model in relation to some observed data (Shipley, 2000). SEM procedures combine principles from factor analysis, principal component analysis, regression, and path analysis (Raykov & Marcoulides, 2006). A major strength of the SEM approach in psychological research is that it can be used to combine several of these simpler data analysis methods in one single analysis, removing the need to conduct analyses in multiple steps (Kline, 2004). Thus, genomic SEM may allow researchers to

uncover the genetic basis of complex traits and how these factors influence the number and severity of psychiatric symptoms for many different disorders (Grotzinger et al., 2019).

”While there are several advantages to applying an SEM approach in psychological research, it is also important to note the limitations associated with this approach. Two of the main limitations of SEM are as follows. First, it is often challenging to determine the direction of associations between variables in the model, which can render the modelling inappropriate for making causal inferences. This means that using the SEM approach to identify risk factors associated with a particular psychiatric diagnosis can be challenging and inappropriate for identifying individual factors. Secondly, for each unique SEM model the power analysis can be complex and unreliable, as these calculations are known to be strongly affected by both item reliability and scale length for individual variables in the model (Wang and Remtulla., 2021). Therefore, determining sample sizes based on prior work in the field may be unreliable, even when the same model has been tested previously.

In order to account for these limitations, best practise guidelines for using SEM in psychological research include employing a conservative approach to sensitivity analyses and ensuring that the psychometric properties and construct validities of scale scores for all measured variables are well documented in scientific reporting (Morrison et al., 2017). Additionally, where data is not normally distributed, including certain correction factors (e.g. standard error estimates) and using estimation methods such as the unweighted least squares technique (rather than the more commonly employed maximum likelihood method for parameter estimation) can minimise the issues associated with small sample sizes and weak empirical relationships between variables in SEM (Werner and Schermelleh-Engle., 2010).”

2.4.4. Modelling *OXTR* Genotype, CU score and CD symptoms using SEM (chapter 5)

In the study reported in Chapter 5, DNA from saliva samples was used and extracted within 7 days of collection using the Oragene OG-500 Kit. DNA quality cut-off was a 260/280 ratio above 1.8. DNA was stored at -80°C immediately. Genotyping and genomic imputation was conducted using the Illumina Infinium Global Screening Array V3.0 + PsychChip kit by the Department of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy at Goethe University, Frankfurt as previously published (Yousaf et al., 2020). This included rigorous data-cleaning procedures and imputation of missing genotypes, detection/confirmation of gender and chromosomal anomalies, relatedness and population structure (ancestry), detection of batch effects, Mendelian error detection and duplication error detection. Quality thresholds are published elsewhere (Yousaf et al., 2020). SNPs within +/- 2kb of the *OXTR* gene were exported. Analysis of linkage disequilibrium was also computed using LDlink (Machiela & Chanock, 2015). The *semPlot* and *lavaan* packages in R (*version 3.6.0*) were used for SEM fitting and analysis. A full path diagram of all variables included in the SEM is shown in Figure 17.

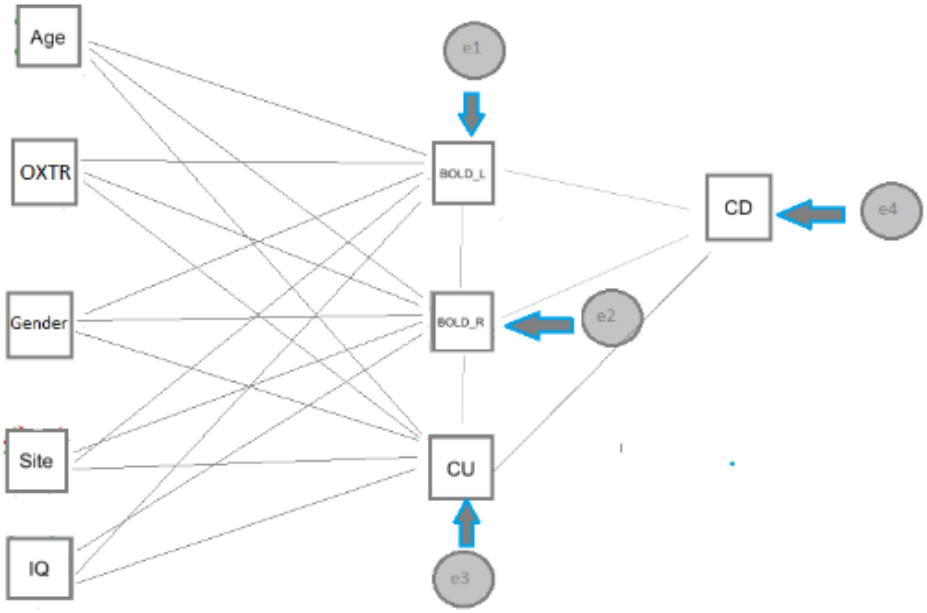


Figure 17. Full Path Diagram for Structural Equation Modelling of CD Symptom Variation (Chapter 5)

Linear regressions were used to model the associations between CD symptom score, ICU score and amygdala response. The model also included an expression term that accounted for the covariance between CU traits and BOLD response and a term representing the covariance between contrasts across the left and right amygdalae. The SEM also included parameter estimates for the residual variance of all endogenous variables (i.e., CD symptom score, CU-trait score and BOLD response in the amygdala).

As recommended (Hu & Bentler, 1999), two statistical measures were used to describe the effectiveness of each model. These were the comparative fix index (CFI), and the standardized root mean square error approximation (RMSEA). The CFI compares the fit of the hypothesized model to a "worst fitting" model (also known as the 'baseline model'). RMSEA is an absolute fix index and so gives an indication of the difference between the hypothesized model and a

perfect model. I used the recommended values (Hu & Bentler, 1999) of RMSEA <.06 and CFI >.95 to indicate a reasonable model-data fit.

2.5 The FemNAT-CD Project

2.5.1 Overview and Aims

The data analysed for this thesis were collected during the FemNAT-CD study ((Freitag et al., 2018) see www.femnat-cd.eu). This project aimed to investigate the central role of emotion processing in the neurobiology and treatment of adolescent female CD, and was a collaboration between 17 sites across eight European countries (Germany, Greece, Hungary, Ireland, the Netherlands, Spain, Switzerland and the United Kingdom). Participant recruitment and data collection were completed between January 2014 and February 2018. In total 1827 participants were involved, including 880 youth with CD (61% female) and 947 TD youth (65% female). Both cross-sectional and longitudinal data collection was completed including clinical interviews, self-report and carer/parent-report questionnaires, cognitive tasks, neurophysiological assessments, genetic and epigenetic measures (via saliva and blood DNA sample collection) and structural and functional MRI acquisition. The following section of this thesis provides detailed information about the recruitment, testing process, and data collection for those measures used within analysis for the three reported studies in this thesis.

2.5.2 Recruitment Process

Data collection was carried out at 12 of the European sites; Goethe Universität Frankfurt, Uniklinikum Aachen, Stichling VU-VUMC, Universität Heidelberg, Universität Basel, the University of Southampton, the University of Birmingham, Trinity College Dublin, Fundacio Mutua De Terrassa Per A La Docencia (Recerca Biomedica, Social Fundacio Privada Catalana),

Fundacion Vasca De Innovacion E Investigacion Sanitarias, Szegedim Tudomanyegyetem and the National and Kapodistrian University of Athens. Participants were recruited from a range of sources including youth offending and mental health support services, local clinics, special education and mainstream schools and youth programmes and public engagement (outreach) events.

2.5.3 Eligibility and Exclusion Criteria

Data were collected from both male and female participants aged between 9-18 years. Inclusion criteria were that participants had a total IQ ≥ 70 and that they had no current or past indications of genetic syndromes, neurological disorders, or traumatic brain injury. Additionally, participants could not have a present or past diagnosis of ASD or schizophrenia and could not have a sibling already taking part in the study. For participants recruited to the TD group it was also required that they did not currently meet the minimum symptom threshold for any DSM-5 psychiatric disorders and that they did not have a history of being diagnosed with any externalising disorder, bipolar disorder or mania. 'Case' participants were required to meet one of the following three criteria; (i) aged 13 or over and meeting diagnostic criteria for ODD with two or more co-morbid CD symptoms, (ii) aged 9-12 and meeting the diagnostic criteria for ODD with at least one CD symptom present, or, (iii) meeting the DSM-5 CD criteria. Finally, all participants were required to attend the first experimental session with a parent or carer-giver who was also willing to partake in the study.

2.5.4. Informed Consent and Data Collection Procedure

Prior to any data collection, all participants underwent initial screening to confirm that they met the study's inclusion criteria *(see above*) and to ensure that there were no contraindications to

them taking part in MRI scanning (e.g. metal in the body). Participants gave written consent to partake in the study and for participant under the age of 16 (at UK sites) parents/care-givers consent was also obtained (parental consent was obtained for all participants at German sites). All data collection was performed within six months of obtaining participants' informed consent.

The study consisted of two sessions. In the first session semi-structured Kiddie Schedule for Affective Disorders and Schizophrenia – Present and Lifetime Version (KSADS-PL; (Kaufman, Birmaher, Brent, & Rao, 1997)) diagnostic interviews were conducted to assess for CD and other psychiatric symptoms. These interviews were conducted individually- with participants and their parents/caregivers being interviewed separately. The first session also involved participants completing an IQ test questionnaire, using the Wechsler Abbreviated Scale of Intelligence (WASI; (Wechsler, 1999) or the Wechsler Intelligence Scale for Children, Fourth Edition (WISC-IV; Wechsler, 2003) at UK/German sites respectively, and additional questionnaires (measuring levels of CU traits, pubertal development status and other health information). Participant also underwent neuropsychological tests and psychophysiological measures. Finally, participants gave saliva DNA samples and completed the MRI safety screening form. The total duration of this session was 3-4 hours.

The second session was predominantly MRI data collection. A T1-weighted structural scan and data from various fMRI tasks were collected, but for this thesis only the structural scan and emotional face processing task are relevant. Participants were also given the opportunity to complete any questionnaires that they had omitted in session 1. The average duration of this session was 1.5-2 hours.

2.5.5 Clinical/Behavioural Data Assessments

The following assessment instruments were used to collect clinical and demographic information:

Kiddie Schedule for Affective Disorders and Schizophrenia – Present and Lifetime Version (K-SADS-PL)

Current and history of psychiatric disorders (affective, psychotic, anxiety, behavioural, substance abuse, and other psychiatric disorders) were assessed by separate semi-structured diagnostic interviews using The Schedule for Affective Disorders and Schizophrenia for school-age children - Present and Lifetime version (K-SADS-PL) with participants and their parents and / or caretaker(s) who were interviewed separately. All interviews were conducted by trained masters- and doctoral-level staff. Interrater reliability (Cohen's $\kappa = .91$) and rater agreement (95%) of current CD symptoms were high. For current and past ADHD, oppositional defiant disorder (ODD), depressive and posttraumatic stress disorder, interrater reliabilities (Cohen's $\kappa = .50$ - .95) and rater agreements (92 - 95%) were moderate to high (Kaufman et al., 1997).

Inventory of Callous-Unemotional Traits (ICU), Parent-Report Version

The ICU (Essau, Sasagawa, & Frick, 2006) was used to assess participants' levels of CU traits through parent/carer reporting. This measure is a 24-item questionnaire, with 8 items belonging to each of the three subscales (callous, uncaring and unemotional). Parents/Carers were asked to indicate on a 4-point scale whether or not each of the items was applicable the participant. A score of 0 indicated that they considered the statement in the item was '*not at all true*', whereas a score of 3 indicated that it was '*definitely true'.*

Wechsler Intelligence Scale (WASI, WAIS, WISC)

At the English-speaking data collection sites, the vocabulary and matrix reasoning tests from the WASI-I (Wechsler, 1999) were used to estimate IQ. The remaining sites used the vocabulary, block design and matrix reasoning subscales of the WISC (for participants <17 years old; Wechsler, 2003) or the WAIS (for participants who aged 17-18 years; Wechsler, 2011).

Pubertal Development Scale (PDS)

Pubertal status was assessed via self-report using the Pubertal Development Scale (PDS) (Petersen, Crockett, Richards, & Boxer, 1988) asking about pubertal growth (e.g. changes in body hair, voice or breast development) with four response options (not yet started, barely started, definitely started, seems complete) resulting in a 5-level categorical scale (0 = pre-pubertal, 1 = early-pubertal, 2 = mid-pubertal, 3 = late-pubertal, 4 = post-pubertal).

Semi-structured Psychosocial and Medical History.

Psychosocial and medical risk factors were assessed using the Medical History, a semi-structured clinical interview conducted by trained masters- and doctoral-level staff designed to assess current and past environmental influences and exposure to risk factors of children and adolescents. Parents and/or caregivers were interviewed regarding; 1. Pregnancy and birth history, 2. Specific risk factors during early development, 3. Developmental milestones, 4. Nursery and kindergarten, 5. School career, 6. Chronic medical problems, 7. Parental education status, 8. Information about the family, such as single parenting, and 9. Psychiatric disorders in family.

Socio-economic status.

SES was assessed based on parental income, education and occupation. Assessments were based on the International Standard Classification of Occupations (González-Galarzo & García, 2012) and the International Classification of Education (ISCED; UNESCO Institute for Statistics, 2015). Human rater and computer-based ratings were combined into a factor score using Principal Component Analysis (PCA). A clear one-dimensional structure underlying the different measures could be corroborated using Confirmatory Factor Analysis (CFI=.995; RMSEA=0.035). Reliability (internal consistency) of the composite SES score was acceptable (Cronbach's Alpha =.74). The validity of the SES score could be verified considering significant correlations with external criteria (e.g., cognitive abilities [r=.33,p<.01], community violence [r=-.28, p<.01]). Due to potential economic variation on the country level, SES was centred and scaled within each country, in order to obtain an indicator of relative socioeconomic position.

2.5.6 Site Qualification Procedures

To ensure inter-site consistency, clinical assessment inter-rater reliability (IRR) assessments were completed for eight K-SADS-PL interviews (five CD subjects and three healthy controls). Either diagnostic interviews were filmed and coded by members from different sites or a second rater attended the diagnostic interview. Additionally, when obtaining questionnaire data all sites involved in the FemNAT-CD project confirmed they had used authorized translations of the PDS and YPI.

All functional and structural MRI scans were acquired using Siemens 3T (Frankfurt and Southampton: Tim Trio; Aachen: Prisma) or Philips 3T (Birmingham: Achieva) scanners, using a 20- or 32-channel head coil. To ensure consistency of MRI scanning parameters between sites, image acquisition sequences and experimental protocols were standardized. Prior to data

collection, each site also underwent a qualification procedure which including scanning with phantoms and healthy volunteers not included in study. The three stages of this process were: (i) scanning an American College of Radiology (ACR; Chen, Wan, Wai, & Liu, 2004) phantom; (ii) completing a scan of a functional Biomedical Informatics Research Network (FBIRN; (Glover et al., 2012) phantom; and (iii) scanning a human volunteer. Once collected, the data were reviewed by an MRI physicist at the University of Birmingham. To make the scanning procedures comparable between sites, each site adjusted their scanning parameters according to the physicist's recommendations.

2.5.7 Ethical approval

All experimental protocols employed during the FEMNAT-CD study were conducted in-line with the current legal regulations of the European Union, national legislation, and the Declaration of Helsinki. All procedures were approved by the relevant national ethical committees for each experimental site prior to commencement of data collection. The ethical committees from which approval was obtained are as follows; the Ethics Committee of the RWTH Aachen University Hospital (EK027/14) for the Aachen site, the medical faculty of Goethe University Frankfurt for the Frankfurt site, the NHS Research Ethics Committee for Birmingham and Southampton sites (NRES Committee West Midlands, Edgbaston; REC reference 13/WM/0483).

2.5.8 Imputation of Missing Data

The Institute of Medical Biometry and Statistics (IMBI) was a member of the FemNAT-CD consortium, and statisticians from this institute performed imputation of missing data for all

measures. The text below is provided by the IMBI to explain how data were imputed. It is a standard text approved for use in all FemNAT-CD publications. Please note that the procedure for PDS imputation is described separately, as this was performed before a decision was made to impute data for other measures.

Missing values of the PDS score were imputed based on the whole FemNAT-CD sample. It has been shown that missing data for a multi-item instrument is best treated by item-level imputation (Eekhout et al., 2014). Thus, missing values of the separate items were imputed first and then the total scores were calculated based on these imputed items. The imputation was done in SAS® version 9.4 using the PROC MI procedure. Imputation by fully conditional specification (FCS) was used - this offers a flexible method to specify the multivariate imputation model for arbitrary missing patterns including both categorical and continuous variables (Liu & De, 2015). As the items are measured at an ordinal level, the logistic regression method is specified in the FCS statement. For imputation diagnostics, distribution of the observed and imputed items and scores were checked. The imputation of the PDS items was done separately in males and in females as the measure included a number of sex specific items: item 2 (females and males) and items 4, 5a of the form for females or items 4, 5 of the form for males were imputed respectively. The following variables were included in the imputation model: sex specific items of the PDS as mentioned above and the two remaining PDS items (items 1 and 3), age at PDS and age at informed consent (to impute age at PDS if missing), weight, case/control status, site, and migration status.

Imputation for all other measures was conducted separately, using the same procedure as described above. The following variables were included in the imputation model: all items of the respective questionnaire, age, IQ, group (case/control), sex (male/female), site, comorbid

diagnoses (PTSD, ADHD, ODD, depression, anxiety), and items of other questionnaires if correlated with at least one of the items with ≥0.4. For imputation diagnostics, distribution of the observed and imputed items and scores were checked.

CHAPTER 3-*SLC25A24* GENE METHYLATION AND GRAY MATTER VOLUME IN FEMALES WITH AND WITHOUT CONDUCT DISORDER: AN EXPLORATORY EPIGENETIC NEUROIMAGING STUDY

3.1 Introduction

3.1.1 Introduction to CD and CU traits

CD is a psychiatric disorder of childhood and adolescence characterized by persistent antisocial behaviours (i.e., violence towards others or animals, destruction of property, theft, and serious rule violations), which significantly impact the individual's social, academic, or occupational functioning (APA, 2013). There is considerable variation in the possible combinations of symptoms that could lead to a CD diagnosis (Nock, Kazdin, Hiripi, & Kessler, 2006). Therefore, to identify more homogeneous subgroups of youth with CD, several subtyping approaches are included within the DSM-V (APA, 2013). One approach focuses on the LPE specifier, which indexes CU traits (i.e., reduced empathy, callousness, a lack of guilt, and shallow effect). This specifier designates a particularly impaired subgroup of youth with CD who are at increased risk of developing psychopathy in adulthood (Frick, Ray, Thornton, & Kahn, 2014; Viding & Eamon J McCrory, 2018). Levels of CU traits show moderate stability from adolescence to adulthood (Loney, Huntenburg, Counts-Allan, & Schmeelk, 2007) and are also a predictor of more severe antisocial and aggressive behaviours both in adolescence and adulthood (Marcus, 2017). In this context, understanding the aetiology of these CU traits in adolescents with CD is an important step towards identifying risk factors for a subgroup of youth with CD who are particularly susceptible to poorer outcomes in adulthood (Walters, 2020).

Research shows that both genetic and environmental risk factors are implicated in the development of conduct problems or CD (Polderman et al., 2015; Salvatore & Dick, 2018), with

around 50% of the variance in CD risk attributable to heritable genetic influences (Salvatore & Dick, 2018). Crucially, twin studies indicate that youth with CD symptomatology and high versus low levels of CU traits are characterized by different environmental and genetic risk vulnerabilities (Viding & McCrory, 2018). Indeed, Viding et al. (2005) demonstrated that antisocial behaviour in youth with CD symptomatology and high levels of CU traits is highly heritable (0.76), whereas in youth with CD symptomatology and low levels of CU traits it is moderately heritable (0.64) and more influenced by environmental factors (Viding et al., 2005). Along with CU traits, sex is an important factor to consider in youth with CD in relation to genetic vulnerability for this disorder. Indeed, heritability estimates for antisocial behaviour in youth with CD are higher in males than females (Caspi, Taylor, Moffitt, & Plomin, 2000). Furthermore, in males with CD and high levels of CU traits, heritable factors explain a high proportion of the variance in antisocial behaviour (Viding et al., 2005). Conversely, antisocial behaviour in females with conduct problems (CP) and high levels of CU traits was shown to be entirely explained by environmental factors in one study (Fontaine, Rijsdijk, McCrory, & Viding, 2010). These data suggest sex differences in the biological mechanisms underlying antisocial behaviour in youth with CD depending on their levels of CU traits.

3.1.2 Gene–environment interplay in CD development

A key question in CD research is how genetic and environmental risk factors interact at the molecular level in relation to CU trait phenotypes (Fairchild et al., 2019). One candidate mechanism is via epigenetic changes in the form of DNA methylation, which involves addition of a methyl group at a specific genomic location (Anastasiadi, Esteve-Codina, & Piferrer, 2018). Depending on the pattern, location, and level of methylation within or proximal to the gene's coding sequence, gene expression may be suppressed or amplified (Anastasiadi et al., 2018). The

genetic variation of an individual is also an important factor to consider in understanding how environmental factors are translated into methylation signatures. Recent research has highlighted that individual differences in heritable factors may influence methylation signatures (van Dongen et al., 2018) and thus gene regulation. These genetic variants that can affect DNA methylation are known as methylation quantitative trait loci (mQTLs) and may be further useful markers for genetic influence on gene regulation (Barker et al., 2018).

Altered regulation of genes expressed in brain tissues and/or implicated in behaviour, may explain how methylation levels mechanistically mediate environmental influences, e.g. adverse life experiences to subsequent risk for CD (Veroude et al., 2016) and CU traits (Henry, Pingault, Boivin, Rijsdijk, & Viding, 2016). A recent study suggests that exposure to adverse prenatal environmental factors has a large effect on the brain epigenome, and that epigenetic effects associated with brain development are also sex-specific (Mattern et al., 2019).

Epigenetic studies of youth with CD or sub-clinical CP have provided initial evidence that DNA methylation patterns may mediate environmental factors associated with antisocial behaviour (Chiocchetti, in preparation; Gescher et al., 2018). In males with CD, methylation of the *OXTR* gene correlates positively with CU traits (Dadds et al., 2014). Similarly, in a mixed-sex study, higher methylation of *OXTR* at birth was associated with higher CU traits in adolescence for participants with low levels of anxiety (Cecil et al., 2014). Alterations in the expression of genes that govern the oxytocin system, as a result of epigenetic modifications, may thus play an important biological role in the development of CD and CU traits (Cecil et al., 2014; Dadds et al., 2014). A recent small-scale epigenetic neuroimaging study on males with CD showed that *OXTR* methylation and levels of CU traits interacted to predict frontoparietal hyperactivity and weaker amygdalo-frontoparietal connectivity in males during a face processing

task (Aghajani et al., 2018). This is consistent with previous reports of abnormalities in this circuitry in CD (e.g., (Gillespie et al., 2017)) and the fact that *OXTR* is highly expressed in both limbic and cortical brain tissues (Quintana et al., 2019). Interestingly, a fundamentally opposite association between brain functional connectivity and level of CU traits was observed in CD as compared to TD youth (Aghajani et al., 2018).

3.1.3 Study aims

To expand current knowledge on epigenetics in CD and limited research on females with CD, I adopted an exploratory approach and conducted the first EWAS with salivary DNA data on females with CD and varying levels of CU traits. As previous research in psychiatric disorders has demonstrated differential methylation according to diagnostic status (Lin et al., 2018) and level of CU traits (Dadds et al., 2014; Moul, Dobson-Stone, Brennan, Hawes, & Dadds, 2013), I first examined the main effects of CD diagnostic status and level of CU traits. Secondly, studies from the FemNAT-CD group (Raschle et al., 2018) and others (Aghajani et al., 2018) have demonstrated an inverse association between biomarkers and the level of CU traits in clinical groups as compared to TD populations. Thus, I investigated whether there was a CDxCU traits interaction effect on DNA methylation. The relationship between CU traits and methylation level has been demonstrated in individuals with CD (Cecil et al., 2014; Dadds et al., 2014) but the nature and direction of this relationship in TD youth is unknown. Finally, to investigate whether these methylation changes co-incidence with altered brain development, I related the methylation data to GMV as measured using voxel-based morphometry (VBM).

3.2 Methods and Materials

3.2.1 Participants

Fifty-one females with CD (mean age = 14.9, SD = 1.7) and 59 TD females (mean age = 14.7, SD = 2.4), recruited across five sites, were included as a subsample of the FemNAT-CD study (Freitag et al., 2018) (see Appendix Table A2 for details). This study was conducted according to the legal regulations outlined by the European Union, national legislation, and the Declaration of Helsinki. For each site, written informed consent was obtained from all participants and their parents, in accordance with the site-specific ethical requirements. In addition to standard FemNAT-CD inclusion and exclusion criteria (see Supplementary materials), participants were required to be non-smokers, be medication-free, and have good quality saliva-DNA and structural MRI data. Participants were included in the CD group if they either; (a) met the DSM-5 criteria for a diagnosis of CD; (b) were 9–12 years old, met the criteria for a diagnosis of ODD and also had at least one current symptom of CD; or (c) were aged >12 years, met the criteria for ODD and also had at least 2 current CD symptoms. All TD participants had no diagnosable psychiatric disorders and no history of externalizing disorders (ADHD, ODD). The participants were aged 9–18 years and groups were matched on PDS, performance IQ, ethnicity, and data-collection site (Table 3).

Table 3. Demographic and Clinical Characteristics of the participants (n=110)

Demographic	CD (n=51)		TD (n=59)		*P (t-test)*	Wilcoxon's *p*
	M	*SD*	*M*	*SD*		
Age	14.9	1.73	14.7	*2.38*	0.67	0.961
PDS	3.98	1.05	4.07	0.98	0.651	0.692
Total IQ	94.7	12.2	100.05	10.2	0.013	0.007
Perf. IQ	93.7	14.8	98.83	12.7	0.062	0.091
Verbal IQ	93.6	19.2	101	12.9	0.023	0.004
Clinical						
ADHD Symptoms	0.22	0.42	0.14	0.34	0.24	0.28
GAD Symptoms	0.24	0.55	0	0.29	0.008	0.004
MDD Symptoms	0.5	0.7	0	0	<.001	<.001
ICU total	29.6	11.4	17.6	9.02	<.001	<.001
ICU callous	*10.2*	*5.38*	*4.65*	*3.8*	<.001	<.001
ICU uncaring	*13.1*	*5.27*	*8.37*	*4.54*	<.001	<.001
ICU unemotional	*6.31*	*3.59*	*4.93*	*2.74*	<.001	0.04

3.2.2 Clinical and Psychometric Measures

Detailed information about these measures is provided in previous work (Rogers et al., 2019). Briefly, trained staff interviewed the participants and their parents (or caregivers) separately using the Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime version (K-SADS-PL (Kaufman et al., 1997)) to assess for CD and other DSM-IV-TR psychiatric disorders. Supplementary questions from the K-SADS-PL (e.g. for ODD/ ADHD) were completed if key items were endorsed during the initial screening. CU traits were assessed using the parent-version ICU (Frick, Cornell, Barry, Bodin, & Dane, 2003). Total, verbal and performance IQ were assessed using the Wechsler Abbreviated Scale of Intelligence (Wechsler, 2011) in the UK and the Wechsler Intelligence Scale for Children, Fifth Edition (Wechsler, 2014) at other sites. Pubertal status was determined using the PDS (Petersen, Crockett, Richards, & Boxer, 1988) completed by the participants (if aged >12 years) or by the parents/caregivers (for participants ≤ 12 years).

3.2.3 Genome-wide Methylation Data Pre-processing

DNA was extracted from saliva within 7 days of collection using the Oragene OG-500 Kit. DNA quality cutoff was a 260/280 ratio above 1.8. DNA was stored at −80 °C immediately. Genome-wide methylation was measured using the Illumina Infinium HumanMethylationEPIC BeadChip Array at Life & Brain GmbH, Bonn, Germany. Pre-processing was performed in R *version 3.6.0* (Team R, 2019). Raw .idat files were pre-processed with the minfi (Aryee et al., 2014) package (version1.32.0) following standard parameter settings (see Appendix A1 for full pre-processing R script). I removed failed and noisy probes as suggested (McCall & Almudevar, 2012), and also

probes spanning an SNP with an SNP147 database annotated MAF > 10%[1]. Finally, cross-reactive probes were eliminated. Between-array normalization was completed using the *preprocessFunnorm()* function (Fortin et al., 2014) included in the minfi package, following standard recommendations. This unsupervised method uses control probes to identify unwanted variation. It then extends the idea of quantile normalization to regresses out components of variation captured by these control probes (Fortin et al., 2014). This has been shown to be an effective method for removing positional effects (Jiao et al., 2018). I used ANOVA testing in the normalized methylation data to ensure there were no residual batch effects. As an additional check, I also extracted the first principal component of the methylation data and performed pairwise T-tests (with Tukey's correction for multiple testing) across the batches to confirm there were no correlations between the batch IDs and M values.

Heat maps and hierarchical clustering plots based on the Euclidean distance of the top 2000 loci selected by variance in methylation were generated to visually check for outliers and batch effects (Appendix A3). The methylation M-values were calculated based on the log-transformed ratio of methylated to unmethylated signal-intensities for each locus in line with previous research (Wilke, Holland, Altaye, & Gaser, 2008) and I ensured these M values were normally distributed across the differentially methylated region (Appendix A4). Probes were mapped to their genomic region using the human reference genome hg19.

[1] When genotype is not being included in the DNA methylation analysis it is important to remove probes where common SNPs may affect the measured value of methylation. This is because the functional relevance of the values which are obtained cannot be adequately determined without also knowing the precise details of the genetic variant which has undergone methylation. This stage of pre-processing was completed in-line with standard recommendation to drop the probes that contain either a SNP at the CpG interrogation or at the single nucleotide extension (Fortin and Hansen, 2014)."

3.2.4 MRI Acquisition

T1-weighted structural scans were collected at five research sites using MRI scanners all operating with 3 T fields (either Siemens or Philips manufactured) and harmonized acquisition sequences (see refs. (Raschle et al., 2018; Rogers et al., 2019) and Supplementary materials).

3.2.5 Pre-processing of the Neuroimaging Data

Consistent with previous work (Raschle et al., 2018), SPM12 (www.fil.ion.ucl.ac.uk/spm), Computational Anatomy 12 (CAT-12: http://dbm.neuro.uni-jena.de/cat/) and template-o-matic (Wilke et al., 2008)) toolboxes were used to pre-process MRI data (see Supplementary materials).

3.2.5 Genome-wide Methylation Statistical Analysis

To examine the associations between CD diagnostic status, level of CU traits and genome-wide methylation, I employed linear regression modelling: M-values for each CpG site was modelled as a function of CD status, CU traits (total ICU score), and the CDxCU traits interaction effect. Corrections for the effects of age and hormonal contraceptive use were included in the model. SES was not included as a covariate in the DNA methylation analysis on statistical and conceptual grounds. From a statistical point of view, when using a group design where participants are not randomly allocated to the groups (here CD vs TD), one cannot control or covary out the influence of a third variable (here SES) that might relate to group status. There is indeed agreement in the field of statistics that this approach renders interpretation of results problematic (Miller and Chapman, 2001). From a conceptual point of view, nuisance variables such as SES may be critical elements in a complex causal chain contributing to CD. Indeed, we

know that CD is associated with lower SES. Evidence also shows that low SES is associated with increased stress in children (Lupien et al., 2001), which in turn may increase the risk of CD in predisposed persons. In this case, SES is part of the complex causal chain leading to CD. By controlling for this variable, we would have removed one or more of the factors contributing to CD and dampen/wiped out any potential influence of CD on our results."

To identify components of extraneous variation due to unmodelled or unknown latent variables, surrogate variable analysis in R (sva package, "leek" method selected) was performed and the two factors identified were included in the final model as covariates. The effect sizes and p-value of each predictor (CD-case status, CU-trait levels and CDxCU) were calculated using the suggested Bayesian approach as implemented in the minfi ebayes function. P-values were then submitted to the Bumphunter algorithm (Jaffe et al., 2012) to identify differentially methylated regions (DMRs). I specified different coefficients from the linear regression modelling in the arguments of the Bumphunter function to test separately for: (i) the main effect of CD diagnosis, (ii) the main effect of CU score, and (iii) a CD × CU interaction effect on methylation, while controlling for the main effects of the other two factors. QQ plots were generated to confirm appropriate model fits for each EWAS model (see Appendix A5). Correction for multiple testing using the false discovery rate (FDR (Genovese & Wasserman, 2006)) was done across the individual probes tested as recommended (Li, Xie, Le Pape, & Dye, 2015).

3.2.7 VBM Analysis

Since I identified a significant DMR associated with the group-by-CU traits interaction effect on methylation level, I employed the GLM framework to explore the association between GMV and

average M-value across probes within the respective DMR. No DMR associated with main effects for CD or CU-traits was identified.

Specifically, GMV was analysed on a voxel-by-voxel basis, via multiple regressions. PDS, SES, total intracranial volume (TIV), scanning site (dummy coded), and total IQ were included as covariates of no interest. Unlike in the epigenetic analysis, I include SES as a covariate here to allow me to investigate the association between methylation and GMV across the full cohort without the potential confounding effects of SES on GMV that are independent of methylation. At a whole-brain level, inferences were made using a statistical threshold of $p< 0.05$ after FWE correction for multiple comparisons. I also investigated associations between GMV and M-value in four regions of interest (ROIs, bilaterally) where the identified gene of interest *SLC25A24* is highly expressed (Genotype-Tissue Expression (Consortium, 2013) GTEx project database, see Appendix A6), namely the amygdala, hippocampus, basal ganglia and cerebellum (Appendix A7). Masks of these regions were defined based on the Talairach Daemon database using the WFU PickAtlas tool in SPM12 (Lancaster et al., 2000). The MarsBAR toolbox was used to extract mean-cluster and peak-voxel GMV values from significant clusters for each participant. All brain imaging coordinates are reported in standardized MNI space.

3.3 Results

3.3.1 Participant Characteristics

As per matching on PDS and performance IQ, CD and TD females did not differ in terms of age, puberty, ethnicity, site and performance IQ, but the CD group had lower full-scale IQs than the TD group ($p = 0.013$). The number of ADHD symptoms did not differ between groups, but individuals with CD had significantly more symptoms of GAD and MDD than the TD

participants. Females with CD also had higher total ICU and ICU subscale scores (p<.001, see Table 3).

3.3.2 Power Calculation

While I acknowledge that the sample size is rather small for a genome-wide approach, power analysis using the online calculation tool *epigenetics.essex.ac.uk/shiny/EPICDNAmPowerCalcs* confirmed that my analysis with a sample size of n = 110 participants conferred each CpG site tested with ~80% power to detect a difference in methylation at the recommended level for the EPIC array (p< 6.21e−05). Two other recent studies have similarly adopted a genome-wide approach to investigating DNA methylation in relation to aggressive behaviours in youth, both using a sample size <n = 100 [48, 49].

3.3.3 Identification of Differentially Methylated Regions

At the single probe level, DNA-methylation was not predicted by case-control status or level of CU traits (at a significance level of pFDR < 0.05). However, the CDxCU traits interaction significantly predicted differential methylation at one genomic region on chromosome 1 (hg19 chr1: 108,735,312–108,735,893, FDR = 0.004), spanning eight probes. The interaction was driven by a positive association between CU traits and methylation of the respective probes in females with CD (Pearson $r_{(49)}$ = 0.39, p = 0.006), but a negative association between CU traits and methylation in TD females (Pearson $r_{(57)}$ =−0.27, p = 0.042). The slopes of these correlations differed significantly (Z = 2.48, p = 0.007). The region identified includes exon 1 of the solute carrier *SLC25A24* gene (see Figure 18).

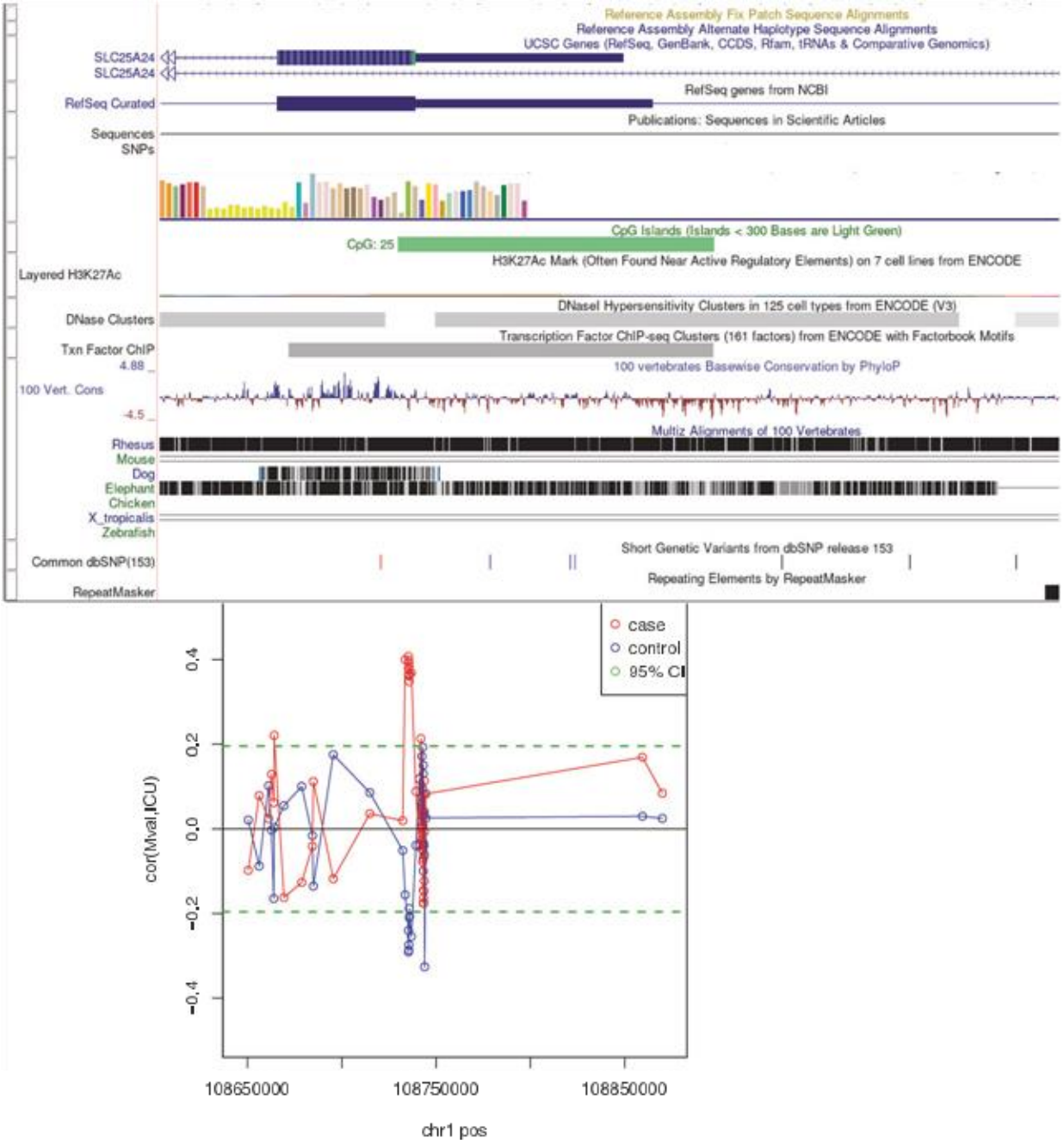


Figure 18. (Top) UCSC Genome Browser Illustration showing stacked annotation tracks beneath the genomic coordinates of the DMR (Bottom) scatter plot of DMR methylation- CU correlation vs. position

It is important to note that these methylation findings do not include the methylation values at common SNPs, as these were removed during the pre-processing stage of this analysis, thus my findings should be considered in light of this limitation.

3.3.4 Association between Methylation and GMV

I then tested whether the *SLC25A24* methylation levels observed for the interaction effect of CDxCU traits was also associated with GMV in any brain region. After correction for multiple comparisons, no significant (i.e. pFWE < 0.05) positive or negative associations between the average M-value of the *SLC25A24*-DMR and GMV were detected (in analysis across the whole cohort). However, given the exploratory nature of this study, I report findings at a more liberal significance level of $p< 0.001$ uncorrected with an extent threshold of $k= 72$ voxels empirically determined according to random field theory (Hayasaka & Nichols, 2004; Worsley et al., 1996). At this level I observed a negative association with *SLC25A24* methylation M-value for GMV in several clusters within the brain (please see Appendix Table A8), indicating that higher *SLC25A24* methylation is associated with lower GMV in these regions. I identified these clusters in multiple brain regions including the SFG, dlPFC, supramarginal gyrus, the secondary visual cortex in the left hemisphere, and the ventral PCC and secondary visual cortex in the right hemisphere. All coordinates are reported in MNI space. Mean cluster GMV values were extracted for each participant and then plotted against the average methylation M-value across the DMR on chromosome 1 (i.e. exon 1 of gene *SLC25A24* (see Figure 19)). Across all regions, in both CD and TD groups, there was a negative association between GMV and the mean exon 1 *SLC25A24* M-value.

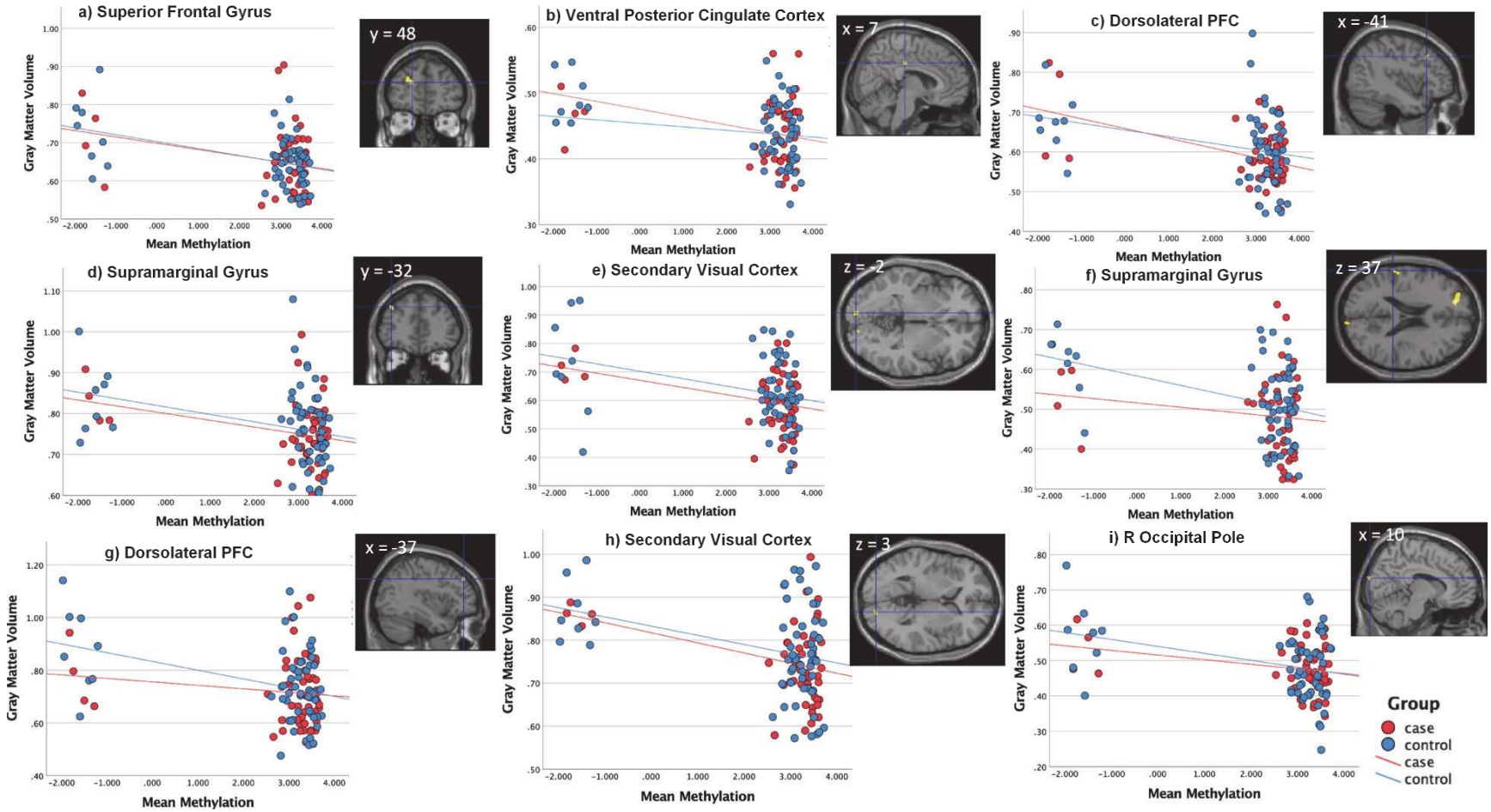


Figure 19. Mean GMV values in the cluster significantly associated with methylation for $p < 0.001$, size > 72 voxels were extracted for each participant and then plotted against the average methylation M-value across the DMR on chr1 (*SLC25A24* gene)

3.3.5 ROI Analysis

No significant positive or negative association between *SLC25A24* methylation and GMV could be detected in the amygdala, hippocampus, basal ganglia or cerebellum ROIs. (Please see Appendix A7) for 3D visualization of the four brain regions tested as ROIs.)

3.3.6 Post-hoc Testing of *OXTR* Methylation

We did not observe a significant association between CU traits and methylation at any of the 12 CpG sites on the *OXTR* gene for which I had DNA methylation data. Even when the significance threshold was reduced to a nominal level of $p< 0.001$, uncorrected, the main effect of CU traits was not significant for any of the individual sites, or for this region as a whole.

3.4 Discussion

To my knowledge, this is the first EWAS and epigenetic neuroimaging study in females with CD. First, I examined the main effects of CD group status, level of CU traits and their interaction on saliva-based DNA methylation. My analyses revealed that in CD and TD females there is a fundamentally opposite pattern of association between CU traits and methylation at a chromosome 1 genomic region, spanning exon 1 of the *SLC25A24* gene. Second, I related the identified DMR to GMV, both in multiple brain regions implicated in CD and CU traits and in a whole-brain exploratory analysis. GMV in regions including the SFG, dlPFC and supramarginal gyrus was negatively correlated with methylation levels, however, these neuroimaging findings did not reach the minimum threshold for significance.

3.4.1 Genome-wide Methylation

We found a significant CD × CU traits interaction effect on methylation level in exon 1 of the *SLC25A24* gene, whereby methylation level was positively correlated with CU traits in CD participants, but negatively correlated with CU traits in TD controls. Elevated methylation at the first exon and promoter regions of genes has been demonstrated to decrease the expression of the respective gene (Brenet et al., 2011; S. Li et al., 2018). Thus, my results indicate that in adolescent females with CD, higher levels of CU traits are associated with reduced *SLC25A24* gene expression, whereas in TD females, CU traits are positively associated with gene expression.

SLC25A24, a member of a solute-carrier gene family (He et al., 2009), is involved in adenosine triphosphate (ATP)-mediated Calcium buffering at the mitochondrial matrix and is potentially involved in protecting cells against oxidative stress-induced cell death. In mitochondria, ATP production is associated with the production of free oxidative radicals. These cellular redox scavengers, as well as nutrition-derived antioxidants, are crucial to neutralize these free radicals (Fraunberger, Scola, Laliberte, Duong, & Andreazza, 2016). As the brain accounts for 25% of the body's total energy expenditure (Herculano-Houzel, 2012), impaired mitochondrial function, as suggested by a reduced expression of *SLC25A24*, may lead to higher rates of cell death due to oxidative stress (Kramer & Bressan, 2018) and thus leave neuronal cells especially vulnerable to oxidative damage (de Oliveira, Ferreira Lima, & El-Bacha, 2012). Increased cell death, due to an impaired redox-scavenger system in the brain's mitochondria, may also, at least partially, explain the association I observed with GMV. Furthermore, unbalanced energy provision and reduced Calcium homeostasis in neurons may result in impaired functioning and ultimately lead to

neurodegeneration (Kramer & Bressan, 2018). Accordingly, mitochondrial dysfunction has been suggested to be associated with several neurodevelopmental disorders, including ASD (Chiocchetti et al., 2014; Varga et al., 2018) and ADHD (Hwang et al., 2017). Reduced expression of the *SLC25A24* gene has been reported in the thalamus and motor cortex of patients with ASD and hypothesized to be associated with the impairments in sensory processing and response inhibition observed in this population (Anitha et al., 2012).

As discussed, deficient mitochondrial functioning is a possible consequence of increased methylation and the resulting decreased expression of the *SLC25A24* gene. Given that mitochondria work alongside the mitochondrial-bound monoamine oxidase A (MAO-A) enzyme to break down catecholaminergic neurotransmitters (Floris et al., 2020), altered functioning of either component in the degradation process may contribute to abnormally high or low levels of neurotransmitters in the brain (Godar, Fite, McFarlin, & Bortolato, 2016). Importantly, atypical levels of neurotransmitters have previously been associated with both CD (Fairchild et al., 2019) and CU traits (Moul et al., 2013). Both elevated *SLC25A24* methylation and variants of the MAO-A enzyme may contribute to disrupted catecholamine catabolism. This is reported to be the biological means by which variation of the *MAOA* gene contributes to the affective (e.g., emotion dysregulation) and behavioural (e.g., reactive aggression) features of females with CD (Holz et al., 2016). Thus, *SLC25A24* gene hypermethylation may also result in behavioural patterns associated with atypical levels of neurotransmitters in the brain in a similar way to that reported for variants of the MAO-A enzyme, which have previously been linked to aggressive/violent behaviours in both animals (Bortolato, Floris, & Shih, 2018) and humans (Kolla & Houle, 2019).

Environmental risk factors and *SLC25A24* methylation Childhood maltreatment, a key factor known to influence DNA methylation (Cecil, Zhang, & Nolte, 2020), has been shown to interact with *MAOA* gene variants to predict aggression in both sexes (Byrd & Manuck, 2014). In females, the high activity allele has been shown to confer a risk for aggressive behaviour following childhood maltreatment (Byrd & Manuck, 2014), but see ref. (Ducci et al., 2008). Future studies should further investigate the relationship between childhood maltreatment and methylation to determine whether experiences of child maltreatment alter DNA methylation levels and thereby increase the risk for aggressive behaviours.

More generally, mitochondrial dysfunction has been linked to exposure to environmental stressors (Bennuri, Rose, & Frye, 2019). Mitochondria are key components of the human body's stress response system, providing intra-cellular energy and synthesizing stress hormones and neurotransmitters central to stress responding (Picard, McEwen, Epel, & Sandi, 2018). Experimental manipulation of mitochondrial function has been shown to influence physiological and behavioural responses to psychological stress (Picard et al., 2018). Crucially, there is evidence that epigenetic markers of stress exposure are mitochondrially regulated (Picard et al., 2018). Thus, reduced expression in genes governing mitochondrial function, such as *SLC25A24*, may arbitrate how environmental factors result in epigenetic modifications (Aon, Cortassa, Juhaszova, & Sollott, 2016).

Individuals with CD are more likely to have experienced 'stressful' early life environments and thus to have elevated stress biomarkers associated with psychiatric symptoms (Horn, Leve, Levitt, & Fisher, 2019). CU traits may be another factor that moderates the association between

environmental risk factors and the individual's biological stress response (Hawes, Brennan, & Dadds, 2009). Consequently, the combination of CD diagnostic status and level of CU traits may influence epigenetic markers associated with stress exposure. Altered methylation across genes in the energy metabolism system may represent an adaptive response to these variations. Thus, rather than being a unique marker of one stressor, I postulate that *SLC25A24* gene methylation may reflect the cumulative effect of exposure to multiple early-life environmental factors triggering the biological stress response system.

3.4.2 Epigenetic Neuroimaging Data

Our neuroimaging analysis revealed trend-level negative associations between *SLC25A24* methylation values and GMV in several brain regions, namely, the SFG, dlPFC, supramarginal gyrus and secondary visual cortex in the left hemisphere, and the ventral PCC and secondary visual cortex in the right hemisphere.

These results may suggest that higher levels of *SLC25A24* gene methylation is linked to a reduction in GMV in these regions. This finding would be consistent with the theory that increased methylation has a silencing effect on the gene, leading to impaired mitochondrial function (and thus a reduced capacity for energy production and growth) during brain development. Many of the regions where reduced GMV was observed, such as the SFG, dlPFC, the supramarginal gyrus and the ventral PCC, are involved in higher cognitive functions, such as working memory (du Boisgueheneuc et al., 2006), as well as socio-cognitive processes such as affective empathy, which have been shown to be impaired in CD (Fairchild et al., 2019; Martin-Key, Brown, & Fairchild, 2017). For example, a recent meta-analysis of 13 VBM studies found

that youth with CP had significantly reduced GMV in the left medial SFG (Rogers & De Brito, 2016). Atypical cortical thickness and functional connectivity have also been reported in adults with psychopathy in several brain regions across the frontal cortices (Yang et al., 2012) and deficits in cortical folding in these regions are also reported in youth with CD (Hyatt, Haney-Caron, & Stevens, 2012).

In youth with CD, greater levels of methylation were observed in association with higher CU traits and greater levels of methylation were also related to reductions in GMV at trend level. In TD youth, we see the inverse pattern (with individuals with higher CU traits having higher GMV in the observed brain regions). I speculate that in individuals with CD and high CU traits this increased methylation and the associated higher levels of oxidative stress during energy production contributes to a higher rate of neuronal death during neuronal pruning, and subsequently leads to a reduction in GMV in the observed brain regions in this group. However, currently, the underlying factors contributing to this mechanism are unknown, and further research with more highly powered studies is needed to determine whether the suggestive negative relationship between GMV and methylation that I observed here holds true in larger samples.

3.4.3 Post-hoc Testing of *OXTR* Methylation

The fact that other studies have found an association between CU traits and methylation of the *OXTR* gene (e.g. refs. (Cecil et al., 2014; Dadds et al., 2014)), but I did not, can be explained by a number of factors. For example, this may be related to methodological differences between this study and previous studies, such as the use of different measures of CU traits (i.e., ICU here, but

others (Aghajani et al., 2018) have used the Youth Psychopathic Traits Inventory (YPI (Pechorro, Ribeiro da Silva, Andershed, Rijo, & Abrunhosa Goncalves, 2016)) or other different investigative approaches, i.e., candidate gene vs. EWAS. Additionally, I focused on females only, which contrasts with previous studies that have relied on male-only or mixed-sex samples.

3.4.4 Strengths and limitations

As the first study integrating epigenetic and neuroimaging data from females with CD, this work is an important contribution to our understanding of the biological factors implicated in CD and CU traits in females. Using multi-site data allowed for a larger sample size than would have been possible at a single site, as CD females are difficult to recruit. Furthermore, as data were collected as part of the FemNAT-CD project, the sample is well characterized, with all participants undergoing thorough assessment for psychiatric disorders and symptoms using a reliable measure based on DSM-IV-TR criteria. Finally, the two groups did not differ on PDS, performance IQ, ADHD symptoms, site, and ethnicity, minimizing the potential confounding effects of these factors.

Nevertheless, this study has limitations. First, the sample size is relatively small. As mentioned above, power analysis confirmed this analysis with a sample size of n = 110 participants conferred each CpG site tested with ~80% power to detect a difference in methylation at the recommended level for the EPIC array ($p < 6.21e{-}05$). This power allowed me to detect moderate-to-large effects, however smaller effects ($f < 0.35$) on genome-wide methylation levels or GMV were not detectable with this study design. Also, I only had data on childhood maltreatment for a small subset of participants (n = 31), so I was unable to include this

information in my analysis. Second, while several previous studies report concordance of DNA methylation across saliva and brain tissues (e.g., (Braun et al., 2019)), tissue specific epigenetic modifications have also been reported (Gutierrez-Arcelus et al., 2015). Thus, it is possible that the differential methylation in salivary DNA demonstrated in this study does not accurately reflect brain-level methylation and might thus be specific to buccal cells only. I also did not correct for cell composition in the salivary DNA samples. Third, as the methylation findings I report do not include the methylation at common SNPs, I do not yet know whether the methylation differences I observe are themselves genetically influenced. Additionally, although participants were asked to refrain from substance use prior to experimental sessions participants were not tested to verify this thus variations in levels and types of substances used by participants may have affected both the epigenetic and imaging findings reported here Finally, due to funding limitations, I chose to focus solely on investigating genome-wide methylation in females. I felt this would maximise the novelty of my work and add to the knowledge base in this particularly under-researched group. However, as I only included female participants the findings may not apply to males with CD, as research indicates sex-specific influences of environmental and genetic factors on CD and CU traits (Fontaine et al., 2010; Viding et al., 2005). Thus, similar studies in males and mixed-sex samples will be an important area of future research to investigate whether these mechanisms are sex-specific.

3.4.5 Conclusions

Methylation of the *SLC25A24* gene was significantly associated with CU traits in both females with CD and TD females but in a fundamentally opposing pattern. Given its essential role in energy metabolism, *SLC25A24* is a key component of the biological stress response system. I

postulate that the combination of the individual's level of CU traits and the number of stressful early life experiences may epigenetically modify the *SLC25A24* gene thus influencing its functionality. Furthermore, I detected negative trends between *SLC25A24* methylation values and GMV in several brain regions, many of which have also been implicated in CD and CU traits. While my findings are preliminary and need to be replicated in larger samples, they provide novel evidence that CU traits in females are associated with methylation levels in a fundamentally different way in CD and TD groups, which in turn relates to observable variations in GMV in the brain.

CHAPTER 4- INVESTIGATING THE ASSOCIATION BETWEEN BRAIN ACTIVATION DURING EMOTIONAL FACE PROCESSING AND METHYLATION OF THE *SLC25A24* GENE

4.1 Introduction

4.1.1 Introduction to CD and CU traits

CD is a psychiatric diagnosis given to youth under the age of 18 who exhibit persistent antisocial behaviours (APA, 2013). These behaviours have a significant impact on the individual's social, academic and/or professional lives and when left untreated present a huge cost to society (Scott, Knapp, Henderson, & Maughan, 2001). Due to widespread variation in the combinations of symptoms observed in individuals with CD (Nock, Kazdin, Hiripi, & Kessler, 2006), several sub-typing methods have been developed by clinicians and researchers to identify more homogeneous subgroups of youth with CD. In this study, I focus on the commonly used method for sub-typing individuals with CD according to their levels of CU traits (i.e. lack of empathy, shallow affect, and lack of guilt/remorse), which are captured under the LPE specifier in the DSM-V (APA, 2013). Recent work has shown that neuroimaging markers in CD also vary between the sexes (e.g. (Rogers et al., 2019; Smaragdi et al., 2017)). In this context, this study on females is an important addition to this field of research, which has mostly relied exclusively on male samples.

4.1.2 DNA Methylation of *SLC25A24*- Theoretical Effect on Functional Brain Activity

Exposure to particular environments and other lifestyle factors may affect levels of DNA methylation (Martin & Fry, 2018). These alterations in DNA methylation may then subsequently affect brain development and structure (Fagiolini, Jensen, & Champagne, 2009). Indeed, research in recent years supports the theory that epigenetic modifications such as DNA methylation play a

key part in governing both the potency and plasticity of developing networks of neurons throughout the lifespan (Tognini, Napoli, & Pizzorusso, 2015). The effects on gene expression that result from this process demonstrate a biological mechanism whereby early environmental experiences can become embedded in an individual's genetic material, leading to long-term changes in neurobiology and behaviour. In chapter 3, I previously reported that the combination of CD group diagnostic status and level of CU traits was associated with differential methylation in a region on chromosome one, encompassing the *SLC25A24* gene.

Specifically, I showed that in females with CD, greater levels of CU traits were associated with higher methylation levels of this gene, whereas in TD females, greater levels of CU traits were related to lower levels of methylation of the *SLC25A24* gene. I suggested that increased levels of DNA methylation in this region, (e.g. as a function of increasing CU traits in participants with CD) was likely to represent a silencing effect on the expression of this gene (*see chapter 3*).

In chapter 3, I also explored how methylation of the *SLC25A24* gene was related to GMV. As this gene is central to cell energy metabolism (Palmieri, 2013) and expressed in both cortical and sub-cortical brain tissues (Gutierrez-Aguilar & Baines, 2013), I expected that reduced expression of *SLC25A24* (due to elevated levels of DNA methylation) might influence GMV. My results showed a negative association, albeit non-significant, between methylation of the *SLC25A24* gene and GMV in several brain regions, including the SFG, dlPFC, supramarginal gyrus, secondary visual cortex and ventral PCC. Subsequently, I concluded that in regions where the gene is in general active the increased methylation might limit expression of the gene. This would, in turn, reduce the availability of energy for growth during crucial developmental periods and thus contribute to reduced volume in the associated region.

Accordingly, given recent meta-analytic evidence that GMV abnormalities in CD can also be accompanied by functional abnormalities in the same regions (Raschle, Menks, Fehlbaum, Tshomba, & Stadler, 2015), I hypothesized that elevated methylation of the *SLC25A24* gene may also be associated with brain response in regions where *SLC25A24* is expressed, and which have previously been implicated in research examining emotional processes in CD. Here, I explicitly investigated the association between *SLC25A24* methylation and brain response as measured with fMRI during an emotional face processing task. Specifically, I investigated the association between brain response to both neutral and negative (fearful/angry) emotional faces and *SLC25A24* methylation. As the *SLC25A24* gene plays a key role in energy metabolism, and as higher energy needs are associated with negative emotion processing (Deli, 2020; Deli, & Kisvárday, 2020), this gene may be particularly relevant to the emotional processing deficits associated with CD and CU traits (Blair, 2014).

4.1.3 Brain Regions Associated with Emotional Processing

Emotional processing activates a broad range of subcortical and cortical regions such as the amygdala (LeDoux, 2007), hippocampus (Buchanan, 2007), basal ganglia (Cheung, Lee, Yip, King, & Li, 2006), insula (Rodriguez et al., 2019), ACC (Stevens, Hurley, & Taber, 2011) and prefrontal cortices (Zinchenko, Yaple, & Arsalidou, 2018). Research also indicates that the primary and associative visual cortices and primary auditory cortices play a role in managing sensory information salient to emotional processing (Kropf, Syan, Minuzzi, & Frey, 2019). The amygdala is one of the most studied brain regions in emotion research (Pessoa & Adolphs, 2010) and plays its key role in emotional processing by coordinating the function of cortical networks, which determine the biological salience of affective visual stimuli (Baxter & Croxson, 2012).

Increased activity in the hippocampus has also been reported during various emotion-related processes including emotional perception (Lindquist, Wager, Kober, Bliss-Moreau, & Barrett, 2012), emotional memory encoding (Zhu et al., 2019) and emotional regulation. The basal ganglia has been linked to emotional speech processing (Paulmann, Ott, & Kotz, 2011) and the globus pallidus (which forms part of the basal ganglia) is a region consistently activated in response to emotional facial cues of disgust (Murphy et al, 2003). Indeed, studies in patients with Parkinson's disease show that neurodegeneration in this brain region is linked to a reduced ability to recognise facial expressions of disgust (Suzuki, Hoshino, Shigemasu, & Kawamura, 2006). The insula has reciprocal connections to multiple regions involved in emotional processing and is considered as the main area where information from internal and external sources is integrated via subjective emotional awareness (Gasquoine, 2014). Finally, the ACC (Stevens et al., 2011) and PFC (Dixon, Thiruchselvam, Todd, & Christoff, 2017) are shown to be activated during emotional regulation. In children with conduct problems, reduced response in the ACC has also been linked to lower empathy to another person's pain (Lockwood et al, 2013). Both the ACC and PFC are involved in top-down regulation of the aforementioned limbic regions (Stevens et al., 2011) and dysfunction in these top-down regulatory networks has been suggested to contribute to the emotional dsyregulation observed in other psychiatric disorders, such as ADHD (Petrovic & Castellanos, 2016).

A 2009 meta-anlaysis of fMRI studies involving emotional face processing tasks with mixed-sex healthy control participants also highlighted that responses in differing brain regions play key roles in relation to neural processing of specific emotions or particular emotional contrasts (e.g. different activation for contrasts involving 2 different emotions as compared to an emotional vs. neutral contrast) (Fusar-Poli et al., 2009). For example, activation in the bilateral amygdala and

the fusiform and medial frontal gyri were most common in response to viewing fearful faces (Fusar-Poli et al., 2009). However, the neural activations associated with processing angry faces were principally increased responses in the left insula and right inferior occipital gyrus (Fusar-Poli et al., 2009).

4.1.4 Brain Response During Emotional Processing: CD, CU Traits and Sex Effects

Most fMRI studies on emotion processing in CD have used tasks in which the participants are presented with emotionally salient images such as pictures of human faces depicting emotional expressions (Fairchild et al., 2014), or visual stimuli intended to initiate an empathic response (e.g., an image of another person in pain (Singer et al., 2004)). Meta-analyses indicate that youth with CD, as compared to TD youth, are characterised by lower responses to these stimuli in brain regions implicated in affective processing, such as the amygdala, ACC, medial PFC, dlPFC, temporal pole and ventral striatum (Alegria et al., 2016; Noordermeer, Luman, & Oosterlaan, 2016; Raschle et al., 2019). Importantly, CU traits have also been shown to moderate brain response to emotional stimuli (Viding et al., 2012). For example, a recent study reported that CU traits were negatively correlated with amygdala-ventral ACC connectivity in male youth during an emotional face-processing task (Ewbank et al., 2018). In a recent meta-analysis (Alegria, Radua, & Rubia, 2016), which only included studies adopting a whole-brain approach in their analyses, higher levels of CU traits were related to reduced brain response in the ventromedial PFC, thalamus and ventral striatum, but elevated response in the caudate and dorsolateral PFC, during emotion processing tasks (Alegria et al., 2016). A number of studies focusing on a priori regions of interest have also shown that CU traits are negatively correlated with brain response in the ACC, amygdala and insula during emotional processing (Yoder, Lahey, & Decety, 2016).

These findings are thought to explain the lower emotional reactivity observed in youth with CD and high levels of CU traits (Northam & Dadds, 2020). Finally, there is good evidence from healthy youth that sex influences the neural correlates of emotion processing (Domes et al., 2010). Although much of the previous research in individuals with CD has focused on males (Freitag et al., 2018), sex-specific trends have been reported on some emotional measures including empathic processing. For example, females with CD are reported to score higher than males on measures of empathic concern and personal distress (Arango-Tobón, Pinilla Monsalve, Rosa, Orejarena Serrano, & Carmona Cardona, 2020) and across females with and without CD there is a negative association between emotional empathic ability and number of disruptive behavioural symptoms, whereas in males a positive association is reported (Arango-Tobón et al., 2020). In addition, studies testing male-only and female-only participants have reported dissimilar findings on amygdala responses to facial stimuli. Specifically, increased amygdala response to human faces has been observed in adult males with violent behaviours (Pardini & Phillips, 2010) and male youth with CD and low CU (Viding et al., 2012), but a negative association between number of lifetime CD symptoms and amygdala response to threatening facial expressions has been reported in females (Fairchild et al., 2014). A positive association between amygdala response to fearful faces and CU traits has also been reported solely in females (De Brito et al., in preparation). Taken together, these data indicate that in youth with CD the response of the neuro-circuity involved in emotion processing varies according to both the individuals' level of CU traits and sex.

4.1.5 Aims and Hypotheses

In this study of females with CD and TD females, I examined the association between DNA methylation and brain response, as measured by fMRI when viewing emotional faces. In line with previous studies, I first examined brain response to faces in general by modelling the contrast of all faces vs. fixation (e.g. (Fairchild et al., 2014)). Then, I considered the responses to particular emotional facial expressions by modelling the following contrasts: (i) angry > neutral and, (ii) fearful > neutral faces. I hypothesized that elevated methylation of the *SLC25A24* gene may be associated with level of response in 4 regions of interest (ROIs). These were selected based on two criteria. Firstly, that they were brain regions in which the *SLC25A24* gene is expressed and secondly, that the region had previously been implicated in emotional processing studies in CD. The ROIs were the hippocampus, amygdala, hypothalamus and basal ganglia. I did not specify the direction of the association in my hypothesis, as previous studies investigating the relationship between DNA methylation and brain function have demonstrated mixed results (as discussed in more detail in section 4.4.1). Additionally, given that higher levels of methylation of the *SLC25A24* gene was associated with a reduction in GMV in several other brain regions in previous work (Farrow et al., 2021), and as organizational differences in the brain are closely linked to functional variations (Batista-Garcia-Ramo & Fernandez-Verdecia, 2018), I performed whole-brain exploratory analysis to investigate whether there were other regions where brain response to emotional faces was associated with *SLC25A24* gene methylation levels. Due to funding limitations, in this study I focus solely on female youth, in order to add to the knowledge base in this particularly under-researched group.

4.2 Methods

4.2.1 Participants

Thirty-one females with a diagnosis of CD (mean age=15.2, SD=1.7) and thirty-one TD females (mean age=14.9, SD=2.4) recruited across four sites (see Appendix B1), were included as a subsample of the FemNAT-CD study (Freitag et al., 2018). In addition to standard FemNAT-CD inclusion and exclusion criteria (see (Rogers et al., 2019)), participants were required to be non-smokers, be medication-free, and have good quality saliva-DNA and useable fMRI data from the emotional face processing task (Passamonti., et al., 2012).

4.2.2 Clinical and Psychometric Measures

Detailed information about the procedures used to collect all clinical and psychometric data is provided in previous publications from the FemNAT-CD consortium (Raschle et al., 2018; Rogers et al., 2019). Briefly, trained research staff interviewed participants and their parents/caregivers separately using the K-SADS-PL (Kaufman, Birmaher, Brent, & Rao, 1997) to assess CD symptoms and screen for other DSM-IV-TR psychiatric disorders. Additional questions from the supplementary sections of the K-SADS-PL (e.g., for ODD/ADHD) were included only if the initial screening indicated additional psychiatric symptoms. CU-trait scores were obtained using the parent-version ICU (Essau, Sasagawa, & Frick, 2006). Total IQ scores were assessed using the Wechsler Abbreviated Scale of Intelligence (WASI; Wechsler, 2011) in the UK, and the Wechsler Intelligence Scale for Children, Fifth Edition (WISC-V; Wechsler, 2014) at other sites. Values for the Verbal and Performance IQ subscales were also computed from these measures. Pubertal status was determined using the PDS (Petersen et al., 1988), this was completed by participants aged 12 years or older, or by the parents/caregivers where participants were younger than 12.

4.2.3 Genome-wide Methylation Data Analysis

Using the methods described in chapter 3 (and (Farrow et al., 2021)), saliva DNA samples were collected from all participants and genome-wide DNA methylation data from the EPIC array was obtained and analysed for these samples. In a sub-sample of the original participants, I re-ran the methylation analysis using the *Bumphunter* algorithm (Jaffe et al., 2012) in R software *version 3.6.0* (Team R, 2019) using the same parameters and statistical significance thresholds as described in chapter 3 (and (Farrow et al., 2021)). Once again, in this sub-sample (which included only those participants from the initial analysis for whom good quality fMRI data were available) I found one DMR on chromosome one was significantly associated with the interaction effect of CD diagnostic status and level of CU traits (see Results section below). As before, this DMR corresponded to the location of gene *SLC25A24*. I then investigated whether there was an association between the methylation values for gene *SLC25A24* (represented by the average M-value across all CpG sites in the DMR) and functional brain activation, as measured by the emotional face-processing task (see below).

4.2.4 fMRI Task Description

The paradigm was an adapted version of one used previously in male (Passamonti, Fairchild, et al., 2012) and female (Fairchild et al., 2014) youth with CD (see Figure 20). Briefly, participants were presented with an image of an emotional (angry, fearful or neutral) face and were asked to indicate by a button press whether the face was male or female. These images were presented in 17.5s blocks consisting of 5 faces and 5 fixation crosses. For each emotional condition participants were shown 12 blocks of faces and the images were counterbalanced across subjects. The total duration of the task was 10 minutes 30 seconds.

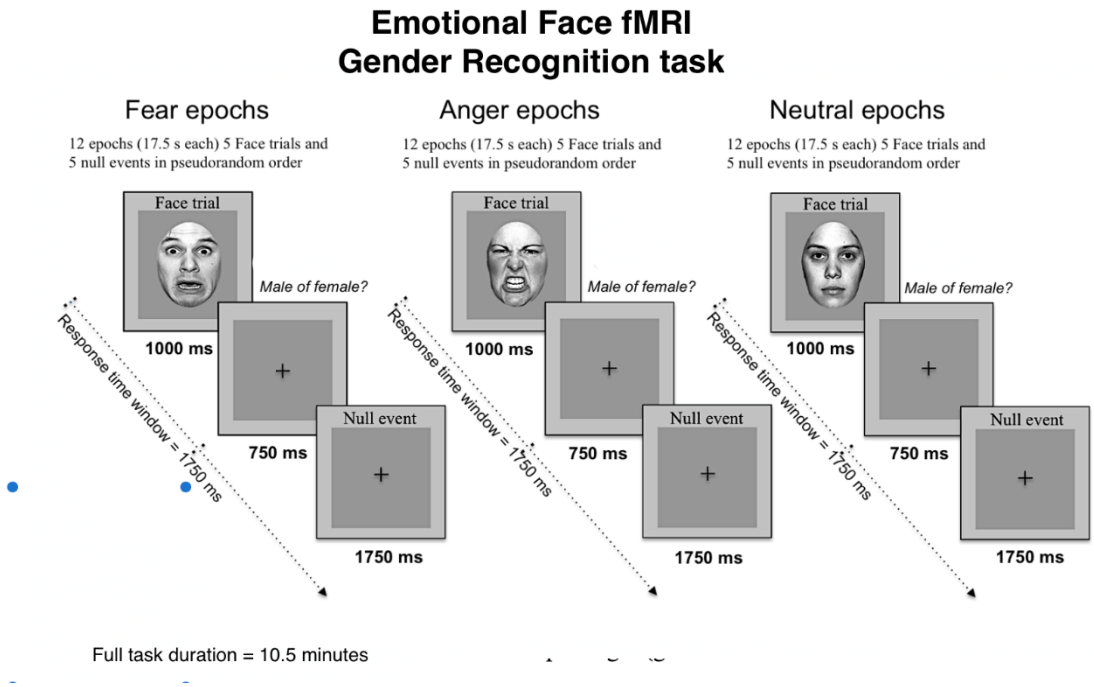


Figure 20. Emotional Face processing task - design based on Passamonti et al (Passamonti et al., 2012)

4.2.5 Image Acquisition and Pre-processing

All data were collected using Siemens or Phillips 3T MRI scanners across four European sites taking part in the FemNAT-CD project (Freitag et al., 2018). All sites underwent screening checks and quality control procedures before commencement of data collection. Acquisition of data across different sites was performed in accordance with standard operating procedures designed by the FemNAT-CD project's neuroimaging working group. As previously reported (Rogers et al., 2019), rigorous quality control procedures and matched experimental protocols across the various locations qualify these data for use in multi-site analysis

The SPM12 (University College London, London, UK; http://www.fil.ion.ucl.ac.uk/spm/doc/manual.pdf) and ART imaging toolboxes (Neuroimaging Tools and Resources Collaboratory; https://www.nitrc.org/projects/artifact_detect/) were used during the pre-processing stages of the fMRI data analysis. The pre-processing steps included (i) Realignment and unwarping of functional data with reference to the participant's first scan image, (ii) Co-registration of scans to a reference structural T1 image, (iii) Normalisation of images into standard space using a DARTEL template (created based on sample-specific tissue probability map (TPM)), (iv) Smoothing of data with a 6-mm full width at half maximum Gaussian kernel and finally (v) using the ART toolbox I identified individual regressors associated with motion and variation in mean signal intensity. These regressors were then included in the first-level analysis model.

4.2.6 Statistical Analysis

The SPM12 (University College London, London, UK; http://www.fil.ion.ucl.ac.uk/spm/doc/manual.pdf) toolbox was also used for statistical analysis of the fMRI data. At the first

level, I generated contrast images for each participant, representing a directional difference between two emotional-face conditions, or between the observed BOLD response to an emotional-face condition and the fixation cross. The five contrasts generated were: all faces>fixation, anger>neutral, fear>neutral, anger>fixation, fear>fixation.

The second-level analysis involved assessing the difference in BOLD signal intensity change associated with the five contrasts of interest across the whole sample. Using a linear regression model, I examined if BOLD signal intensity was significantly associated with methylation of the *SLC25A24* gene. Age, total IQ, SES and data collection site were included as covariates of no interest in the model. I conducted both a whole-brain exploratory analysis and targeted region of interest (ROI) analyses. For the whole-brain analysis, I adopted a significance threshold of $p<0.005$, cluster-size corrected to a minimum cluster size 20 voxels, to be consistent with previous fMRI studies on youth with CD symptomatology (Marsh et al., 2013). This extent threshold was determined using the SPM12 minimum k value which is calculated based on the projected distribution of cluster sizes under the null hypothesis when there is no activation in any of the voxels in the cluster. The minimal cluster size to control for family-wise error rate (FWER) can also be estimated from the sampling distribution of the largest null hypothesis cluster size among supra-threshold voxels within the search area (Woo et al., 2014). The key limitation of cluster level thresholding is that it gives low spatial specificity for clusters much larger than the estimated extent threshold (Nichols., 2012), however this approach does effectively account for the fact that voxel activations are not independent of the activation of the adjacent voxels(Heller et al., 2006; Wager et al., 2007). This is especially relevant for spatially-smoothed data (Woo et al., 2014). This significance threshold has been demonstrated to be

suitable for investigating complex emotional phenomena, striking a good balance between types I and II errors in fMRI analyses (Lieberman & Cunningham, 2009).”

For the ROI analyses, four regions were selected which fulfilled the following three criteria; (i) areas of high *SLC25A24* gene expression, (ii) significance demonstrated in at least one published fMRI study focusing on CU traits or CD and, (iii) previously reported to be implicated in responding to emotional face stimuli in relation to CD (e.g. in (Alegria et al., 2016)). These regions were the hippocampus, amygdala, basal ganglia and hypothalamus. For the ROIs analyses I used a statistical threshold of $p<.05$ FWE-corrected. Bilateral masks for these regions were anatomically defined based on the Automated Anatomical Labelling (AAL) atlas of the WFU PickAtlas (Maldjian, Laurienti, Kraft, & Burdette, 2003).

4.3 Results

4.3.1 Characteristics of the Participants

Females with CD and TD females did not differ in terms of age or PDS, but the CD group scored significantly lower than the TD group in measures of total ($p=.004$), performance ($p=.018$), and verbal IQ (all $p< .001$) (Table 4). SES was also significantly lower in the CD group ($p=.003$). There were significant between-group differences in total ICU scores ($p=.001$), and for the callous ($p=.003$) and uncaring ($p=.001$) sub-scales, with the CD group scoring higher than the TD group. The average number of ADHD ($p<.001$) and MDD ($p=0.005$) symptoms was significantly higher in CD participants than in TD participants, however there was no significant difference in the number of Anxiety (GAD) symptoms in the groups (see Table 4 for details).

For all participants, behavioural data for the task was checked to ensure they had actively engaged with the task (i.e. >80% accuracy in responses to gender question). There was no significant difference in the behavioural data between CD and TD groups for either task accuracy (*p=.057*) or reaction time (*p=0.28*).

Table 4: *Descriptive statistics for n=62 participants*

Demographic	CD (n=31)		TD (n=31)		*P (t-test)*	Wilcoxon's
	M	*SD*	*M*	*SD*		*p*
Age	15.22	1.72	14.87	2.39	.511	.758
PDS	4.156	.920	4.07	1.01	.718	.714
SES	-0.38	0.76	0.22	0.72	.003	.003
Total IQ	91.72	10.6	99.13	9.32	.005	.004
Perf. IQ	90.56	14.5	98.9	11.4	.015	.018
Verbal IQ	92.9	11.4	99.4	13.8	.024	.028
Clinical						
ADHD Symptoms	1.25	1.63	.03	.18	<.001	<.001
MDD Symptoms	1.75	1.85	0.03	.18	<.001	<.001
GAD Symptoms	.20	0.41	0	0	.03	.04
ICU total	28.34	11.2	18.4	10.5	<.001	.001
ICU callous	9.16	6.01	5.20	4.45	.004	.003
ICU uncaring	12.97	4.84	8.60	4.92	<.001	.001
ICU unemotional	6.219	3.77	4.60	2.94	.064	.08

4.3.2 Genome-wide Methylation Data Analysis (in n=62 sample)

In chapter 3, I reported that the interaction term for CD diagnostic status and CU-trait score was associated with the level of DNA methylation in a genomic region on chromosome 1 which corresponded to the position of the *SLC25A24* gene (hg19 chr1: 108,735,312-108,735,893). In this sub-sample of the original participants (n=62), I used the same analysis model, and once again obtained a DMR (*p=.003 FDR-corrected*) associated with the combined CD x CU interaction, which corresponded to the location of gene *SLC25A24* (hg19 chr1: 108,735,353-108,735,893). I then examined the correlation between CU-trait score and methylation in this region separately in the two groups. Consistent with the results from the original sample of 110 participants, in these 62 participants, I observed a positive correlation between methylation and level of CU traits in females with CD (Pearson $r_{(31)}$= .48, p=.042) and a negative correlation in the TD group (Pearson $r_{(31)}$= -.26, p=.042). I also confirmed that these correlations were significantly different from each other (Z=-2.99, p=.003), as shown in Figure 21.

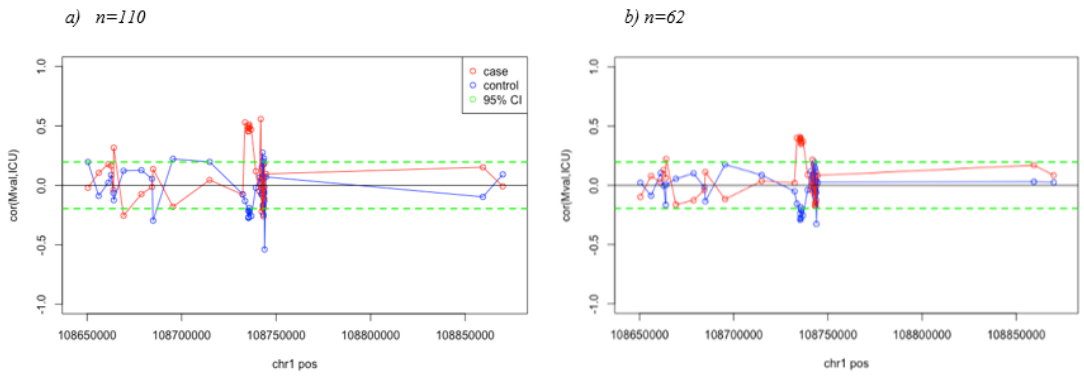


Figure 21. Differentially methylation region on chromosome 1 for (left) n=110 and (right) n=62 female youth with and without CD

4.3.3 fMRI Results

All faces>fixation

Whole brain analysis revealed a *positive* association between methylation and BOLD response in four clusters within the right hemisphere, including the ventral caudate and three smaller clusters in the para-hippocampal region, the superior temporal cortex (STC) and from the STC extending into the mid-temporal region (Figure 22). The ROI analysis did not reveal significant associations (positive or negative) between *SLC25A24* average methylation and BOLD response in any of a priori regions.

Anger>neutral.

For this contrast, whole brain analysis revealed a *positive* association between methylation and BOLD response in four clusters in the left hemisphere. Specifically, those clusters were in the angular gyrus/AI, occipital lobe (primary visual regions), occipital lobe extending into calcarine cortex and calcarine cortex only (Figure 23). As for the all faces>fixation contrast, the ROI analysis did not reveal significant associations (positive or negative) between *SLC25A24* average methylation and BOLD response in any of the a priori regions I examined.

Fear>neutral.

At whole-brain level, a *positive* association between methylation and BOLD response was observed in three clusters, two of which were in the right hemisphere. These were clusters in the ventral posterior cingulate and the SFG, and in the left hemisphere, I observed a cluster in the dorsal ACC (Figure 24). The ROI analyses again did not reveal any associations (positive or negative) between *SLC25A24* methylation and BOLD response.

Table 5: Regions showing main effect at *P*< 0.005 cluster-size corrected, size > 20 voxels for All Faces > Fixation, Anger>Neutral, Fear>Neutral contrasts

Contrast	Brain region	BA	L/R	Peak Voxel	k	Z	Cluster corrected P-value
All Faces> Fixation	Para-hippocampal	36	R	33 -42 0	26	4.02	<.001
	Caudate	48	R	21 15 15	49	3.67	<.001
	STG	44	R	54 6 -9	27	3.57	<.001
	Mid Temporal	13	R	42 -6 -9	24	3.47	<.001
Anger>Neutral	IFG	45	L	-27 30 6	30	4.30	<.001
	Angular Gyrus	39	L	-54 -63 12	38	3.34	<.001
	Occipital	17	L	-15 -81 0	30	3.33	<.001
	Vent. Post. Cing	23	L	-12 -57 6	24	3.46	.001
Fear>Neutral	Vent. Post. Cing.	23	R	9 -36 27	41	3.41	<.001
	Pre-central G/	6	R	21 12 36	32	3.32	<.001
	Dorsal ACC	6	L	-6 12 39	36	3.28	0.001
						3.23	0.001

Notes: STG = Superior Temporal Gyrus, IFG = Inferior Frontal Gyrus, Vent. Post. Cing = Ventral Posterior Cingulate Cortex, ACC = Anterior Cingulate Cortex

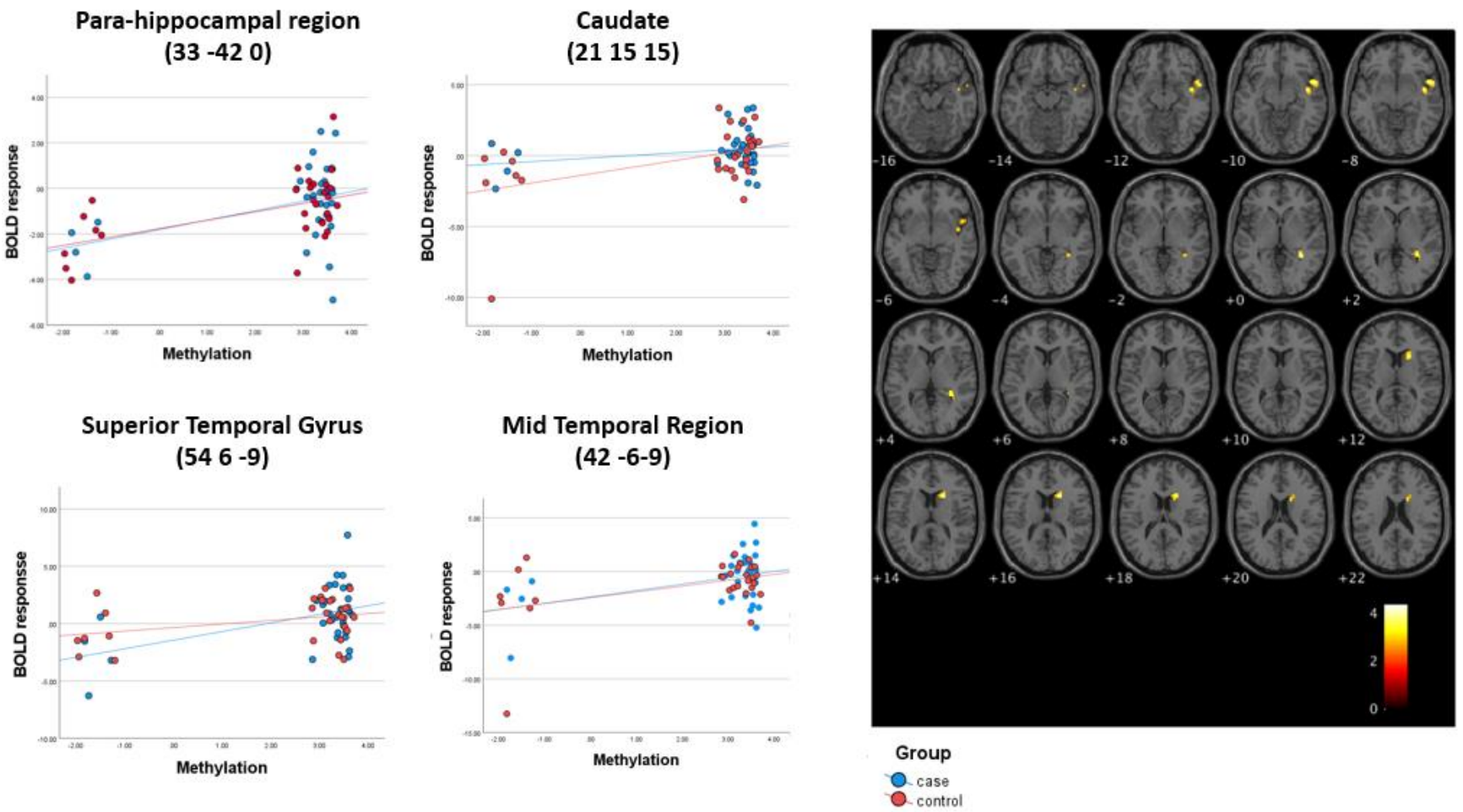


Figure 22. All faces vs. fixation significant clusters and scatter plots of correlation between methylation and BOLD response. Differential activation in response to faces compared to a fixation cross was associated with methylation in multiple regions.

N.B. The colour of the highlighted voxels indicates the magnitude of the t-statistic, which is an indicator of the level of BOLD signal activation in the region, as indicated in the colour bar on a scale from 0 = Black/Red (low) – 4 =Yellow/White (High).

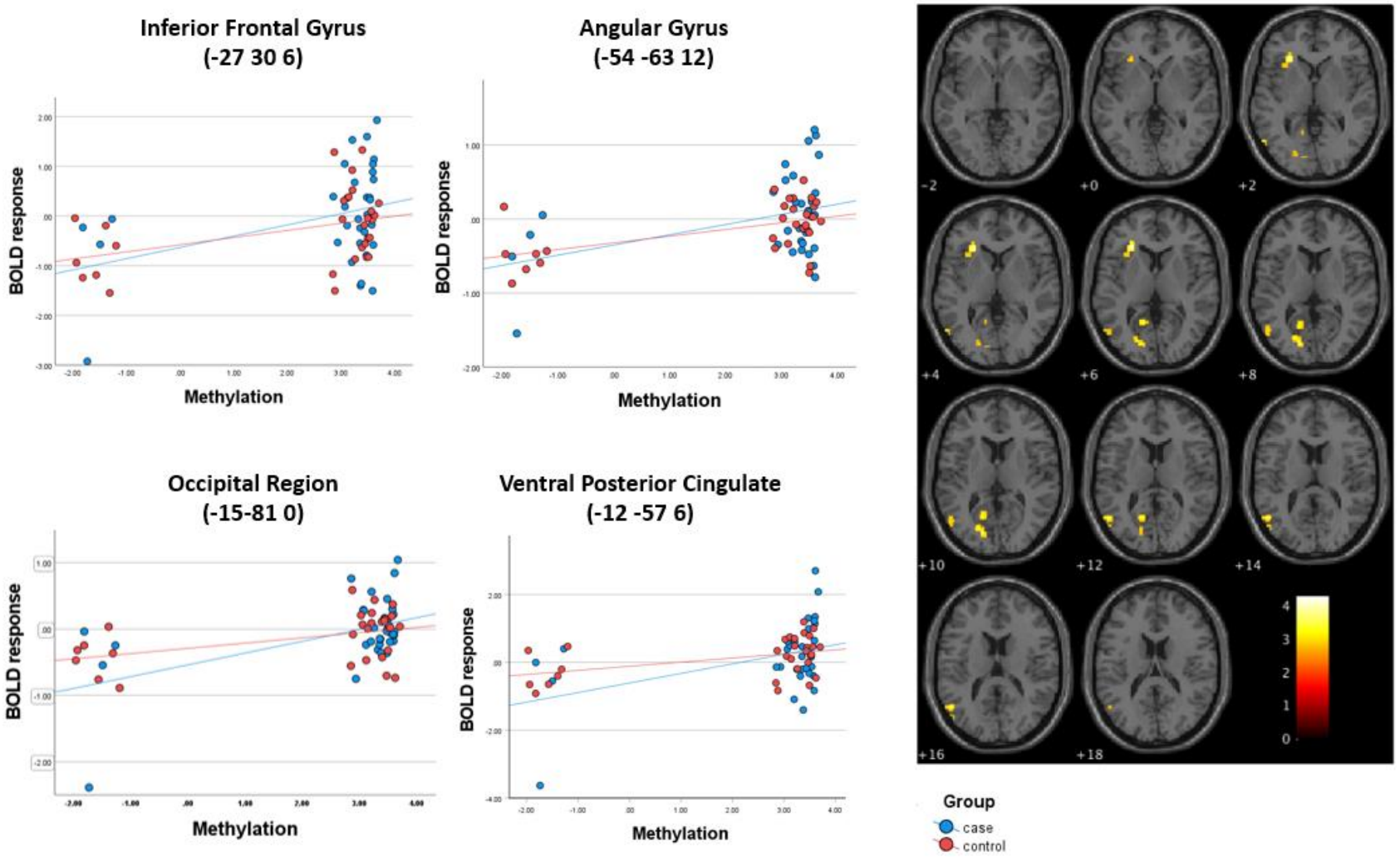


Figure 23. Anger vs. neutral significant clusters and scatter plots of correlation between methylation and BOLD response. Differential activation in response to angry as compared to neutral-emotion faces was associated with methylation multiple regions. *N.B. The colour of the highlighted voxels indicates the magnitude*

of the t-statistic, which is an indicator the level of BOLD signal activation in the region, as indicated in the colour bar on a scale from 0 = Black/Red (low) – 4 =Yellow/White (High).

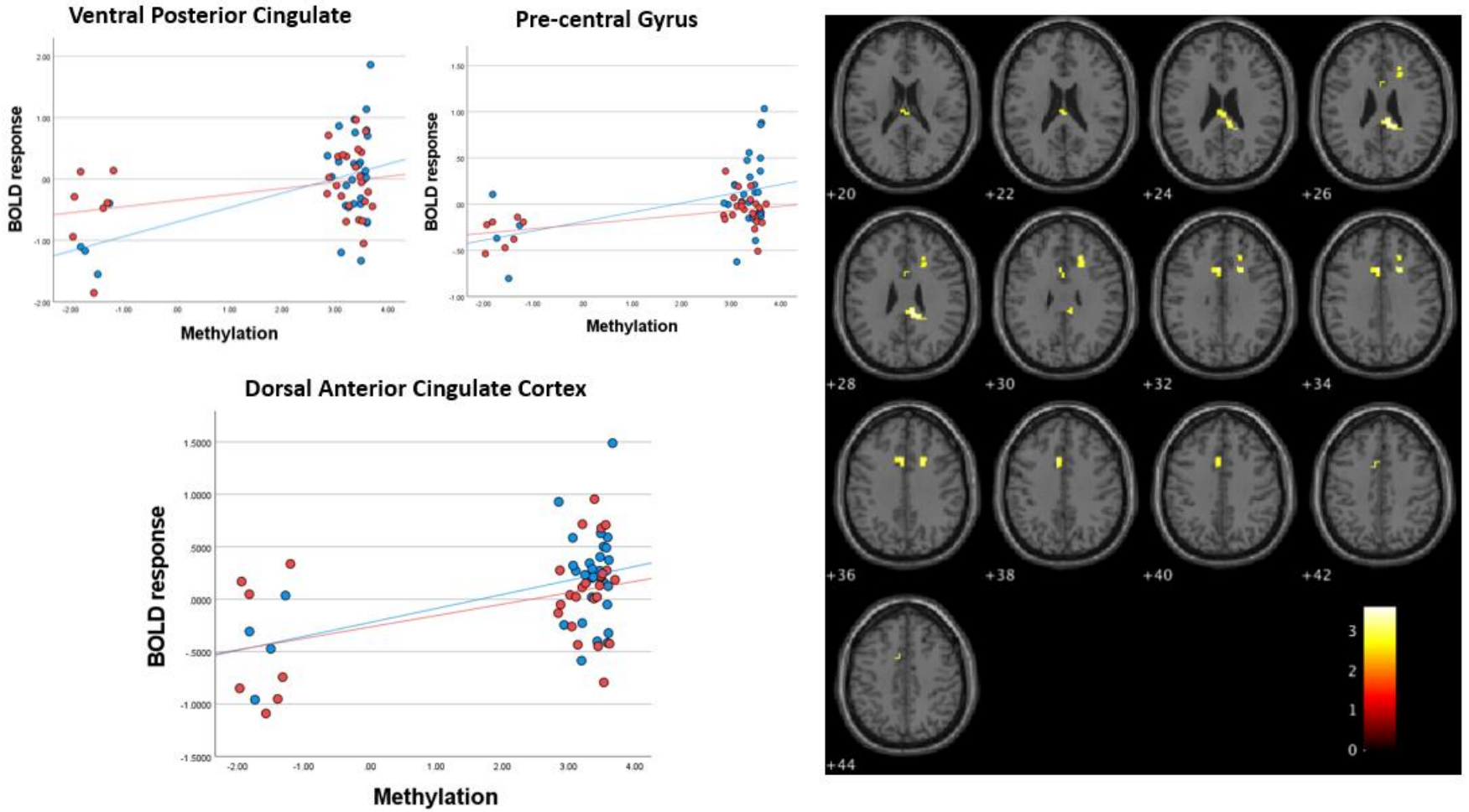


Figure 24. All fear vs. neutral significant clusters and scatter plots of correlation between methylation and BOLD response. Differential activation in response to fearful as compared to neutral-emotion faces was associated with methylation in multiple regions.

N.B. The colour of the highlighted voxels indicates the magnitude of the t-statistic, which is an indicator the level of BOLD signal activation in the region, as indicated in the colour bar on a scale from 0 = Black/Red (low) – 4 =Yellow/White (High).

4.4 Discussion

This study aimed to investigate the association between methylation level of the *SLC25A24* gene and brain response during an emotional face processing task. Both ROI and whole-brain exploratory analyses were performed. I first tested the hypothesis that elevated methylation of the *SLC25A24* gene would be associated with brain response in regions where the *SLC25A24* gene is expressed and which have also been implicated in emotional processing in CD (i.e., ROIs of the hippocampus, amygdala, hypothalamus, basal ganglia). In the ROI analysis, I found no significant association between methylation level and brain response in any of these regions for either the all faces>fixation, anger> neutral or fear>neutral contrasts. Secondly, as higher methylation of the *SLC25A24* gene was associated with a reduction in GMV in several brain regions in previous work (chapter 3), I also conducted a whole-brain exploratory analysis to investigate the association between brain response and *SLC25A24* methylation across the whole brain. Across the three contrasts, this analysis revealed positive associations between methylation level and brain response in both cortical and subcortical regions. Interestingly, for the all faces>fixation contrast I did observe that brain response in the caudate (part of the basal ganglia) was positively associated with methylation of the *SLC25A24* gene, therefore partially supporting the first hypothesis. These findings will now be discussed in more detail by considering four key themes. Firstly, how we can interpret the fact that I observe only positive associations between *SLC25A24* methylation and brain response. Secondly, I will discuss the differences in brain regions activated in response to the separate face contrasts. Thirdly, I will focus on the distinct patterns of association between methylation and brain markers I observe in CD vs. TD youth. Finally, I suggest that the findings point towards an opposite association

between CU traits and brain responses in females with CD, compared to that reported in previous studies in males with CD.

4.4.1 Positive Associations between Methylation and Brain Response

Across the three contrasts I examined, I found only *positive* associations between methylation level of *SLC25A24* and brain response. Previously, only a limited number of studies have examined the association between DNA methylation and task-related brain response with functional MRI data and most of these adopted the candidate gene approach as opposed to examining genome-wide methylation (Wheater et al., 2020). For example, while several studies have reported a positive association between *SLC6A4* gene methylation and amygdala response during emotional face processing tasks (Ismaylova et al., 2018; Nikolova et al., 2014; Schneider et al., 2017; Swartz et al., 2017), there are also inconsistent findings for several genes. For instance, PFC activation during working memory tasks has been both positively (Walton et al., 2014) and negatively (Ursini et al., 2011) associated with methylation of the *COMT* gene, while the effect of *BDNF* methylation on PFC activation in working memory tasks appear to be opposite in individuals with and without early-life experiences of hypoxia (Ursini et al., 2016) genes.

Because of the inconsistencies in previous studies, it was difficult to formulate general hypotheses for the direction of association between methylation and brain response based on previous experimental findings. In addition, predicting this association from a theoretical viewpoint is also not trivial, as the nature of this relationship depends on several factors (Wheater et al., 2020). These include features such as the specific biological function of the gene

in question, the location of the differentially methylated region and the brain region in which activation is being measured (as different brain tissues are known to have unique methylation signatures (Ladd-Acosta et al., 2007). For example, elevated methylation in the promotor regions of genes and on 'CpG islands' areas have been shown to have a silencing effect (Brenet et al., 2011). Therefore, increased methylation in this region of a gene will have an opposite effect on brain activity when this modification occurs on a gene involved in maintaining/establishing neuronal pathways compared to that same epigenetic modification on a gene which catalyses neuronal death/pruning (Hwang, Aromolaran, & Zukin, 2017; Jobe & Zhao, 2017).

Additionally, although the association between increased methylation and promotor silencing is the most commonly suggested mechanism of action for this epigenetic modification (Medvedeva et al., 2014), in recent studies hypermethylation has been associated with un-altered or upregulated gene expression (Wan et al., 2015). The reported direction of association between methylation of a single gene and brain function are mixed and appear to depend on the specific brain region where response is recorded and also interacts with other genetic variation (genotype). For example, while *BDNF* gene methylation is reported to be positively correlated with activity in the ACC (Moser et al., 2015), methylation level at this gene also interacts with *BDNF* genotype to determine the direction of association with prefrontal lobe function (Ursini et al., 2016). Furthermore, *BDNF* methylation is reported to be negatively correlated with hippocampal activation in animal models of caregiving (Roth, Matt, Chen, & Blaze, 2014). These findings demonstrate that the direction of association between methylation and brain response may vary, even for the same gene.

In this study, I used methylation data from exon 1 of the *SLC25A24* gene, which is involved in mitochondrial function. Decreased expression of this gene has been linked to mitochondrial dysfunction and a decrease in cell energy production (Babenko, Smagin, Galyamina, Kovalenko, & Kudryavtseva, 2018). As methylation of the first exon is most commonly accompanied by down-regulation of the modified gene (Brenet et al., 2011), my findings of positive correlations between *SLC25A24* methylation and BOLD response during emotional face processing suggests a mechanism whereby elevated methylation inhibits the expression of this gene, which, in turn, appears to relate to an increased level of brain response in a given region. This is biologically plausible, as *SLC25A24* is widely expressed in neuronal tissues (Hawrylycz et al., 2012) and elevated methylation of this gene is associated with decreased cell energy production (Babenko et al., 2018). Thus, this low energy availability may impede efficient neuronal function and may mean a higher volume of blood is needed in a certain brain region to produce the same energetic effect (Hillman et al., 2015), hence potentially accounting for the observed increased BOLD response when viewing negative (i.e., angry and fearful) emotional faces. This interpretation is in-line with previous suggestions that elevated response in a specific brain region on viewing facial stimuli indicates a higher level of 'inefficient' internal processing (Hillman et al., 2015). In this context, my results suggest that reduced expression of gene *SLC25A24* and the consistent pattern of increased response in multiple brain regions during the processing of emotional faces is indicative of more effortful (thus less effective) processing of facial expressions in these regions in individuals who have higher levels of methylation in this region.

4.4.2 Interpreting Specific Regions Activated for the Different Face Contrasts

All faces>Fixation contrast findings: For the all faces>fixation contrast, *SLC25A24* methylation level was positively associated with brain response in four regions. The largest cluster was in the ventral caudate. This region forms part of the basal ganglia, one of the ROIs in which I hypothesized there may be an association between methylation and brain response during emotional face processing. While the hypothesis that methylation would be associated with response to emotional faces in the ROI as a whole was not supported, these findings in the ventral caudate indicate a more localized effect of increased activation in this region in relation to elevated methylation. It is likely that this effect was obscured as I used the whole and larger region of the basal ganglia as an ROI. Increased BOLD response in the caudate has previously been associated with a wide range of processes and disorders – from reward learning in TD populations (e.g. (Haruno et al., 2004)) to manic states of bipolar disorder (Blumberg et al., 2000). Abnormalities in the caudate are also associated with impaired affective decision-making in the most clearly articulated neurobiological model of CU traits (Blair, 2013).

Anger>Neutral contrast findings: For the angry>neutral contrast, *SLC25A24* methylation level was related to brain response in the left AI, left occipital lobe and the left calcarine cortex. These regions have all been previously associated with the processing of angry facial expressions in healthy populations (Somerville, Fani, & McClure-Tone, 2011). Increased response to emotional faces in the AI has also previously been reported in females with CD (Fairchild et al., 2019), and dysfunction in this region is consistent with findings of lowered empathic accuracy (Martin-Key, Allison, & Fairchild, 2020) and poor emotion recognition (Decety, Michalska, Akitsuki, & Lahey, 2009) in this group.

Fear>neutral contrast findings: In the fear>neutral face contrast, *SLC25A24* methylation level was positively correlated with brain response in the left dorsal ACC, right PCC and right SFG. These regions are all implicated in the processing of fearful facial expression in healthy individuals (Somerville et al., 2011) and in this group the ACC also plays an important role in modulating fear expression and responding (Etkin, Egner, & Kalisch, 2011). I previously reported a negative association between GMV in the left SFG and methylation (chapter 3), but given that the brain responses during emotional processing are in the right hemisphere, these structural and functional findings are unlikely to be directly linked.

4.4.3 Distinct Patterns of Association between Methylation and Brain Markers in CD vs. TD youth

As mentioned in the first study of this thesis, methylation of the SLC25A24 gene has an opposite direction of association with level of CU traits in female youth with CD, as compared to that observed in TD youth. Several other examples of differential gene/brain/behaviour associations in psychiatric as compared to healthy control groups have been documented (e.g. (Aghajani et al., 2018; Choi et al., 2015; Pereira et al., 2017; Raznahan et al., 2009). For example, Aghajani et al (2018) reported that elevated OXTR methylation and higher levels of CU traits predicted increased cortical activity but lower amygdalo-cortical connectivity in male participants with CD (Aghajani et al., 2018), but the opposite patterns were observed in TD youth (see Figure 5). Also, epigenetic modifications of the BDNF gene associated with an increase in serum BDNF concentration have been associated with reduced white matter tract integrity in MDD (Choi et al., 2015), but this association is the reversed in healthy controls. Similarly, group-by-genotype interaction effects have been reported in patients with ASD vs. healthy controls, for the effect of

an SNP of the BDNF gene on cortical volume and surface area in frontal brain regions (Raznahan et al., 2009). Specifically, in the ASD group, cortical volume was greater in BDNF gene met allele carriers compared to BDNF val-allele homozygotes, whereas the opposite direction of association was seen in controls (Raznahan et al., 2009). However, the mechanisms underlying these differences remain poorly understood (Kirsch, 2015; Pine, Ernst, & Leibenluft, 2010). One theory suggests that differential gene/environment interactions are at work in healthy and clinical populations, and that these subsequently catalyse distinct biobehavioural outcomes (Kirsch, 2015; Mechelli et al., 2012). These findings suggest that the results obtained in this study may not specific to CD and may also be observed in various psychiatric disorders. This would be in-line with previous observations that, in some cases, neurobiological abnormalities appear to cut across the conventional diagnostic categories of psychiatry (Pereira et al., 2017).

4.4.4 Association between Methylation, CU and Brain Responses in Females

Much of the previous research on CD, CU traits and/or methylation in relation to neuroimaging markers involves only male participants (Freitag et al., 2018), thus it is difficult to draw direct comparisons to my findings from a female-only sample. However, two recent neuroimaging studies from the FemNAT-CD group report that the association between CU traits and brain markers (amygdala response and white matter integrity) appears to be opposite in females and males with CD (De Brito et al, in preparation; (Villemonteix et al., 2021)). Specifically, De Brito et al observed a positive association between amygdala response to fearful faces in females with CD and high CU traits (De Brito et al, in preparation), which is opposite to the previously reported findings in males with CD (e.g., Viding et al, 2012). In a DTI study, Villemonteix et al (2021) showed that axial diffusivity in the left uncinate fasciculus was positively associated with CU traits in males (Villemonteix et al., 2021), while the opposite pattern was noted in females. Similarly, the findings in this study for angry and fear suggest that there might be an opposite association between CU traits and brain response in males and females. I observed only positive associations between methylation (which is positively correlated with CU traits in the CD group) and brain response in females, which is opposite to findings in males with antisocial behaviours (Dotterer et al., 2020) where CU traits have generally been found to be negatively associated with brain response during emotion processing.

In response to angry faces, I showed that *SLC25A24* methylation level was positively related to brain response in the bilateral AI, left occipital lobe and the left calcarine cortex. Atypical brain response during emotional face processing in these regions has previously been reported in males with CD (Alegria et al., 2016), but a reduction in response has generally been reported in this group (e.g. De Brito et al, 2021; Villemonteix et al, 2021). Additionally, previous research has

demonstrated reduced activation in the occipital lobe in males with CD, compared to their TD peers, when viewing fearful facial expressions (Sethi, O'Nions, McCrory, Bird, & Viding, 2018). fMRI studies in male with CD have also identified reduced response in occipital regions during empathy tasks (Alegria et al., 2016). Interestingly, in a recent study on a mixed-sex sample, lowered response in the calcarine cortex during emotional face processing was also reported for the CD group compared to the TD group, but only after correcting for the differences in eye-gaze behaviour between the two groups (Menks et al., 2021).

4.4.5 Recognition of Facial Expressions and the CD Phenotype

The capacity to accurately interpret and respond to facial cues relating to negative emotions, particularly fear and sadness, is understood to be an important precursor for socialization and prosocial behaviours and it has been suggested that lacking these abilities may contribute to antisocial and aggressive behaviours that are instrumental in nature (Blair, Budhani, Colledge, & Scott, 2005). In-line with this, previous studies have shown that youth with CD and high levels of CU traits have a reduced ability to recognise fearful facial expressions (Fairchild, Van Goozen, Calder, Stollery, & Goodyer, 2009; Fairchild et al., 2010). Studies in male youth have demonstrated that impaired ability to recognise fearful expressions might be associated with reduced response in the amygdala (Jones, Laurens, Herba, Barker, & Viding, 2009; Marsh et al., 2008). Also, in adult males with psychopathy, both the amygdala and fusiform gyrus are reported to show reduced response to fearful faces (Deeley et al., 2006; Dolan & Fullam, 2009).

There is growing evidence from healthy participants to suggest that the neural correlates of fear recognition are sex-specific (Kempton et al., 2009) and correspondingly my results in female youth with CD appear to differ from the findings mentioned above in males. Specifically, I observed elevated response in the left dorsal ACC, right PCC and right SFG in female youth with CD and greater CU (i.e. higher *SLC25A24* methylation) in response to fearful faces. This may suggest that, while the same networks are implicated in impaired emotional face processing in both males and females with CD, the dysfunction may be different in nature. While confirmation in mixed-sex participant groups is needed, these findings, considered together with the patterns of reduced brain responses in males with CD, tentatively suggest that the association between brain response and CU traits in youths with CD might be sex-specific.

4.4.6 Strengths and Limitations

There are several strengths to this study. It is the first on females with CD to combine genome-wide epigenetic and fMRI data, thereby significantly increasing our understanding of the association between epigenetic modifications and brain response in this population. Secondly, I re-ran the original DNA methylation analysis model with the sub-sample of 62 participants and again identified the same DMR, confirming the M-values for gene *SLC25A24* calculated in my previous work were a robust finding and valid to be used in this subsample. Third, the sample was very well characterized, with all participants undergoing thorough semi-structured interviews for psychiatric disorders and symptoms based on DSM-IV-TR criteria. Finally, the groups were matched on important demographic variables (e.g., age, puberty, ethnicity) thereby reducing the likelihood of these factors confounding my results.

There are, however, some limitations that should be noted. Firstly, the sample size of 62 participants is small and thus only allowed me to detect effect sizes > .34 with a minimum of 80% power, as calculated through sensitivity analysis in G*power (Faul, Erdfelder, Buchner, & Lang, 2009). Although, the sample size is similar to the only other epigenetic neuroimaging study on CD (Aghajani et al., 2018), this analysis was underpowered to investigate the association between *SLC25A24* methylation and brain response separately in the CD and TD groups, which made my results more difficult to interpret. Moreover, the fact that I chose to focus on methylation of a single gene rather than investigate the association between genome-wide methylation and brain response means some of the limitations inherent to traditional candidate-gene studies (e.g. see (Duncan et al., 2019) also apply to this work. For example, it has been shown for other psychiatric disorders (e.g. MDD; Border et al., 2019, Schizophrenia; Collins et al., 2012) that alterations of individual regions of the genome contribute to only a small fraction of an the variation in an observed phenotype.

Secondly, because I relied on methylation levels derived from salivary DNA rather than brain-derived DNA, I cannot be certain that the methylation data precisely parallel methylation patterns in brain tissues. However, several studies have demonstrated concordance between peripheral-tissue methylation and that of brain tissues (e.g.(Braun et al., 2019), but see (Gutierrez-Arcelus et al., 2015)).

Thirdly, the CD and TD participant groups do show significant differences in comorbidity rates for other psychiatric symptoms (i.e. ADHD and MDD symptomology), thus I cannot rule out the possibility that these are acting as confounding variables in my analyses. However, I determined that controlling for these variables would not be appropriate, as CD is commonly associated with these comorbid diagnoses, especially in females. Additionally, although participants were asked

to refrain from substance use prior to experimental sessions participants were not tested to verify this thus variations in levels and types of substances used by participants may have affected both the epigenetic and imaging findings reported here.

Fourthly, we did not include as ROIs some of the key regions known to be activated during emotional face processing, for example the fusiform gyrus, insula and ACC (Fusar-Poli et al., 2009). These regions were excluded because there was no reported expression of the *SLC25A24* gene in the GTEx database in these areas. However, as our analysis approach did not include these key regions, this work is unable to shed any light on how variation in DNA methylation is related to the differential activation in these regions during emotional face processing in relation to CD and/or CU traits.

Finally, due to funding the sample size was limited therefore I chose to focus specifically on female participants to maximise the impact and novelty of my work, as females with CD are under-researched (Freitag et al., 2018). However, as this study includes only females, I could not test if my findings were sex-specific, as have previously been reported in neuroimaging studies investigating CD and CU traits (e.g. (De Brito et al., in preparation; Rogers et al., 2019; Villemonteix et al., 2021)). Studies with larger, mixed-sex participant groups will be an important direction for future research in this field.

4.4.7 Summary and Conclusions

Overall, these results indicate that levels of methylation of the *SLC25A24* gene are positively associated with brain response in several regions, including the caudate, para-hippocampal regions, STC and AI, on viewing emotional face stimuli. I also observed emotion-specific positive relationships between methylation and brain response in several cortical and subcortical

regions during an emotional face-processing task. I observed no significant association between *SLC25A24* methylation and BOLD response in the ROIs of the hippocampus, amygdala, hypothalamus or basal ganglia, either in response to human faces in general, or for emotionally contrasting facial stimuli. However, whole-brain analysis did indicate activation in the caudate (part of the basal ganglia) was positively associated with methylation for the all faces>fixation contrast. These findings demonstrate different directions of association between CU traits, methylation and brain response in females with CD vs. TD females, in-line with previous work documenting distinct patterns of associations between brain markers, epigenetic factors and behavioural measures in healthy control vs. psychiatric groups. Finally, those findings, which differ from those of previous studies on males with CD, support the idea that the association between brain response and CU traits might be sex-specific and of opposite direction.

CHAPTER 5 – STRUCTURAL EQUATION MODELLING TO UNCOVER THE RELATIONSHIP BETWEEN *OXTR* GENOTYPE, SEX, CU TRAITS, AMYGDALA ACTIVATION AND NUMBER OF CD SYMPTOMS

5.1 Introduction

5.1.1 Overviews of CD and CU traits

Individuals with a psychiatric diagnosis of CD form a heterogeneous population characterised by recurrent displays of rule-breaking behaviours that contravene the rights of others and neglect social norms (APA, 2013). These behaviours have a high cost to society through increased rates of violent crime, recidivism and the economic burden associated with prison and rehabilitation services (Anderson & Kiehl, 2014). In particular, youth with CD and high levels of CU traits (i.e., lack of guilt/remorse, shallow emotionality and deficient empathy) display more severe and enduring patterns of antisocial behaviours, compared to those with CD and low levels of CU traits (Frick & Viding, 2009). As a result, the most recent edition of the DSM-V includes a LPE specifier (APA, 2013) to identify youth with CD and high CU traits who are at greater risk of poorer long-term outcomes than youth with CD and low levels of CU traits. The affective and neurocognitive profiles of youth with CD and high CU traits also show similarities to those observed in adults with psychopathic personalities (Blair, 2013). Therefore, research on this specific subgroup of youth with CD is critical to increase our understanding of the developmental risks for psychopathy and to develop effective prevention and treatment efforts.

5.1.2 Emotional Face processing, Amygdala Activation and CU traits in CD

Impairments in emotional processing, specifically atypical responses to threatening cues such as angry or fearful faces, have repeatedly been shown in adolescents with CD (Fairchild et al.,

2019; Martin-Key et al., 2018). The amygdala is one of the brain regions that has most commonly been implicated in impaired emotional face processing in this population as recently shown in several meta-analyses of fMRI studies in this population (Alegria et al., 2016; Noordermeer, Luman, & Oosterlaan, 2016; Raschle et al., 2015). Thus, a prominent neurocognitive model posits that youth with CD display distinct patterns of amygdala response to threatening cues according to their levels of CU traits (Blair, 2013). Specifically, research has shown that youth with CD and high CU traits are characterized by amygdala hypo-responsivity to fearful faces compared to TD youth (Jones, Laurens, Herba, Barker, & Viding, 2009; Marsh et al., 2008) whereas those with CD and low levels of CU traits show increased amygdala response compared to both TD youth and those with CD and high CU traits (Sebastian et al., 2014; Viding et al., 2012). There is further evidence from youth studies adopting a variable-centred approach that antisocial behaviour is positively associated with amygdala response to emotional faces (both threatening, such as anger and fear, and happy faces) traits (e.g. (Dotterer et al., 2020; Lozier, Cardinale, VanMeter, & Marsh, 2014)).

However, some studies have reported no evidence for an association between brain responses to emotional faces and level of CU traits. For example, Passamonti et al (2010) observed no correlation between level of CU traits and brain response in the amygdala (or any brain regions), either across their whole participant sample (which included youth with childhood-onset CD, youth with adolescent-onset CD and control youth) or in individual groups (Passamonti et al., 2010). Similarly, a recent community-based study on youth aged 11-15 years reported that higher levels of antisocial behaviour were associated with increased right amygdala activity to emotional faces, but that there was no relationship between amygdala reactivity to faces and

level of CU traits (Dotterer, Hyde, Swartz, Hariri, & Williamson, 2017). Overall, these findings demonstrate that, given the amygdala plays is a central component in the current model of CD, further research is needed to fully understand whether, and if so how, CU traits is related patterns of amygdala activation within youth with CD.

5.1.3 The Oxytocin System and Emotional Processing

Recent studies have also demonstrated that varying levels of certain neurohormones in the brain, which subsequently affect functionality of specific brain regions, may be a significant factor contributing to individual differences in emotional processing (Ali, Begum, & Reza, 2018). In humans, the neurohormone Oxytocin is strongly implicated in social and emotional behaviour (Puglia, Lillard, Morris, & Connelly, 2015), and is thus potentially relevant to both CD and CU traits (Cecil et al., 2014; Dadds et al., 2014; Dadds & Rhodes, 2008). Oxytocin is a neurohypophysial peptide with a 9 amino-acid structure, synthesized by neurons in the hypothalamus (Russell et al., 2018). The Oxytocin system is governed by three main structural genes: the principal gene coding for Oxytocin (*OXT*) - this gene is also involved in coding for neuronal pentraxins, which are heavily involved in synapse formation and plasticity-, the *OXTR* gene and the central Oxytocin secretion gene (*CD38*).

Oxytocin primarily exerts its effects through the *OXTR* gene (Kraaijenvanger et al., 2019). The *OXTR* gene is located on chromosome 3 and includes three introns and four exons (Inoue et al., 1994). Inter-individual variation in levels of expression of the *OXTR* gene between people may be partly explained by SNPs of this gene. SNPs occur when the DNA sequence between two individuals differs by a single nucleotide (adenine, thymine, cytosine or guanine). Additionally, some SNPs are more likely than others to play a governing role in the function of a gene due to

their specific type (for example, if they influence the structure of a transcription factor binding site) and their location. Specifically, SNPs located in the 3'untranslated region (3'UTR) have been shown to have a key role in gene expression differences between individuals (Kim & Bartel, 2009) as they interfere with polyadenylation, regulatory protein-miRNA and miRNA-miRNA processes (Arnold, Ellwanger, Hartsperger, Pfeufer, & Stumpflen, 2012) and thus influence mRNA stability and translation. Two 3'UTR variants of *OXTR* that have previously been in investigated in relation to social behaviour and cognition include rs1042778 (Creswell et al., 2015; Wade, Hoffmann, & Jenkins, 2015) and rs6770632 (Lerer et al., 2008; Malik et al., 2012; Zhang, Liu, Chen, & Zhang, 2020). In particular, rs6770632 previously been linked to aggressive behaviour in females (Malik et al., 2012). Additionally, research has also highlighted two *OXTR* SNPs that are specifically linked to structural and functional differences in the emotional circuitry of the brain, these are rs2254298 (Tost et al., 2010; Tost et al., 2011) and rs53576 (Furman, Chen, & Gotlib, 2011; Inoue et al., 2010). Interestingly, rs53576 is an intronic variant between exon 4 and 5 known to be associated with CU traits (Ezpeleta et al., 2019) and also amygdala structure and function (Furman et al., 2011; H. Inoue et al., 2010). Specifically, the rs53576 A-allele has been significantly associated with impaired empathy (particularly observed in youth with CD and high CU traits (Blair, 2013) and increased emotional reactivity (Baribeau et al., 2017) (observed particularly in youth with CD and low levels of CU traits (Masi et al., 2014).

Experimental manipulation of Oxytocin levels has been shown to influence brain responses to social cues such as emotional faces (Domes et al., 2010) and, in particular, previous research in healthy adult populations has shown that epigenetic modifications to the *OXTR* gene are

uniquely associated with differences in brain responses to emotional faces (Puglia et al., 2015). Imbalances in the oxytocinergic system have also been linked to atypical amygdala responses during socio-affective processing (Puglia et al., 2015). Specifically, increased methylation of the *OXTR* gene, a biological marker of reduced gene expression, has been shown to be significantly associated with increased amygdala response to both angry and fearful faces (Puglia et al., 2015). Research has also demonstrated that amygdala response to emotional faces is mediated by *OXTR* genotype in clinical populations, such as, for example, patients with Schizophrenia (Haram et al., 2016).

In summary, there is evidence that variations in oxytocin levels influence key socio-affective behaviours, including emotional recognition, emotional responding and emotional learning (Bartz, Zaki, Bolger, & Ochsner, 2011; Kirsch, 2015). Therefore, this neurohormone is likely of great relevance in both CD and CU traits (Cecil et al., 2014; Dadds et al., 2014; Dadds & Rhodes, 2008). Accordingly, genotype of the *OXTR* gene has been specifically linked to severe conduct problems and high CU in youth (Beitchman et al., 2012; Dadds, Moul, et al., 2014) and epigenetic modifications of this gene have also been associated with high levels of CU traits (Cecil et al., 2014; Dadds, Moul, et al., 2014). These findings indicate that genes involved in governing the Oxytocin system, in particular the *OXTR* gene, may play an important role in contributing to the socio-affective impairments observed in both conduct problems (Salvatore & Dick, 2018) and CU traits (Moore et al., 2020). Furthermore, dysregulation of the oxytocin neuropeptide system in interaction with stress system measures has been reported specifically for females with CD in another study from the FemNAT-CD group (Bernhard et al., 2021), indicating that this neurohormone may produce sex-specific effects within youth with CD. Take

together, these findings provide further grounds to investigate how the oxytocin system relates to CD and CU traits in females.

5.1.4 Emotional Processing in CD and the *OXTR* System: Sex Matters.

The majority of studies examining the neural correlates of emotional processing in CD have focussed on males (Fairchild et al., 2019; Freitag et al., 2018) thus limiting our understanding of the neural correlates of emotional processing in females with CD and varying levels of CU traits. However, a small emerging body of studies have demonstrated sex-effects on both brain structure (Smaragdi et al., 2017) and function (Cao, Sun, Dong, Yao, & Huang, 2018). For example, a recent surface-based morphometry study from the FemNAT-CD consortium reported several sex effects in frontal regions, such that males with CD had lower cortical thickness in the supramarginal gyrus, but females with CD presented with higher cortical thickness, as compared to their TD counterparts (Smaragdi et al., 2017) In contrast, in the frontal gyrus high gyrification was observed in males with CD, but gyrification was significantly reduced in females with CD, as compared to their TD counterparts. A recent resting-state fMRI study that directly compared spontaneous brain activity in males vs. females with CD reported higher levels of activity in males in the insula and left putamen, but significantly reduced activity in the left ACC, left frontal gyrus and the left temporal gyrus in males compared to the females (Cao et al., 2018).

Taken together, these findings indicate that males and females with CD might be characterised by distinct structural and functional brain abnormalities that need to be considered if we are to increase of understanding of the pathophysiology of CD to, in turn, develop sex-specific prevention and treatment efforts (Fairchild et al., 2014; Fairchild et al., 2019; Freitag, 2018).

Oxytocin has also been suggested to have sex-specific effects on social behaviour through sex differences in the mechanisms by which oxytocin regulates brain activity (Duarte-Guterman et al., 2020). In-line with this, sex differences in the effects of endogenous oxytocin administration on brain activity have been reported in studies investigating amygdala responses to human faces (Gao et al., 2016). In particular, oxytocin administration has been repeatedly shown to reduce amygdala activation to fearful stimuli in males (Kirsch et al., 2005; Labuschagne et al., 2010), but associated with the opposite effect in females (Bernhard et al., 2016).

5.1.5 Aims of Study and Research Questions

Given the current evidence that level of CU traits, sex and genetic variation associated with the *OXTR* gene are important in understanding brain responses to emotional faces in both healthy and clinical populations, this study aimed to shed light on the nature of the associations between these factors and investigate whether particular combinations of factors predicted variation in CD symptomology. Specifically, I aimed to investigate which SNP to brain to behaviour associations contribute most to the variation in numbers of CD symptoms. Thus, the research aims were to use an SEM approach to investigate whether allelic variation in the *OXTR* gene was associated with number of CD symptoms in a large mixed-sex sample of youth, and to examine whether this association was mediated through participants' level of amygdala response during emotional face processing or CU trait score.

Genotyping data from individual SNPs on the *OXTR* gene were analysed in conjunction with amygdala Blood Oxygen Level Dependant (BOLD) signal data obtained from an emotional face processing task previously used in both males (Passamonti et al., 2010) and females with CD (Fairchild et al., 2014). This was used to investigate whether brain response to threating (i.e., angry and fearful) faces varies according to *OXTR* genotypes, level of CU traits or sex, and if so

whether these factors are directly or indirectly associated with variation in the number of CD symptoms. The two SNPs selected were rs53576 (as it has been previously implicated in relation to both CU traits (Ezpeleta et al., 2019) and amygdala structure and function (Furman et al., 2011; H. Inoue et al., 2010)), and rs6770632 (as it has previously been implicated in aggression (Malik et al, 2012)). Consistent with previous work in this field (da Cunha-Bang, Fisher, Hjordt, Holst, & Knudsen, 2019; Waller et al., 2016), I used an SEM approach to investigate the associations between these variables. The sample included both youth with a clinical diagnosis of CD and TD youth, thus representing a wide range of CD symptoms. I also created an overall composite risk score for *OXTR* based on the summary statistics of the EAGLE GWAS study on aggression, incorporating data from 34 SNPs of this gene. I used this risk score in the SEM to determine whether, at gene-level, variation of the *OXTR* gene was significantly associated with number of CD symptoms. My main hypothesis was that *OXTR* genotype would be associated with amygdala response to emotional faces, which would in turn be related to the participant's number of CD symptoms. For the composite *OXTR* score, I predicted a positive association (as the magnitude of this score was calculated based on weightings taken from a meta-analysis identifying 'risk' alleles associated with aggression (Pappa et al., 2016). I also predicted that some of the variance in number of CD symptoms would be due to direct variation in *OXTR*, but that there would also be an indirect effect via pathways involving other factors, such as the individual's level of CU traits (Fairchild et al., 2019; Moore et al., 2019; Viding et al., 2012), IQ (Fairchild et al., 2019; Koenen, Caspi, Moffitt, Rijsdijk, & Taylor, 2006) and sex (Meier, Slutske, Heath, & Martin, 2011; Waller et al., 2016).

5.2 Methods

5.2.1 Participants

A sample of 349 youths (189 females; 54.2%) aged 9-18 years (M=13.9; SD=2.6), which included 61 females with CD, 128 TD females, 74 males with CD and 86 TD males, was recruited across four sites, as part of the FemNAT-CD study (Freitag et al., 2018). In addition to standard FemNAT-CD inclusion and exclusion criteria (see (Rogers et al., 2019)), participants were required to have provided saliva-DNA of adequate quality for *OXTR* genotype information to be extracted using the Illumina Infinium Global Screening Array V3.0 + PsychChip kit, and also useable functional MRI data from the emotional face processing task (Passamonti et al., 2008).

5.2.2 Clinical and Psychometric Measures

Detailed information about the procedures used to collect all clinical and psychometric data is provided in previous work by the FemNAT-CD group (Rogers et al., 2019). In brief, trained research staff interviewed participants and their parents/caregivers separately using the Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime version (K-SADS-PL (Kaufman et al., 1997)) to assess CD symptoms and screen for other DSM-IV-TR psychiatric disorders. Further questions from the supplementary sections of the K-SADS-PL (e.g. for ODD/ADHD) were also included if the initial screening identified additional psychiatric symptoms. CU traits scores were obtained using the parent-version of the ICU (Frick, Cornell, Barry, Bodin, & Dane, 2003). Total, verbal and performance IQ scores were obtained using the Wechsler Abbreviated Scale of Intelligence (Wechsler, 2011) in the UK and the Wechsler Intelligence Scale for Children, Fifth Edition (Wechsler, 2014) at other sites. Pubertal status was determined using the PDS (Petersen, Crockett, Richards, & Boxer, 1988), completed

by participants aged 12 years or older, or by the parents/caregivers where participants were younger than 12.

5.2.3 *OXTR* Genotyping Analysis

DNA was extracted from saliva within 7 days of collection using the Oragene OG-500 Kit. DNA quality cutoff was a 260/280 ratio above 1.8. DNA was stored at -80°C immediately. Genotyping, and genomic imputation was conducted using the Illumina Infinium Global Screening Array V3.0 + PsychChip kit by the Department of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy at Goethe University, Frankfurt as previously published (Yousaf et al., 2020). This included rigorous data-cleaning procedures and imputation of missing genotypes, detection/confirmation of gender and chromosomal anomalies, relatedness and population structure (ancestry), detection of batch effects, Mendelian error detection and duplication error detection. Quality thresholds are following date of the art and are published elsewhere (Yousaf et al., 2020). SNPs within +/- 2kb of the *OXTR* gene were exported.

In total, participant data for 34 SNPs were obtained and used to create an overall composite risk-score for the *OXTR* gene by combining the contributions from all 34 SNPs. I calculated effect sizes for the alleles based on log beta values taken from a recent meta-analysis investigating genome-wide associations between different SNPs and levels of aggressive behaviour in children (Pappa et al., 2016). For each SNP, I then calculated the values of the product of the number of minor alleles of an individual at the respective locus (0, 1 or 2) by the beta value weighting published for that SNP. (Please see Appendix C1 for more details).

I also investigated two individual SNPs of interest. These SNPs were selected based on a review of the Literature via Prospecotr; HuGe Navigator and the Pubmed database (the search terms

entered were ("oxytocin*" and/or "OXT" and/or "*OXTR*") and ("conduct disorder", "aggression" and/or "stress"). Initially each SNP was ranked by the number of publications, then the abstracts of papers were examined to determine whether there had been a positive finding (i.e., whether there was evidence supporting an association between the SNP and the behavioural trait captured by the search terms). SNPs were included in the final list if they fulfilled one or more of the following criteria: (i) good evidence of an association with variables of interest based on replicated findings, (ii) multiple sources of weak evidence presented (different association studies, different phenotypes) or (iii) weak (i.e., nominally significant, no replication) evidence including gene x environment interaction effects reported. Two of the SNPs (rs237885 and rs237889) were excluded due to having a SNP call rate < 95% in the current sample. Three SNPs (rs4564970, rs1488467 and rs2254298) had extremely low variability (MAF < 10%), thus were excluded from further analyses. In order to verify their independence, analysis of linkage disequilibrium was computed for the four remaining SNPs (rs6770632, rs53576, rs7632287 and rs4686302) using LDlink (Machiela & Chanock, 2015).

Ultimately, I decided to focus my investigation on the rs6770632 and rs53576 sites. Firstly, the rs6770632 SNP was selected based on previous findings that this SNP is linked to aggression (Malik et al., 2012) and on the biological basis that, as a 3' UTR variant, genotype of this SNP is likely to influence mRNA stability and translation. Furthermore, variations in this functional region of a gene have been repeatedly implicated in psychiatric disorders (e.g. (Jiang, Long, Ling, Huang, & Su, 2017; Karama et al., 2008; Shibayama et al., 2004)). Secondly, I decided to investigate the SNP rs53576 in the SEM model because of recent evidence implicating it in aggression (Hovey et al., 2016), antisocial behaviour (Smearman, Winiarski, Brennan, Najman, & Johnson, 2015) and CU traits in children (Ezpeleta et al., 2019).

5.2.4 fMRI Task

The paradigm was an adapted version of one used previously in male (Passamonti et al., 2012) and female (Fairchild et al., 2014) youth with CD. Briefly, participants were presented with an image of an emotional (angry, fearful or neutral) face and were asked to indicate by a button press whether the face was male or female (Figure 25). These images were presented in 17.5s blocks consisting of 5 faces and 5 fixation crosses. For each emotional condition participants were shown 12 blocks of faces and the images were counterbalanced subjects. The total duration of the task was 10 minutes 30 seconds.

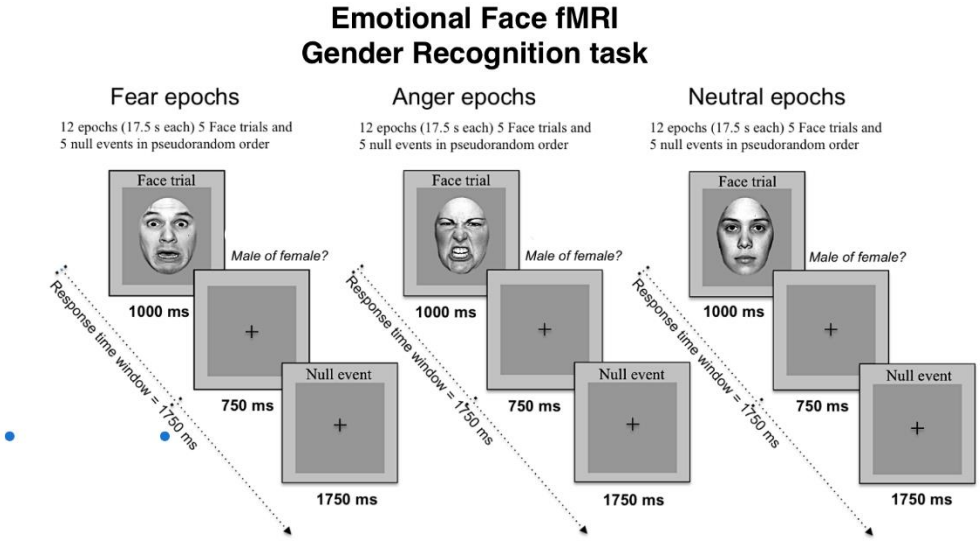


Figure 25. Emotional Face processing task - design based on (Passamonti et al., 2012)

5.2.5 Image Acquisition and Pre-processing

MRI data were collected using Siemens or Phillips 3T MRI scanners across five European sites taking part in the FemNAT-CD project (Freitag et al., 2018), however as one site used a different format for the fMRI task, the final sample only included data from 4 sites. All sites underwent screening checks and quality control procedures before commencement of data collection. Acquisition of data across different sites was performed in accordance with standard operating

procedures designed by the FemNAT-CD project's neuroimaging working group (Raschle et al., 2019; Rogers et al., 2019). As previously reported (Raschle et al., 2019; Rogers et al., 2019), rigorous quality control procedures and matched experimental protocols across the various locations were implemented to enable pooling of the data across the different sites.

The SPM12 (University College London, London, UK; http://www.fil.ion.ucl.ac.uk/spm/doc/manual.pdf) and ART imaging packages (Neuroimaging Tools and Resources Collaboratory; https://www.nitrc.org/projects/artifact_detect/) were used during the pre-processing stages of the fMRI data analysis. The pre-processing steps included; (i) realignment and unwarping of functional data with reference to the participant's first scan image, (ii) co-registration of scans to a reference structural T1-weighted scan, (iii) normalisation of images into standard space using a DARTEL template (created based on sample-specific tissue probability maps (TPMs), using the template-o-matic (TOM8 (Wilke, Holland, Altaye, & Gaser, 2008)) toolbox, (iv) smoothing of data with a 6-mm full width at half maximum Gaussian kernel, and finally, (v) using the ART toolbox to identify individual regressors associated with motion and variation in mean signal intensity. These regressors were then included in the first-level analysis model.

5.2.6 fMRI Analysis

At the first level, I generated contrast images for each participant, representing a directional difference between two emotional-face conditions, or between the observed BOLD response to an emotional-face condition and the fixation cross. The six contrasts generated were: all faces>fixation, anger>fixation, fear>fixation, neutral>fixation, anger>neutral and fear>neutral. The second-level analysis involved running whole-sample T tests to examine the difference in

BOLD signal intensity change associated with the contrasts of interest across all participants (CD and TD). Age, total IQ and data collection site were included as covariates of no interest in the model. I conducted a regions of interest (ROI) analysis focussed on the areas of the left and right amygdalae. Bilateral masks for these regions were anatomically defined based on the Talairach Daemon database using the WFU PickAtlas tool in SPM12 (Maldjian, Laurienti, Kraft, & Burdette, 2003).

BOLD parameter estimates were extracted from the ROIs of the left and right amygdala using the MarsBAR toolbox in SPM12 for all contrasts which elicited amygdala response at a significance threshold of $p<.05$ FWE-corrected (to be consistent with imaging genetics fMRI studies on youth with psychiatric symptomology (Mascarell Maricic et al., 2020)). These values were then exported for analyses in R (*version 3.6.0*). In-line with other recent studies in this field (e.g. (Waller et al., 2016)), and in order to both conserve variability and limit the influence of outliers, all parameter values obtained for imaging variables were 90% winsorized (using the standard functions included with R software) prior to analyses.

5.2.7 Structural Equation Modelling

To test the association between overall *OXTR* risk score, amygdala response, level of CU traits, IQ, age, site, sex and number of CD symptoms, I used the following linear regressions to model CD symptom score, ICU score and amygdala function.

```
model<-'

CD ~ BOLD_L + BOLD_R + OXTR

BOLD_L~OXTR + Age + IQ
BOLD_R~OXTR + Age + IQ
CU~OXTR

Age~~Age
IQ~~IQ
OXTR~~OXTR
BOLD_L~~BOLD_R
BOLD_R~CU
BOLD_L~CU
CU~~CU
CD~~CD'
```

```
model2<-'

CD ~ BOLD_L + BOLD_R + OXTR + Gender

BOLD_L~OXTR + Age + IQ + Gender
BOLD_R~OXTR + Age + IQ + Gender
CU~OXTR

Gender~~Gender
Age~~Age
IQ~~IQ
OXTR~~OXTR
BOLD_L~~BOLD_R
BOLD_R~CU
BOLD_L~CU
CU~~CU
CD~~CD'
```

Figure 26. R code showing linear regressions used to define paths in SEM

Model fitting was performed using the *lavaan* package in R (*version 3.6.0*). I included in the model terms that accounted for the covariance between CU traits and BOLD response and the covariance between contrasts across the left and right amygdala. The model also included parameter estimates for the residual variance of all endogenous variables (i.e., CD symptom score, CU traits score and BOLD response in the amygdala). (See Figure 24 below for full path diagram).

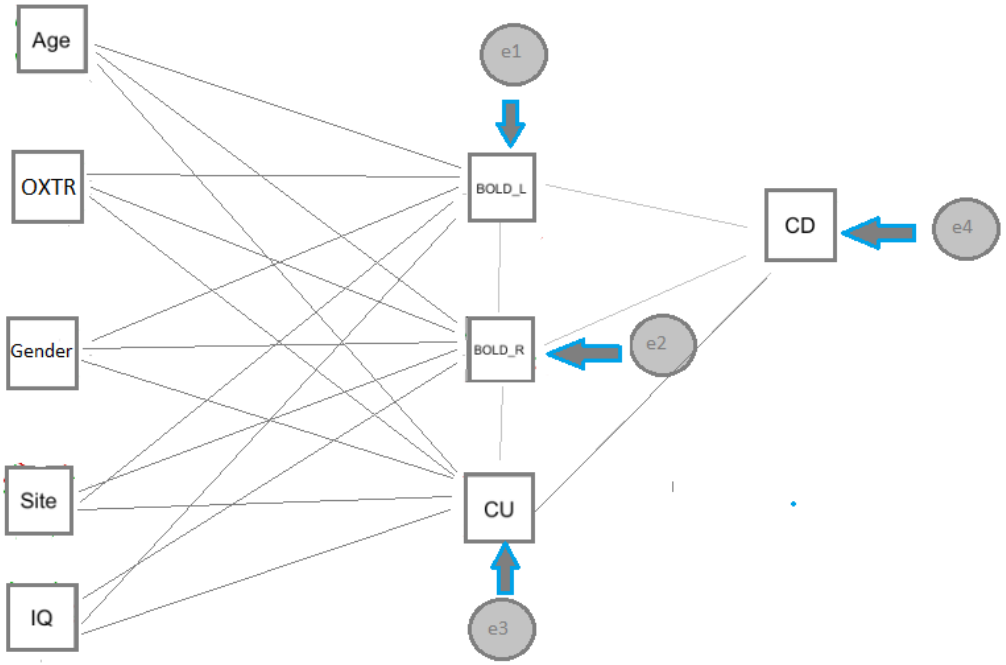


Figure 27. Full Path Diagram for SEM of CD Symptom Variation

I then ran this model to investigate *OXTR* genotype of SNPs rs6770632 and rs53576 and amygdala response for three separate emotional face contrasts (anger>fixation, fear>fixation, neutral>fixation) and for the all faces>fixation contrast. I did not include the anger>neutral or fear>neutral contrasts as there was no significant amygdala activation between these conditions, either across all the participants, or in the CD or TD groups separately. I considered two measures to determine the effectiveness of the model; the comparative fix index (CFI) and the standardized root mean square error approximation (RMSEA). RMSEA is an absolute fix index thus the value gives an indication of the difference between the hypothesized model and a perfect model. CFI relates the fit of the hypothesized model to a baseline model (i.e. the worst fitting

model). As suggested (Hu & Bentler, 1999). Values of RMSEA <.06 and CFI >.95 were considered to indicate a reasonable model-data fit.

5.3 Results

5.3.1 Participant Characteristics

Out of 349 participants, 160 (45.8%) were males and the age ranged from 9-18 years with an average age of 13.9 years (SD=2.64) (Table 6). Across the entire sample the average, scaled value of SES was 0.1 (SD= 1.03) (where 0 is the country-specific national average) and the average value of IQ was 100.7 (SD=12.64), thus on these variables the participants were representative of the wider population. However, individuals with a diagnosis of CD had on average significantly lower IQ scores (p<.001) than the TD group (see Appendix C2). Across the full sample of participants, the number of ADHD symptoms ranged from 0 – 21 (from a possible maximum of 51), with an average of 3.1 (SD=5.8), number of GAD symptoms ranged from 0-7 from a possible maximum of 42), with an average of 0.3 (SD=1.1) and the number of MDD symptoms ranged from 0-29 from a possible maximum of 72), with an average value of 1.6 (SD=4.3).

Across the whole sample, the average number of CD symptoms was 2.1, with values ranging from 0 (in TD participants) to 13 (in participants with CD) from a possible maximum of 15. The average number of CD symptoms was not statistically significantly different between males and females. The average ICU score across the sample was 22.9, with scores ranging from 1 to 66 from a possible maximum of 72. Youths with CD had significantly higher average ICU scores as compared to the TD youth. Across all participants, the average ICU was also significantly higher in males than in females.

Table 6. Demographic, clinical and environmental characteristics of the 349 participants

Male Sex (%)	*160 (45.8%)*
Age, mean (SD; range)	*13.9 (2.6; 9-18)*
PDS, mean (SD; range)	*3.4 (1.2; 1-5)*
SES, mean (SD; range)	*0.1 (1.0; -3.0-2.6)*
IQ, mean (SD; range)	*100.7 (1.2; 68-138)*
ICU score, mean (SD; range)	*22.9 (12.1; 1-66)*
CD Symptoms, mean (SD; range)	*2.1 (2.9;0-13)*
ADHD Symptoms, mean (SD; range)	*3.1(5.8; 0-21)*
GAD Symptoms, mean (SD; range)	*0.3 (1.1;0-7)*
MDD Symptoms, mean (SD; range)	*1.6 (4.3; 0-29)*

The frequency distribution of CD symptoms and CU scores are shown in Figures 28 and 29.

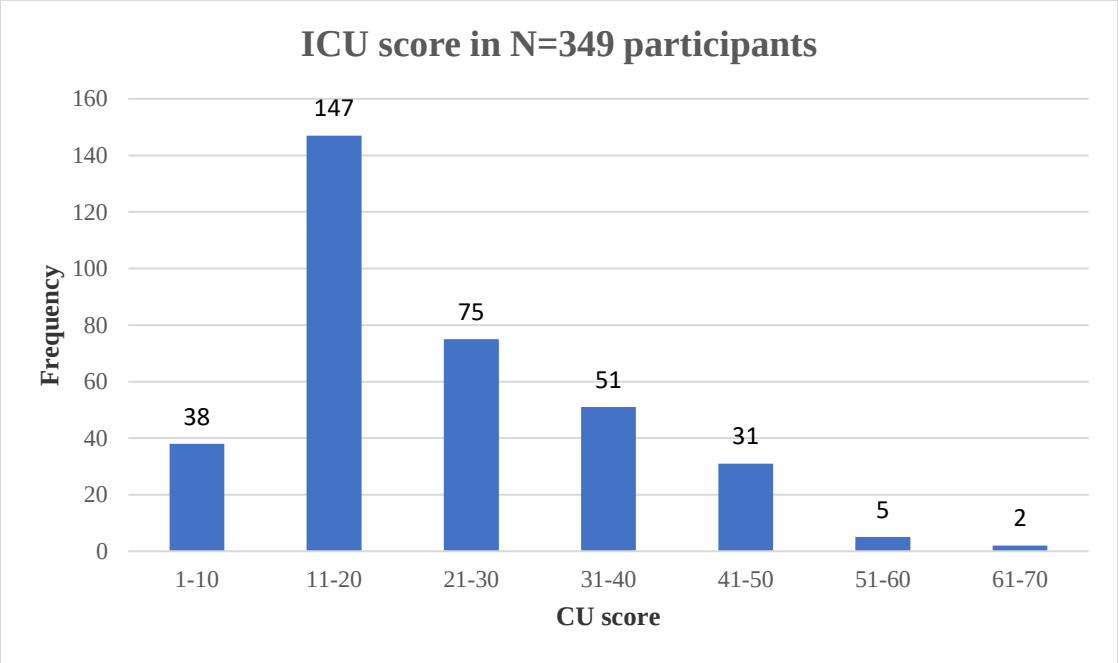


Figure 28. Frequency of ICU score Across All Participants

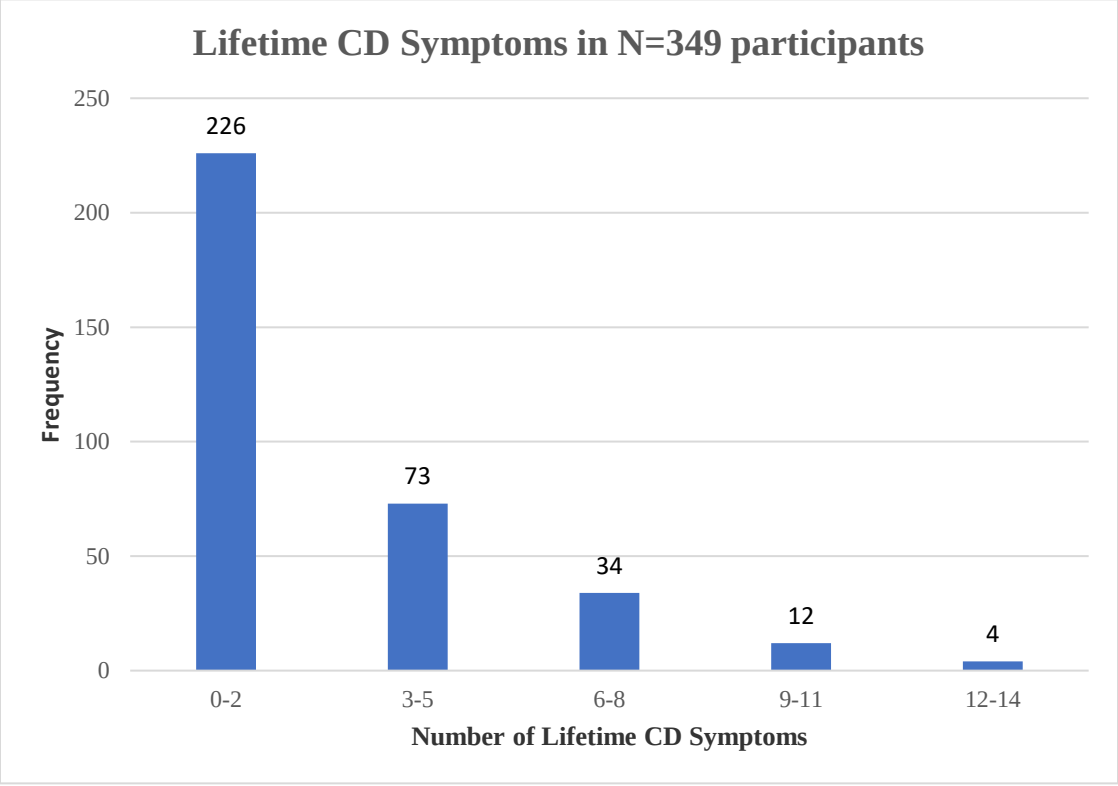


Figure 29. Frequency of Number of CD Symptoms Across All Participants

5.3.2 Correlations between SEM variables

The values of correlation coefficients between all variables included in the SEM model are shown in Appendix C3. In my sample, there were moderate-high positive correlations between bold responses in the left and right amygdalae across the emotional face conditions. There were also significant negative correlations between CD symptom score and SES (Pearson $r_{(347)}$= -.30, p = <.*001*), CD symptom score and total IQ (Pearson $r_{(347)}$= -.35, p = <.*001*), and total IQ and CU score (Pearson $r_{(347)}$= -.23, p = <.*001*) , as well as a significant positive correlation between CD symptom score and CU traits (Pearson $r_{(347)}$= .60, p = <.*001*) (see Figure 30). As expected, there was a significant positive correlation between age and PDS (Pearson $r_{(347)}$= .77, p = <.*001*) and there was a negative correlation between age and total IQ (Pearson $r_{(347)}$= -0.29, p = <.*001*) .

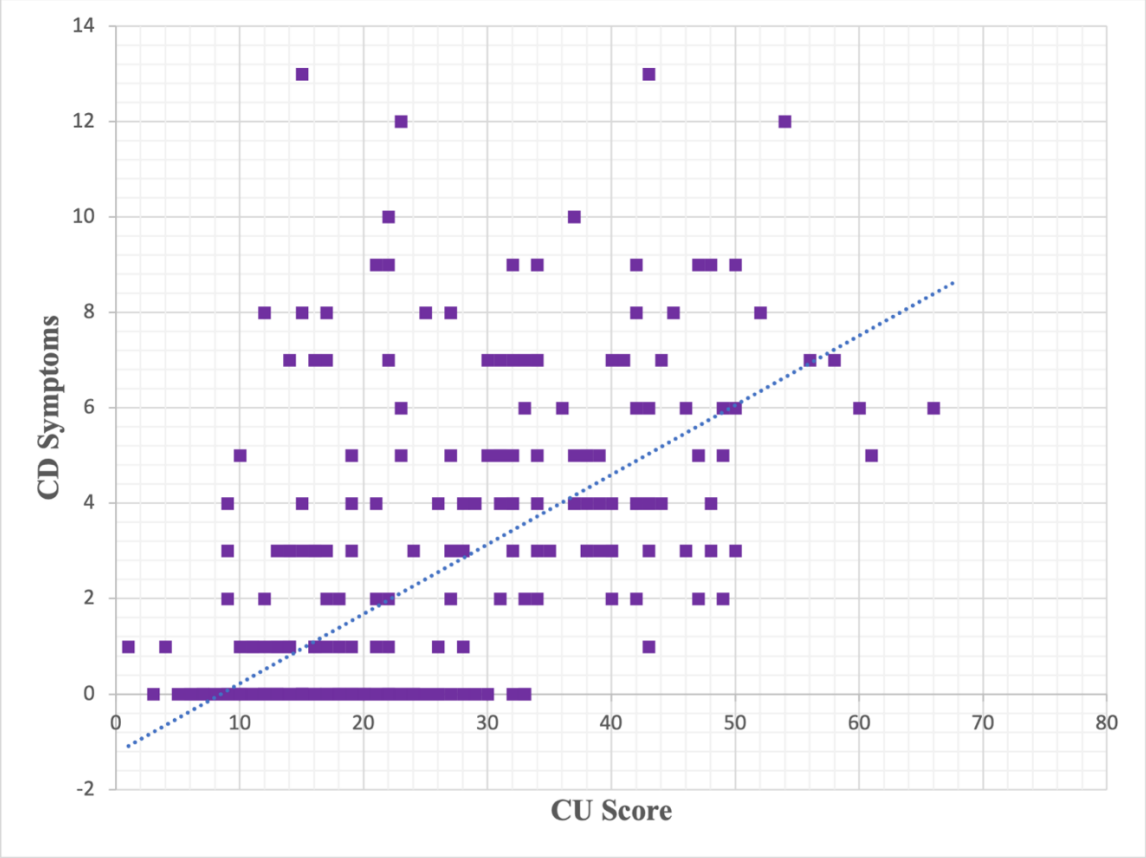


Figure 30. Scatter plot showing the correlation between CD symptoms and CU score across all participants. Correlation (R^2) = 0.60

5.3.3 *OXTR* Genotype Summary

For *OXTR* SNP rs6770632, 8% of participants had a minor allele genotype AA, 34% were heterozygous (i.e., AG/GA) and the remaining 58% were GG homozygotes (minor allele Frequency MAF: 0.24, HWE-p>0.1). For *OXTR* SNP rs53576, 11% were AA homozygotes, 42% were heterozygous and 46% of participants had the GG genotype (MAF: 0.32, HWE-p>0.1). The observed distribution of alleles in this sample conforms to expected values based on Hardy-Weinberg equilibrium calculations. In the sample, there were significantly more males with rs53576 AA genotype than there were females ($p<.01$). Previous studies have reported mixed findings on the sex-distributions of *OXTR* genotype (Andreou, Comasco, Aslund, Nilsson, & Hodgins, 2018; Weisman et al., 2015). There were no significant differences between CD and TD groups in prevalence of genotypes for either of the SNPs. All SNPs were in Hardy–Weinberg equilibrium (See Table 7).

Table 7. Genotype information by sex and group

	Sex		Group		Main effect of Sex		Main effect of Group	
	Male	Female	CD	TD	χ^2	*p*	χ^2	*p*
Across whole sample	160	189	135	214				
Genotype								
OXTR rs6770632 AA	17	11	13	15	2.3	.13	0.65	.42
OXTR rs6770632 AG	47	71	47	71	1.3	.26	0.05	.83
OXTR rs6770632 GG	96	107	75	128	0.11	.74	0.16	.69
OXTR rs53576 AA	24	15	18	21	3.5	.06	0.82	0.37
OXTR rs53576 AG	62	86	56	92	0.66	.42	0.03	.86
OXTR rs53576 GG	74	88	61	101	<.01	.97	0.05	.82

5.3.4 fMRI Results

Across the full mixed-sex sample of participants (n=349), I did not observe any significant differences (increase or decrease) in BOLD response in the amygdala for the anger>neutral or fear>neutral contrasts. However, there was a significant increased response in the left and right amygdalae for the contrast involving all faces vs fixation (See Figure 31) as well as the three separate contrasts including each facial expression vs. fixation (see Appendix C4).

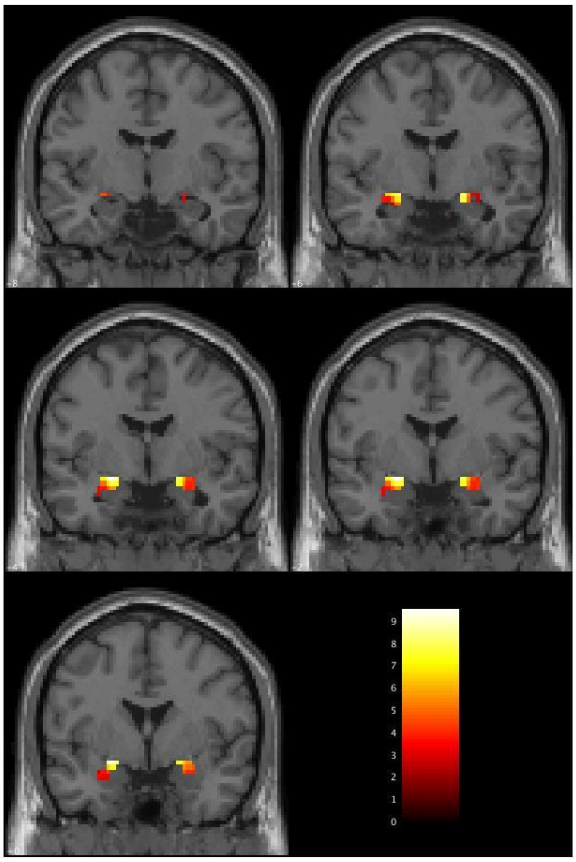


Figure 31. Coronal view of bilateral amygdala clusters activated in response to all faces vs. fixation contrast (slices taken at MNI x=0 to x=8). Results from mixed-sex participant sample (n=349).
N.B. The colour of the highlighted voxels indicates the magnitude of the t-statistic, which is an indicator the level of BOLD signal activation in the region, as shown in the colour bar on a scale from 0 = Black (low) – 5 = Red/Orange(Medium) – 9 =White (High).

Female-Only Sample fMRI Results

In the female-only sample of participants (n=189) my analysis again did not reveal any significant differences (increase or decrease) in BOLD response in the amygdala in the anger>neutral or fear>neutral contrasts. However, there was a significant activation in the left and right amygdalae for the contrast involving all faces vs. fixation and for the three individual facial expression vs. fixation contrasts (See Appendix C5).

Male-Only Sample fMRI Results

Analysis of data from the male-only sample of participants (n=160) also did not show significant differences (increase or decrease) in BOLD response in the amygdala in the anger>neutral or fear>neutral contrasts. As in the females, there was, however, a significant activation in the bilateral amygdalae for the all faces vs. fixation, anger vs. fixation and fear vs. fixation contrasts. For the neutral faces vs. fixation contrast only the cluster in the left amygdala was significant at FWE-corrected level (p=.011), as shown in Figure 32.

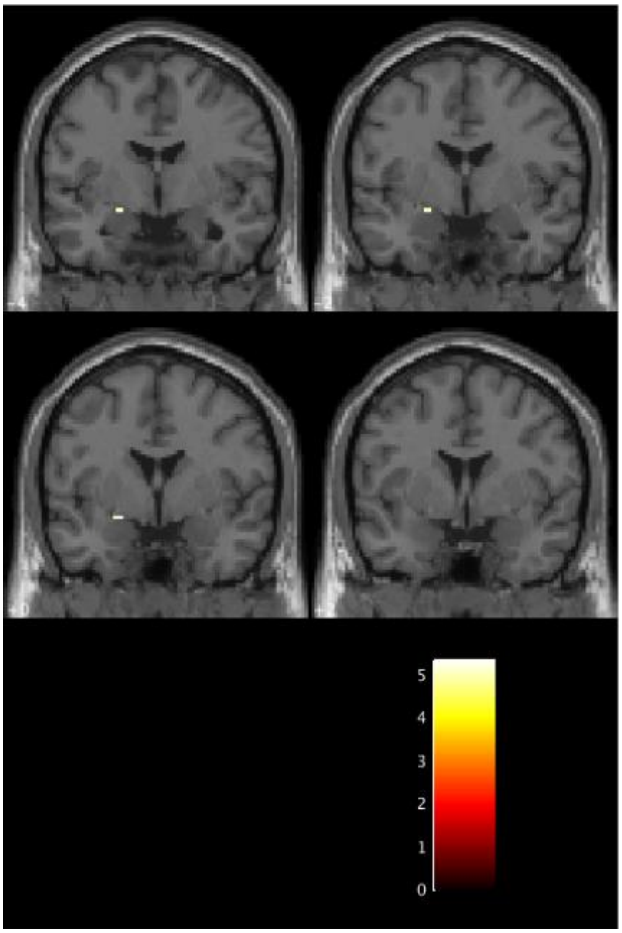


Figure 32. Coronal view of L amygdala clusters activated in response to neutral vs. fixation contrast (slices taken at MNI x=-4 to x=2). Results from male-only participant sample (n=160).
N.B. The colour of the highlighted voxels indicates the level of BOLD signal activation in the region, as indicated in the colour bar on a scale from 0 = Black/Red (low) – 5 =Yellow/White (High)

The full results are shown in Appendix C6.

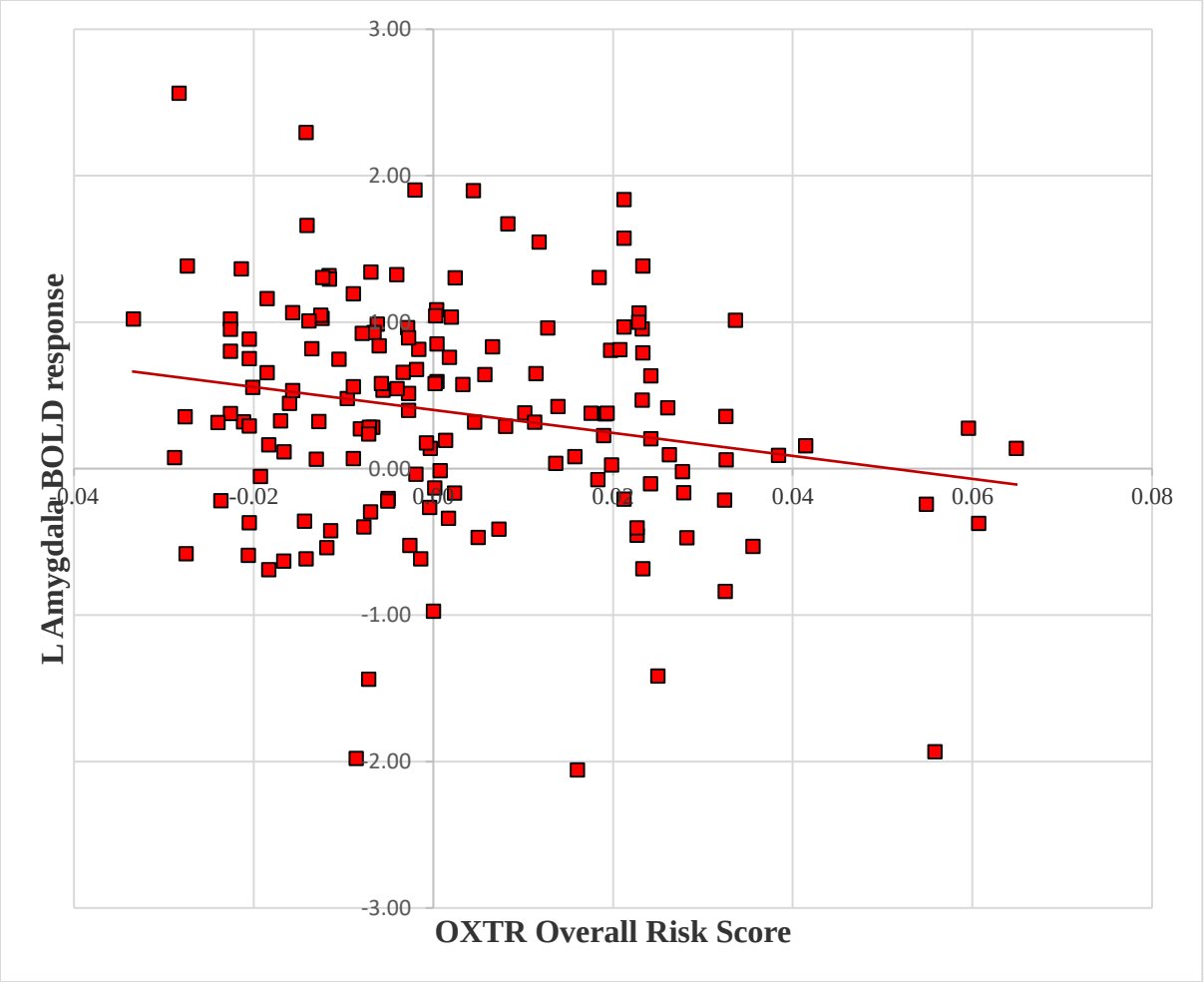


Figure 33. Overall *OXTR* risk score vs. L BOLD amygdala response to Anger>Fixation contrast. correlation (R^2) = -0.204

5.3.5 Structural Equation Modelling

To evaluate the performance of this model in relation to amygdala responses to faces in general, I fitted the SEM using the amygdala responses obtained from the all faces>fixation contrast. Initially, I used the values for each participant for the two individual SNPs independently and I then re-ran the model with the calculated values of the overall risk score for the *OXTR* gene including the 34 SNPs of interest.

Across the full, mixed-sex participant sample (n=349), none of the three SEM models (i.e. with *OXTR* data from rs53576, rs6670632 or overall risk score) were significant for the all faces>fixation contrast (Table 8). Thus, I set out to test the SEM model for the individual emotional face contrasts (i.e., anger>fixation, fear>fixation, neutral>fixation) to check whether genotype of SNPs rs53576 or rs6770632, or overall *OXTR* risk scores were uniquely associated with amygdala response to a particular emotional condition. However, in the mixed-sex participant group I did not find this to be the case for any of the individual contrasts (Table 8). Finally, given evidence that the effects of variation in *OXTR* are sexually dimorphic (Hernandez et al., 2020; Stankova, Eichhammer, Langguth, & Sand, 2012; Wang et al., 2017), I split the participants into male-only and female-only groups and re-ran all SEM models in each sex separately. Those models were also not significant (Table 8). A summary of the CFI, RMSEA and unexplained CD variance from all models tested in shown in Table 8 below.

Table 8. Summary SEM model performance measures: CFI, RMSEA and unexplained CD-symptom variance

***OXTR* measure**	**Contrast**	**Sex**	**CFI**	**RMSEA**	**Unexplained CD variance**
rs6770632	**Anger>Fixation**	F	.486	.114	.60
		M	.470	.110	.59
		Mixed	.479	.112	.59
	Fear>Fixation	F	.443	.118	.61
		M	.438	.118	.60
		Mixed	.441	.118	.59
	Neutral>Fixation	F	.416	.120	.61
		M	.486	.112	.59
		Mixed	.450	.116	.59
	Faces>Fixation	F	.455	.116	.61
		M	.469	.111	.60
		Mixed	.468	.113	.60
rs53576	**Anger>Fixation**	F	.496	.112	.60
		M	.469	.110	.59
		Mixed	.474	.113	.59

	Fear>Fixation	F	.447	.118	.60
		M	.436	.116	.60
		Mixed	.430	.119	.60
	Neutral>Fixation	F	.415	.121	.60
		M	.488	.113	.59
		Mixed	.444	.117	.60
	Faces>Fixation	F	.458	.116	.61
		M	.470	.111	.60
		Mixed	.462	.114	.60
Overall Risk Score	**Anger>Fixation**	F	.454	.116	.61
		M	.492	.108	.59
		Mixed	.479	.112	.59
	Fear>Fixation	F	.453	.116	.61
		M	.440	.117	.60
		Mixed	.441	.118	.59
	Neutral>Fixation	F	.455	.115	.61
		M	.483	.112	.59
		Mixed	.450	.116	.60
	Faces>Fixation	F	.455	.116	.61
		M	.476	.110	.60
		Mixed	.462	.113	.60

Individual Significant Paths within SEM

While none of the SEM models were met the criteria for a good model fit in explaining overall variance in CD symptoms, some of the individual paths between variables were significant and are thus discussed in turn below.

SNP rs6770632 and SNP 53576 genotype

I initially ran the SEM model with genotype values corresponding to the number of A alleles for SNP rs6770632 for each participant. I ran the model firstly across the full sample, and then individually in female-only and male-only samples (see Appendix C7 a) and b)). For both sexes, the all faces>fixation contrast, number of CD symptoms was directly positively associated with CU traits (p<.001) and negatively associated with IQ (p<.001). CU traits were also negatively

associated with IQ (p<.001). For the rs6770632 model in females-only, IQ was positively associated with values of BOLD response for the anger>fixation contrast in both the left (p=.002) and right (p=.016) amygdala. In males, amygdala response for the fear>fixation contrast was significantly associated with Site in both the left (p=.034) and the right (p=.012) hemisphere. All other paths in the rs6770632 model were non-significant (see Appendix C7 a) and b) for diagram and path coefficients).

For the model using *OXTR* genotype values corresponding to the A allele of SNP rs53576, the results for the all faces>fixation contrast were the same as for the rs6770632 model (i.e. number of CD symptoms was directly positively associated with CU (p<.001) and negatively associated with IQ (p<.001), and CU traits were negatively associated with IQ (p<.001) in both sexes). In the female-only model, values of BOLD response for the anger>fixation contrast were again significantly positively associated with IQ in both left (p=.001) and right (p=.011) amygdala. Also, in females-only there was a significant negative association between left amygdala response to angry faces vs. fixation and CU traits (p=.018). All other paths in the rs53576 model were non-significant (see Appendix C7 c) and d) for diagram and path coefficients)).

Neither rs6770632 nor rs53576 *OXTR* genotype was found to have a significant effect on participants' total number of CD symptoms

Overall OXTR Risk Score

For the model using values of overall *OXTR* risk scores and BOLD amygdala response to the all faces>fixation contrast, the results were similar to those for the individual SNPs. Namely, the significant paths were the positive association between number of CD symptoms and CU traits (p<.001), the negative association between CD symptoms and with IQ (p<.001) (Figure 34) and the negative association between CU traits and IQ (p<.001).

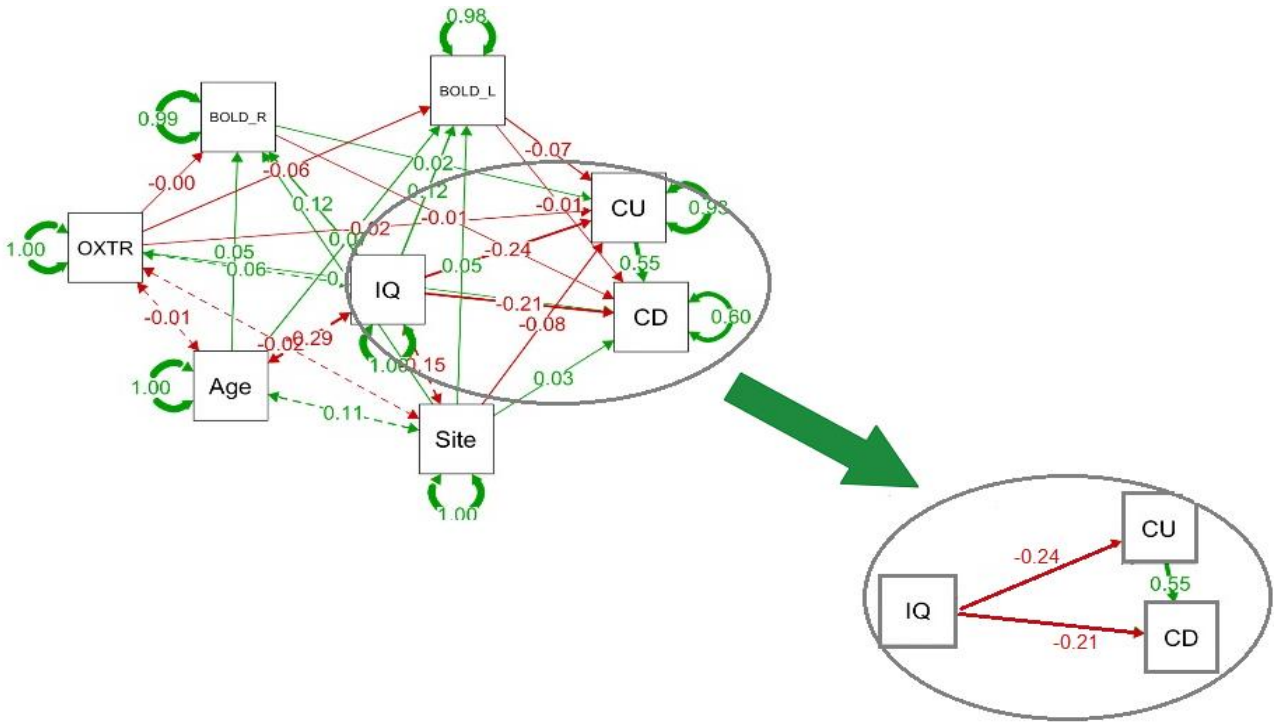


Figure 34. Overall *OXTR* risk score SEM model – Significant paths(p<.001) between IQ, CU and CD highlighted

In this model using overall *OXTR* risk scores, for the all faces>fixation contrast, higher IQ was also positively associated with greater left (p=.032) and right (p=.033) amygdala response across both sexes. When I ran the model in single-sex samples to check for potential sex-specific associations, I observed that, in the female-only subsample, site was also significantly associated with CU traits (p=.001). Interestingly, in the male-only model, the association between right and left amygdala responses and IQ was non-significant (p=0.07), suggesting that this effect in the full, mixed-sex sample was likely being driven by the significant associations in females (L:p=.017, R: p=.020). I next examined the amygdala BOLD response values for the anger>fixation, neutral>fixation and fear>fixation contrasts in separate SEM models. In males-only, the association between site and both left (p=.047) and right (p=.012) amygdala response

for the contrast fear>fixation was significant and *OXTR* risk score was significantly negatively associated with left amygdala BOLD response (p=.008) for the anger>fixation contrast.

5.4 Discussion

The aims of this study were to use an SEM approach to investigate whether allelic variation in the *OXTR* gene was associated with number of CD symptoms in a large mixed-sex sample of youth, and, to examine whether this association was mediated through participants' level of amygdala response during emotional face processing or CU-trait score. I tested a literature-informed model, firstly using genotype values for two individual SNPs of *OXTR,* and then using the same model with calculated values of an overall composite risk score of aggression for the *OXTR* gene, based on 34 SNPs. For all measures of *OXTR* genotype, I initially ran the model across all participants, then in males and females separately. Sex was included as an additional variable when the model was run with all participants.

The findings overall did not support the hypothesis that number of CD symptoms is significantly influenced by direct or indirect effects of *OXTR* genotypic variation. None of the models tested were significant (i.e., the combinations of variables and paths included in these models did not explain a significant amount of the variance in the number of participants' CD symptoms). However, I did identify several individual paths in the models that represented significant association between variables. I will now discuss possible explanations for why those models did not explain a significant amount of the variance in CD symptoms, and then move on to interpreting the individual significant paths within the models. I will conclude by highlighting some of the strengths and limitations of study and suggest how the approach I have used could

be developed to create a more accurate model of the factors influencing variance in CD symptoms in the future.

5.4.1 Variance in CD symptoms

The models tested included paths representing the direct and indirect associations between *OXTR* genotype, CU traits score, age, IQ, sex, site and left and right amygdala responses to emotional faces, and number of CD symptoms. These models did not explain a significant amount of variance in CD symptoms, either when using genotype information from the individual *OXTR* SNPs rs6770632 and rs53576, or when using an overall risk score, calculated using the values from 34 SNPs on the *OXTR* gene. There are many possible reasons why the models did not account for the variation in CD symptoms, but I will focus on discussing three of these. Firstly, it is possible that an individual's *OXTR* genotype is indeed unrelated to the number of CD symptoms they exhibit. Secondly, it may be that the values I use to signify *OXTR* genotype did not accurately captured the variation in the functions of the *OXTR* gene relevant to CD symptoms. Thirdly, I consider whether these models may have been missing key variables needed to demonstrate the nature of the association between *OXTR* genotype and number of CD symptoms.

5.4.2 Is *OXTR* Genotype Really Unrelated to Number of CD Symptoms?

Previous research in adults has demonstrated an association between *OXTR* genotype and levels of antisocial behaviour (Waller et al., 2016) and, as a significant proportion of youth with CD continue to exhibit antisocial behaviours in adulthood (Helgeland, Kjelsberg, & Torgersen, 2005; Ramklint, von Knorring, von Knorring, & Ekselius, 2003), I hypothesized that this link may also

been observable in adolescents. This hypothesis was also based on previous findings that both CU traits (e.g. (Ezpeleta et al., 2019)) and CD symptomatology (e.g. (Malik et al., 2012)) are associated with *OXTR* genotype in youth. Earlier studies have also demonstrated associations between *OXTR* genotype and both amygdala structure (Inoue et al., 2010) and function (Tost et al., 2010) in adolescents, thus, as amygdala dysfunction during emotional face processing has repeatedly been reported in youth with CD (Ewbank et al., 2018; Fairchild et al., 2014; Marsh et al., 2008; Passamonti et al., 2010), this provided further ground to test the aforementioned hypothesis. However, there are several important methodological differences between these earlier studies and my own work, which may explain why I did not observe a significant association between *OXTR* genotype or amygdala response and CD symptoms in these models. Importantly, previous studies (e.g. (Ezpeleta et al., 2019; Malik et al., 2012; Waller et al., 2016)) have predominantly investigated variation in *OXTR* genotype at individual SNPs, with some studies including SNPs for which I did not have data. While some previous work has specifically investigated the two SNPs I tested individually in this study (rs53576 and rs6770632) in relation to antisocial/aggressive behaviour (e.g. Andreou et al., 2018; Malik et al, 2012; Waller et al., 2016), most of the research reports associations between antisocial/aggressive behaviour and other single SNPs. Given evidence of distinct functional effects in relation to different SNPs of the same gene (Shastry, 2009), it may be that genotypic variation in the *OXTR* gene is associated with CD symptoms, but not with the two SNPs I tested. I did also use a composite score to represent overall variation in *OXTR* genotype, as, by including the combined effects of variations at multiple SNPs, genetic risk scores have been reported to more accurately reflect the functional variation of the gene more accurately (Igo, Kinzy, & Cooke Bailey, 2019). However, as this approach is different from previous work on

OXTR and aggression/antisocial behaviour, my findings are not analogous/comparable to those previously reported from single SNP studies. There are also difference between the ages and psychiatric status of participants in previous studies and this present work. For example, some studies have investigated *OXTR* variation in adult participants (Inoue et al., 2010; Tost et al., 2010; Waller et al., 2016) or included only participants with no psychiatric symptoms (Inoue et al., 2010; Tost et al., 2010). Finally, some previous work linking *OXTR* variation to differential social functioning has relied on self-reporting (Tost et al., 2010; Waller et al., 2016) and thus, unlike in this study where CD symptoms were assessed by trained researchers, findings from these studies may be unreliable and limited by participants levels of self-awareness and honesty in their responses (Chan et al, 2009).

5.4.3 Is *OXTR* Risk Score Representative of CD Symptom Risk?

Another possible explanation is that the weightings attributed to the different variants of the overall risk score for *OXTR* did not accurately model risk for CD symptoms. Thus, combining the data from multiple SNPs in this way may have masked the influence of certain key SNPs. This may be particularly relevant given that the values used were taken from previous findings for aggression (Pappa et al., 2016). There are 15 symptoms in the diagnostic criteria for CD and an individual displaying three or more of these symptoms qualifies for a diagnosis of the disorder. These symptoms fall into four categories: aggressive behaviours, destructive behaviours, deceitful behaviours and violations of rules. Thus, given that aggressive behaviours form only one category of CD symptoms, it is possible that a given individual with CD do not fulfil any aggressive symptoms. Because there are currently no published (meta)-analyses of GWAS studies in relation to CD symptoms, I considered aggression to be a suitable proxy to

generate weightings for *OXTR* risk in relation to CD symptoms, but these constructs may not have been similar enough. This would mean that the values I used to identify *OXTR* genotype did not accurately capture the variation in *OXTR* gene function relevant to CD symptoms. Future studies on genotypic variation in relation to CD should ensure that, when calculating combined risk scores, the weightings are representative of the full range of CD symptoms (i.e., aggression, destruction, deceitfulness, and violation of rules).

5.4.4 Additional Sources of Genetic Variation: The Missing SNPs

Additionally, the composite risk score gives a measure of the variation in 34 SNPs of *OXTR*, but there are additional SNPs which may also impact *OXTR* function and expression (Zhang et al., 2015; International HapMap Consortium, 2007), and thus the genetic risk associated with this gene. I chose to include only SNPs within 2kb of the *OXTR* gene, for which data were available for all of my participants, but other studies have used alternative approaches. For example, another recent study investigating *OXTR* genotype in relation to emotional face processing used *imputed* SNP data from a larger, 60k-bp-region around the *OXTR* gene, comprising 75 SNPs (Verhallen et al., 2017). In particular, I did not have data available for two *OXTR* SNPs (rs3796863 and rs401015) that were highlighted in a recent review for being associated with neuronal measures of processing facial emotion/social cues (Tully et al., 2018). Specifically, Sauer et al. (2012) investigated rs3796863 and demonstrated that *OXTR* homozygotic risk allele carriers had greater activation in the left fusiform gyrus in response to visual social stimuli (Sauer, Montag, Worner, Kirsch, & Reuter, 2012) and Montag et al. (2013) reported that allelic variation at rs401015 modulated right amygdala activity to facial cues (direct vs. averted gaze) when participants were under the influence of endogenous oxytocin (Montag, Sauer, Reuter, &

Kirsch, 2013). As I do not include data from these SNPs when calculating the composite risk score in this study, I may have failed to capture some of the genetic variation in *OXTR* associated with emotional face processing. Finally, my calculations were based on an additive effect of individual risk alleles, but this model may be too crude, as it does not include possible interactions between risk variants. Future research may benefit from employing more complex techniques, such as the additive-to-multiplicative linear effect scale (AMLES, (Diaz-Gallo, Brynedal, Westerlind, Sandberg, & Ramskold, 2021)), to thoroughly investigate the additive, multiplicative and intermediate effects of risk variants.

5.4.5 Are Variables Relevant to CD Symptoms Missing from the SEM?

I endeavoured to include in the modelling most of the key variables that have been identified as contributing to CD in previous research. However, in order to maintain statistical power with the available sample, I had to limit the number of paths in the model. Consequently, the models may have been missing key variables that might contribute to the association between *OXTR* genotype and number of CD symptoms. For example, additional factors that may increase risk for CD include childhood maltreatment (Maldjian et al., 2003; Mascarell Maricic et al., 2020) and other environmental influences such as maternal smoking during pregnancy (Boden, Fergusson, & Horwood, 2010; Nigg & Breslau, 2007) and adverse parenting (Hernandez et al., 2020). In particular, previous research in males has demonstrated that *MAOA* genotype is a moderator of the association between maltreatment and psychiatric symptoms (Kim-Cohen et al., 2006), so even if *OXTR* genotype does not directly contribute to increased risk of developing CD symptoms, it may act as a moderator in a similar way to the *MAOA* gene.

5.4.6 Significant Paths within the SEM

In all the models, I observed a significant positive association between CU trait score and number of CD symptoms. The association between higher levels of CU traits and greater severity of conduct problems has been widely reported in studies of youth from both community (e.g. (Frick et al., 2003)) and clinical (e.g. (Enebrink, Andershed, & Langstrom, 2005)) populations. Indeed, the inclusion within the DSM-5 of the CU-based LPE-specifier illustrates the key role CU traits, and their assessment, play in the diagnostic process for identifying youth with a particularly severe form of CD (Frick & White, 2008).

In my models, I also observed a significant negative association between IQ and total number of CD symptoms. The presence of lower IQ scores in individuals with a clinical diagnosis of CD as compared to their TD peers has been reported in multiple studies (e.g. (Lazaratou et al., 2018)). A recent meta-analysis, which synthesized results from over 90 studies, confirmed the overall negative correlation between IQ and CD symptoms (Sánchez de Ribera, Kavish, Katz, Boutwell, & Back, 2019). That meta-analysis also used an SEM approach and showed that IQ accounted for a statistically significant amount of the variance in CD, though the effect size was small (R^2 = .04). My observations that IQ and CU scores are, respectively, negatively, and positively associated with CD symptom variation are thus in line with previous findings.

Sex-specific Association: OXTR and Amygdala Response to Angry Faces

While across the whole sample and in the female-only subsample, I did not find significant effects of *OXTR* variation on amygdala response to emotional faces, in the male-only subsample I did observe a significant negative association between *OXTR* risk score and left amygdala

BOLD response to angry facial expressions. This finding is consistent with the sex-specific effect observed in a recent study, which also reported greater amygdala reactivity to angry faces in adult participants according to *OXTR* genotype (Waller et al., 2016). Waller et al (2016) also showed that T allele homozygotes of *OXTR* rs1042778 displayed higher activity in the right amygdala (Waller et al., 2016) on viewing angry faces, which was positively associated with antisocial behaviour only in male participants.

In mixed-sex adolescent groups, a positive association between amygdala reactivity to angry faces and antisocial behaviours has also been reported for bullying and victimization (Swartz, Carranza, & Knodt, 2019), reactive aggression (Dotterer et al., 2020) and substance abuse (Spechler et al., 2015). Specifically, in males with high anxiety, trait anger scores were shown to correlate positively with bilateral amygdala response to angry faces (Carre, Fisher, Manuck, & Hariri, 2012). Amygdala hyper-response to angry face stimuli has also been reported in mixed-sex participants with a clinical diagnosis of Intermittent Explosive Disorder (McCloskey et al., 2016). In this context, amygdala reactivity has thus been suggested as a potential biomarker for reactive aggression in males (Carre et al., 2012).

However, it must be noted that there is also good evidence that the direction of association of amygdala response to threatening facial stimuli in individuals with antisocial behaviours is moderated by level of CU traits (Jones et al., 2009; Lozier et al., 2014; Sebastian et al., 2014). In studies of adult participants with psychopathy reduced amygdala responses to emotional stimuli has been consistently observed (Poeppl et al., 2019), but see (Deming & Koenigs, 2020). This may mean that if CU traits are not included in analytical models, amygdala reactivity in response

to angry faces in participant with CD samples might appear as not significantly different from TD participants (e.g., (Menks et al., 2021)), as the atypically higher amygdala responses in youth with CD/LCU traits are "cancelled out" by dampened responses in the subgroup of youth with CD and high CU traits. This is another possible explanation for the null findings in the SEM analysis. Future research investigating the associations between genotypic variation and amygdala response to emotional faces should consider testing models in groups with high vs. low levels of CU traits separately to ensure this is not the case. This was not possible in this study due to the nature of this sample (which included a small number of participants with very high CU scores that had a disproportional effect on the overall average). Using a median split to create high and low CU groups would have meant the high CU groups included only 67 participants, and therefore this SEM model would not have been adequately powered to test in this group.

5.4.7 Strengths and Limitations

There are several strengths of this study, including the use of a well-established task for eliciting amygdala response to threatening faces, and novel use of the SEM approach to examine both direct and indirect pathways between *OXTR* genotypic variation, brain (amygdala) response and CD symptoms severity. Furthermore, the inclusion of multi-site data allowed for a larger sample size than would have been possible from a single site, as youth with CD (especially females) are difficult to recruit. Finally, the sample was very well characterized, and all participants (including the TD group) underwent comprehensive assessments for psychiatric disorders and symptoms, in-line with the current DSM-5 criteria.

There are, however, several limitations that should be noted. First, the composite risk score created for the *OXTR* gene is based on weightings taken from a meta-analysis of SNPs associated with aggression (Pappa et al., 2016), not specifically with CD symptoms. Aggressive behaviours only form one subsection of CD symptoms; thus, these weightings may not have been directly applicable to this model for predicting CD symptom severity. Future research aiming to understand the factors contributing to variation in CD symptom severity in relation to genotype should endeavour to calculate composite scores using SNP weightings representative of the full range of possible CD symptoms subsets. Second, I used an additive model of 'risk' alleles for all SNPs, but a dominant-recessive model may more accurately reflect the functional relevance of genetic variants for some polymorphisms. Previous work has shown that additive risk does explain most of the variance in psychiatric conditions (Owen & Williams, 2021)), however by coding each SNP specifically according to the nature of the association between its constituent alleles, future studies may be able to create composite risk scores which more accurately model the variation in function of the *OXTR* gene according to SNP variation. Third, in relation to antisocial behaviours, the functional relevance of *OXTR*, and particular the individual *OXTR* SNPs I studied (rs6770632 and rs53576) is not well understood. Therefore, I cannot rule out the possibility that the significant pathways I observe in models based on data from these SNPs reflect in fact associations with other unknown genetic variants in linkage disequilibrium with these two SNPs (see (Grabitz et al., 2018) for further discussion of issues arising from linkage disequilibrium in genetic studies). Fourth, for many of the SNPs of *OXTR* included in the composite risk score, their interactions with other genetic variability and environment factors have not been researched, and so further work is needed to investigate multi-gene interactive effects and environmental influences on these genotypic variations as well. Finally, the sample

size of the present study is small compared to some imaging genetics designs, which may increase the likelihood of both false positive and false negative findings (Duncan & Keller, 2011) In particlar, genetic effects tend to be very small in psychiatric conditions (Nurnberger et al., 2016) and thus large sample sizes may be required to ensure studies are adequately powered to detect these small effects. However, I did use the interactive Shiny app tool power4SEM (https://sjak.shinyapps.io/power4SEM (Jak, Jorgensen, Verdam, Oort, & Elffers, 2020) to calculate that for this model the full sample size of 349 gave 78.3% power to detect effect sizes above .3 ($d > 0.3$) at a significance level of $p<.05$. (A sample size of 361 would be needed for 80% power to detect d=0.3).

5.4.7 Conclusions

Taken together, my findings do not provide evidence to suggest that genotypic variation of the *OXTR* gene plays a significant role in the pathophysiology of CD symptoms. However, my model did support previous observations that lower IQ and higher CU are both positively associated with the number of CD symptoms in both males and females, and that lower IQ is associated with a higher level of CU traits. My results also provide evidence for sex-differences in the relationships between genetic factors and brain responses during emotional face processing in a region, which has been repeatedly implicated in the pathophysiology of CD. Specifically, I observed that *OXTR* genotype was associated with amygdala response to angry faces in males only. Given the limitations of this study, further research is needed to better understand whether *OXTR* genotype does indeed play a role in the development of CD, either by contributing directly to increased risk, or by moderating the effect of other factors. I have demonstrated that a SEM approach can be used to investigate the links between genetic, clinical and demographic factors

in CD. However, given the limitations discussed above, future studies may need to use more elaborated models incorporating multiple environmental risk factors for CD.

CHAPTER 6: GENERAL DISCUSSION

6.1 Overview

The aim of this thesis was to use genetic, epigenetic and brain imaging data to improve understanding of how those variables are associated with CD symptoms and CU traits in youth. There was a particular focus on investigating the associations between these variables in females with CD because of the paucity of research focusing on this group (Freitag et al., 2018). I found that the association between the epigenetic variation of DNA methylation and level of CU traits has an opposite direction in CD and TD females in the region of gene *SLC25A24*. I also observed that the value of methylation in this region was associated with GMV and BOLD response (at cluster-size corrected level of significance) in several brain regions. Finally, I used an SEM approach to investigate the associations between *OXTR* genotype data, age, site, IQ, sex, CU score and brain response data from the amygdala during emotional face processing to test whether specific combinations of variables were significantly associated with CD symptoms score. I tested all models both in a mixed-sex group of participants with and without clinical diagnoses of CD, and separately in males and females. None of these models explained a significant amount of the variation in CD symptom score, but some individual paths were significant within each model. These included a negative association between IQ and CD symptom score, a positive association between CU score and CD symptom score, as well as a negative association between IQ and CU score. Finally, in males only, there was a significant positive association between *OXTR* genotype and left amygdala response to angry faces.

Table 9 provides a summary of the three studies reported in this thesis.

Table 9. Summary of chapters 3,4 and 5

	Chapter 3	Chapter 4	Chapter 5
Topic(s)	Epigenetic and structural brain imaging	Epigenetic and functional brain imaging	Genetic and functional brain imaging
Sample Size	110 F	62 F	349 (189 F)
Summary of Findings	In adolescent females the direction of association between *SLC25A24* methylation and level of CU traits was dependent on CD diagnostic status. In the CD group, there was positive association between CU traits and methylation. In the TD group, CU traits were negatively associated with methylation. Also, there were trend-level significant findings of negative associations between level of methylation and GMV in several brain regions, including the SFG, dlPFC, supramarginal gyrus, secondary visual cortex and ventral PCC.	In female adolescents, *SLC25A24* methylation was associated with increased brain responses in several cortical and subcortical regions during emotional face processing. Regions differed for the different emotional-face contrasts, however the association between level of methylation and BOLD response was positive for all regions and contrasts.	Evidence did not support the hypothesis that variation in *OXTR* genotype plays a significant role in pathophysiology of CD symptoms. Findings confirmed previously observation of negative association between IQ and CD and positive association between CU score and CD symptom score. Some evidence for sex-specific relationships between genetic factors and brain response/activation.
Power	80% power to detect effect sizes > 0.34	80% power to detect difference in methylation at recommended level for EPIC array (p<6.2e-5)	78.3% power to detect effect sizes > 0.3
Novel Element	First EWAS in females with CD and first to include neuroimaging data	First study to investigate methylation in relation to brain activity in females with CD	First study to use SEM approach to investigate neuroimaging genetic pathways in females with CD

Main Limitation(s)	Data on common SNPs and mQTLs not included	small sample size (for fMRI data only medium/large effects detectable) and data on variables which could influence brain response (e.g. experiences of trauma/maltreatment) was missing for many participants so these could not be controlled for.	*OXTR* SNP weightings taken from meta-analysis of aggression studies (only sub-section of CD symptoms aggressive)
Design	Linear Regression Modelling, Cross-sectional data	Linear Regression Modelling, Cross-sectional data	Structural Equation Modelling, Cross-sectional data
Significance Level of Epigenetic Results	*p*<.05 FDR corrected	*p*<.05 FDR corrected	n/a
Significance Level of Neuroimaging Findings	Cluster-size corrected *p*<.001 (minimum cluster k=72)	Cluster-size corrected *p*<.005 (minimum cluster k=20 voxels)	*p*<.05 FWE-corrected in ROI of the amygdala

6.2 Summary of Main Findings

6.2.1 Chapter 3: *SLC25A24* Gene Methylation and Grey Matter

Chapter 3 presented the findings from an EWAS study using salivary DNA data from female youth to investigate whether the epigenetic modification of DNA methylation was associated with either; (i) the main effect of CD diagnostic status, (ii) level of CU traits, or, (iii) the interaction between these two factors. I did not find associations between CD diagnostic status or level of CU traits and methylation; however, I did identify a region on chromosome one, which was differentially methylated according to the CD x CU interaction effect. This region coincided with the genomic location of the *SLC25A24* gene. This gene is involved in cell energy metabolism and is expressed in brain tissue across multiple cortical and subcortical regions. I did not replicate the previous findings in males of an association between methylation of the *OXTR* gene and CD/CU traits (Aghajani et al., 2018). In the second part of the study, I used VBM to investigate whether participants' average values of methylation across the *SLC25A24* gene were associated with GMV. I tested the hypotheses that greater methylation would be associated with GMV differences in four ROIs and also conducted a whole-brain exploratory analysis. I observed a sub-significant negative association between level of *SLC25A24* methylation and GMV in several clusters across multiple brain regions. These regions included the SFG, dlPFC, supramarginal gyrus, secondary visual cortex and ventral PCC. These are all regions that have been previously implicated in CD and CU traits. I concluded that these findings indicate that the association between methylation and CU traits is opposite in females with CD as comparted to TD females. Furthermore, these findings indicate that the reported differences in methylation may also be associated with volumetric differences across multiple brain regions previously implicated in CD and CU traits.

6.2.2 Chapter 4: Emotional Face Processing and *SLC25A24* Methylation

Chapter 4 built further on chapter 3 by examining the association between methylation level of the *SLC25A24* gene and functional brain response during an emotional face processing task. I performed this analysis in a subset of the participants from chapter 3 for whom good quality fMRI data was available. I first investigated whether differences in BOLD response to faces as compared to a fixation cross (null stimulus) was associated with methylation. I also investigated whether BOLD response to angry/fearful faces, as compared to neutral facial expressions, was associated with methylation. For both analyses I examined BOLD response in four ROIs and across the whole brain. The four ROIs (the hippocampus, amygdala, hypothalamus and basal ganglia) were selected as they are regions where the *SLC25A24* gene is expressed and they have been implicated in emotional processing in relation to CD. I did not observe any significant associations between brain response and methylation in any of the four ROIs, but the whole-brain analysis revealed that higher *SLC25A24* methylation was associated with increased response in several brain regions. This positive association between methylation and brain response was observed for all three contrasts (all faces>fixation, anger>neutral and fear>neutral), but the regions which showed increased activation were contrast specific. These findings demonstrate different directions of association between CU traits, methylation and brain response in females with CD vs. TD females, in-line with previous work documenting distinct patterns of associations between brain markers, (epi)genetic factors and behavioural measures in healthy control vs. psychiatric groups (e.g. Hashimoto et al., 2015). Finally, my findings, which differ from those of previous studies on males with CD (e.g. (Blair, Leibenluft, & Pine, 2014)),

support the idea that the association between brain response and CU traits might be sex-specific and of opposite direction in males and females.

6.2.3 Chapter 5: SEM of *OXTR* Genotype, CU Traits and CD Symptoms

Chapter 5 shifted the focus from investigating the epigenetic modification of DNA methylation to examining genetic variation, namely, genotype of the *OXTR* gene. I measured genotype at two individual SNPs of this gene (rs6770632 and rs53576), but also created an overall composite score, based on values from 34 SNPs across the *OXTR* gene. This composite score was calculated using weightings from a previous meta-analysis on genetic factors associated with aggression (Pappa et al., 2016). I used an SEM approach to test the hypothesis that allelic variation in the *OXTR* gene was associated with number of CD symptoms in a large sample of mixed-sex youth. The SEM approach allowed me to investigate whether there was a direct association between *OXTR* genotype and variation in number of CD symptoms, and also whether there were indirect associations arbitrated by other factors in the model. Variables included in the SEM included; *OXTR* genotype data, age, site, IQ, sex, CU score and brain response data from the amygdala during emotional face processing. Separate models were run with fMRI data representing the amygdala response to the contrasts; (i) all faces>fixation, (ii) anger>fixation and (iii) fear>fixation. Each of these models were run across the full mixed-sex sample and then independently in male-only and female-only groups. The hypothesis that variation in *OXTR* genotype would be associated with total CD symptom score variation was not supported for any of the models. However, the model did replicate previously reported findings, such as the negative association between IQ score and number of CD symptoms. I also observed a positive

association between level of CU traits and CD symptom score, in-line with previous observations of an association between CU traits and more severe conduct problems (Allen, Bird, & Chhoa, 2018). I also noted sex-specific associations between some of the variables in the SEM. In particular, in males only, overall *OXTR* risk-score was significantly negatively associated with left amygdala BOLD response.

6.3 Where Do We Go from Here?

6.3.1 Other FemNAT-CD Study Findings

The data analysed in the three studies presented in this thesis were collected as part of the FemNAT-CD project, a multi-site study focussing on CD in females (Freitag et al., 2018). In order to discuss my studies' findings in context, I must also consider the findings from other recently published neuroimaging studies that used data collected during the FemNAT-CD project (see Table 10). Taken together, the findings of these studies have led to two important conclusions. First, youth with CD show marked differences in brain structure and patterns of brain responses compared to TD youth. Secondly, the nature of both the structural and functional neural correlates of CD are sex-specific.

Table 10. Summary of Neuroimaging Studies from the FemNAT-CD Consortium

Title	**Author(s)**	**Year**	**Participants**	**Key Methods**	**Main Findings**	**CD/TD difference reported**	**Sex-specific effects reported**
Sex Differences in the Relationship between Conduct Disorder and Cortical Structure in Adolescents	Smaragdi et al	2017	N=200 (100 F) CD and TD	Surface-based Morphometry (SBM)	In both sexes, CD was associated with cortical thinning and higher gyrification in the ventro-medial PFC. **Males with CD displayed greater cortical thickness** in the supramarginal gyrus compared with controls, but the opposite pattern was seen in females. Compared to controls males with CD also showed more gyrification and greater surface area in the superior frontal gyrus, but again the	Y	Y

					opposite was seen in females. Results demonstrate both sex and CD diagnostic status effect brain structure.		
Callous-unemotional traits and brain structure: Sex-specific effects in anterior insula of typically-developing youths	Raschle et al	2018	N= 223 TD only	Voxel-Based Morphometry (VBM)	The **Sex x CU-trait interaction** predicted GMV across the mixed-sex TD participants. The interaction was driven by the significant positive correlation between CU traits and bilateral AI volume in males. GMV of the insula explained 19% of the variance CU traits in boys. Results demonstrated that, in TD males, CU traits are related to variations in brain structure.	N	Y

Neural correlates of theory of mind in typically-developing youth: Influence of sex, age and callous-unemotional traits	Gao et al	2019	N=35 (23 F) TD only	fMRI BOLD signal analysis	During a Theory of Mind task males recruited the left temporoparietal junction significantly more than females and CU traits were significantly positively associated with right AI response. Findings demonstrate that the **neural correlates of Theory of Mind in TD youth are sex-specific** and dependent on level of CU traits.	N	Y
Intention attribution and neural processing of laughter in female and male	Martinelli et al	2018	N=112 (55 F) CD and TD	fMRI BOLD signal analysis	Overall **CD participants showed greater activation** in primary auditory processing regions and more hostile ratings of friendly laughter. In males there	Y	Y

adolescents with conduct disorder					was also activation in brain areas implicated in mentalizing/social pain (i.e. mPFC and dACC) in response to mocking/taunting laughter. These findings demonstrate **sex-specific brain function during laughter processing** across CD and TD youth and also show differences in brain response to laughter between CD and TD youth.		
Atypical dorsolateral prefrontal activity in females with conduct disorder during effortful	Raschle et al	2019	N=88 (88 F) CD and TD	fMRI BOLD signal analysis	Significantly **reduced activation** in left dorsolateral PFC and angular gyrus was observed **in the CD group** compared to TD youth during emotion	Y	N

emotion regulation					regulation. Also observed **reduced connectivity** between the left dorsolateral PFC and bilateral putamen, right PFC and amygdala **in CD youth** compared to the TD group during emotional reappraisal. CU traits were negatively associated with behavioural reports of emotional reactivity. Findings demonstrate neural correlates of emotional regulation differ in CD vs. TD.		
White matter microstructure of the extended limbic system in male and female	González-Madruga et al	2020	200 (102 F) CD and TD	Diffusion tensor imaging (DTI)	Overall, compared to the TD group, **CD participants had lower fractional anisotropy**	Y	Y

youth with conduct disorder					and lower hindrance-modulated orientational anisotropy in the right retrosplenial cingulum tract. These **effects were moderated by sex** and driven by the findings in males. In females-only there was no significant difference between CD and TD. These findings again highlight the importance of considering sex when studying the neurobiology of CD.		
White Matter Microstructure in Youths With Conduct Disorder:	Rogers et al	2019	N=298 (162 F) CD and TD	DTI	Compared to the TD group, **CD youth exhibited higher axial diffusivity** in the corpus callosum and lower radial	Y	Y

Effects of Sex and Variation in Callous Traits					diffusivity and mean diffusivity in the anterior thalamic radiation. In the CD group, changes in the internal capsule, fornix, posterior thalamic radiation, and uncinate fasciculus showed **opposite directions of association in males and female**. Also, in the CD group, CU traits predicted the increased axial diffusivity in the corpus callosum and were negatively associated with radial diffusivity in the anterior thalamic radiation.		
Sex matters: association between callous-	Villemonteix et al	2021	N = 124 (59 F) CD only	DTI	The association between brain structure and level of CU traits in youth with	N	Y

unemotional traits and uncinate fasciculus microstructure in youths with conduct disorder	CD was sex-specific. Left uncinate fasciculus axial diffusivity was positively associated with CU traits in males, by the opposite association was seen in females. This sex-specific association between brain structure and level of CU traits in youth with CD highlights the importance of considering sex when investigating brain structure in CD.

Findings from the two studies in TD participants (Gao et al., 2019; Raschle et al., 2018) show that sex and level of CU traits interact to predict both brain structure and function in youth without psychiatric diagnoses. In males-only, there appears to be a significant positive correlation between CU traits and brain volume in the AI (Raschle et al., 2018). These results demonstrate the sexual dimorphism of the neural correlates of CU traits in TD youth and underline the importance of considering sex when investigating the association between CU traits and brain correlates. The results from Raschle and colleagues (2018) also showed an opposite association between CU traits and MRI markers in TD youth compared to that previously reported in clinical groups (e.g. (Caldwell et al., 2019; Cohn et al., 2016)). Observable sex differences in brain function were also reported in TD youth during a 'Theory of Mind' task, specifically, in the left temporoparietal junction (Gao et al., 2019). However, in the aforementioned study no sex differences in activation were detected in either the amygdala or AI (Gao et al., 2019). These findings demonstrate that in TD youth social processing is composed of both shared and sex-specific neural correlates, and that sexual dimorphism is observable in both brain structure (Raschle et al., 2018) and function (Gao et al., 2019). Chapter 3 of this thesis reported a negative association between level of CU traits and methylation of the *SLC25A24* gene in TD females but given the sex specific nature of neurobiological correlates of CU traits in the brain, these findings are likely to differ in males. One previous study has investigated the association between CU traits and epigenetic modifications in a male-only sample (Aghajani et al., 2018), but further studies in mixed-sex participant groups are needed to fully understand the nature of these differences and to determine whether the findings from the existing single-sex studies are replicable.

In youth with CD, it appears that some structural brain abnormalities, such as cortical thinning and higher gyrification in the ventromedial PFC, are shared between males and

females (Smaragdi et al., 2017). However, there is also evidence for some sex-specific structural neural correlates of CD. For example, cortical thickness in the supramarginal gyrus and cortical surface area in the SFG show an opposite direction of association with CD diagnostic status in males as compared to females (Smaragdi et al., 2017).

Findings from the fMRI studies in CD and TD youth listed in Table 10 (Martinelli et al., 2018; Raschle et al., 2019) indicate that there are also sex-specific markers of brain function during tasks involving emotion regulation, and processing of affective cues such as laughter. These findings are in-line with observations presented in chapter 5 of this thesis, where I report some evidence of sex specific associations between *OXTR* genotype (based on a combined risk score of 34 SNPs) and brain response in the amygdala during an emotional face processing task. Taken together, these findings may suggest that a combination of sex and inter-individual variation in genotype for genes associated with neurohormones, like oxytocin, may partially explain the distinct patterns of brain function displayed by males and females with CD during affective processing tasks (Alegria et al., 2016; Martinelli et al., 2018).

There are some noteworthy differences between the methodologies of the studies presented in this thesis and other FemNAT-CD MRI studies summarised in Table 10. For example, I conducted both ROI and whole-brain exploratory analysis to investigate the relationship between methylation and brain activity during an emotional face processing task, whereas Raschle et al (2019) only focused on *a priori* ROIs (Raschle et al., 2019). Further, while Martinelli et al (2018) did perform whole-brain analysis, they did not investigate the interactions of CU traits with other variables in their model (Martinelli et al., 2018), as done in this thesis. While each study used a different task to elicit brain response, meaning that

their findings are not directly comparable, those studies provide evidence of distinct neural correlates in CD as compared to TD youth across a range of affective processes.

Findings from the diffusion MRI studies listed in Table 10 also provide support for distinct structural brain correlates of CD in males and females. For example, in males only, significantly lower fractional anisotrophy and hindrance-modulated orientational anisotrophy in the right retrosplenial cingulum tract were reported in the CD group compared to the TD group (González-Madruga et al., 2020). Additionally, opposite structural differences in white matter across the internal capsule, fornix, posterior thalamic radiation, and uncinate fasciculus were observed for males and females with CD compared to TD youth (Rogers et al., 2019). In the same study, there was also evidence that white matter microstructure was associated with level of CU traits, although the direction of association was region-specific. Namely, a positive correlation between axial diffusivity in the corpus callosum and CU traits was reported, but in the anterior thalamic radiation a negative correlation between level of CU traits and radial diffusivity was observed (Rogers et al., 2019). In the same participant sample, separate analysis also revealed that in youth with CD there is a sex-specific association between CU traits and axial diffusivity in the uncinate fasciculus, with a positive association between CU traits and axial diffusivity demonstrated in males, but a negative association in females (Villemonteix et al., 2021). When taken alongside the findings presented in this thesis, those structural data provide further evidence for differential brain structure in CD as compared to TD youth and also indicate that these associations may be sex-specific. Additionally, they suggest that distinct neural correlates of CU traits exist for male and female youth with CD across multiple brain regions, which may further indicate that the underlying biology and neurodevelopmental origins of CD and CU traits are also sex-specific.

In addition to these neuroimaging findings, several other non-neuroimaging studies from the FemNAT-CD consortium have provided novel neurocognitive evidence of heterogeneity CD (Kohls et al., 2020), and also demonstrated sex differences in both the clinical presentation (Konrad et al., 2021) and neurobiology (Bernhard et al., 2021) of CD. For example, Kohls et al (2020) provided novel insight into the neuropsychological subgroups within youth with CD. Specifically, they noted that less than a quarter of participants with CD showed clinically meaningful impairments in emotional recognition (23%), emotional learning (13%) or emotional regulation (18%) (Kohls et al., 2020). Interestingly, over half of the youth with CD included in the study showed no significant deficits in neuropsychological function at all. These findings demonstrate the importance considering the heterogeneity within CD because it may have implications for the design of individually-tailored interventions, which will likely need to consider both the individuals' level of CU traits and sex (e.g., behavioural interventions aimed at improving emotional processing may only benefit the subgroup of youth with CD for whom these impairments are significant (Kohls et al., 2020).

Additionally, Konrad et al (2021) observed significant sex differences in the clinical presentation of CD symptoms and rates of comorbid psychiatric diagnoses (Konrad et al., 2021). Higher rates of internalising disorders (anxiety disorders, MDDs, PTSD and emotionally unstable personality disorder) were found in girls with CD, while a higher prevalence of comorbid ADHD was reported in boys (Konrad et al., 2021). They also reported evidence of sex-specific symptom presentations and noted that fewer girls had experienced childhood-onset CD symptoms (Konrad et al., 2021). These findings support the 'gender-paradox' hypothesis by demonstrating that, while CD diagnoses are less prevalent in females, they tend to be accompanied by more severe functional impairments and

comorbidities than in males. The findings also provide evidence for a 'delayed-onset' pathway to CD in females, where symptoms are most likely to develop during adolescence (Konrad et al., 2021). Given the findings from another FemNAT-CD study, it is plausible that these findings may be linked directly to the sex-specific neuroendocrine changes the occur during puberty. Bernhard et al (2021) reported sex-specific associations between basal steroid hormone levels and neuropeptides within youth with CD. Specifically, they observed that increased androgen levels in males, and decreased oestrogen in females were predictive of CD group status (Bernhard et al., 2021). Dysregulation of the oxytocin neuropeptide system in interaction with stress system measures was also reported only for females with CD (Bernhard et al., 2021). Finally, Chiocchetti et al investigated DNA methylation in blood samples from CD and TD females and found increased methylation of the *SLITRK* gene in the females with CD, as compared to the TD females. Given the involvement of that gene in modulating neurite growth, it was concluded that this modification might relate to alterations to developing brain networks of females with CD (Chiocchetti et al, in preparation). Again, as this is among the first studies to investigate epigenetic modifications in CD, replication of these findings and/or studies in males with CD or mixed-sex participant groups will be a necessary future development to determine whether these findings represent a reliable shared or sex-specific epigenetic marker of CD.

Taken together, these findings from the FemNAT-CD group show differences in both brain structure and patterns of brain responses between youth with CD and TD youth. The findings also provide evidence that the neural correlates of CD may be accompanied by neuroendocrine and epigenetic changes, and that differences in these markers may be observable between CD and TD groups. However, there is considerable heterogeneity within individuals with CD, and these findings also suggest that both sex and level of CU traits influence the brain markers and neurobiological signatures associated with CD. In the next

section I will further discuss the evidence for the different patterns of association between CU, genetic and brain markers in CD as compared to TD youth, drawing on both the findings from the studies presented in this thesis and those from other recent work.

6.3.2 A Different Relationship between CU, Genetic and Neuroimaging Markers in CD vs. TD Youth

Previous studies have reported significant positive genetic correlations between CU traits and CD in both mixed-sex youth (r_G = .77, (Saunders et al., 2019)) and separately for females (r_G = .65) and males (r_G = .57) (Viding, Frick, & Plomin, 2007)). From this evidence that these two constructs share similarities at a genetic level (in both sexes), we may next consider whether there is overlap in their epigenetic underpinnings. The findings in chapter 3 indicate that this is not the case, as I did not observe main effects of CD or CU traits in same direction on the same gene. Instead, I found that the epigenetic modification of DNA methylation at the *SLC25A24* gene is linked to distinct patterns of CU traits depending on whether the individual has CD or not. Specifically, the direction of the correlation between *SLC25A24* gene methylation and level of CU traits was negative in TD females and positive in females with CD. Another recent study in males (Aghajani et al., 2018) observed that the association between methylation level of the *OXTR* gene and overall neural activation differed depending on both the participants' levels of CU traits and CD diagnostic status (see Figure 31 below). Specifically, they observed that overall neural activity strength (during the emotional face processing task) was differentially associated with *OXTR* methylation level at low, moderate, and high CU levels, in CD versus healthy control participants (Aghajani et al., 2018). These findings highlight the importance of including a control group in imaging genetics studies in psychiatry, as a fundamentally different association may be present between neuroimaging

and genetic markers in healthy control as compared to psychiatric populations (Hashimoto et al., 2015).

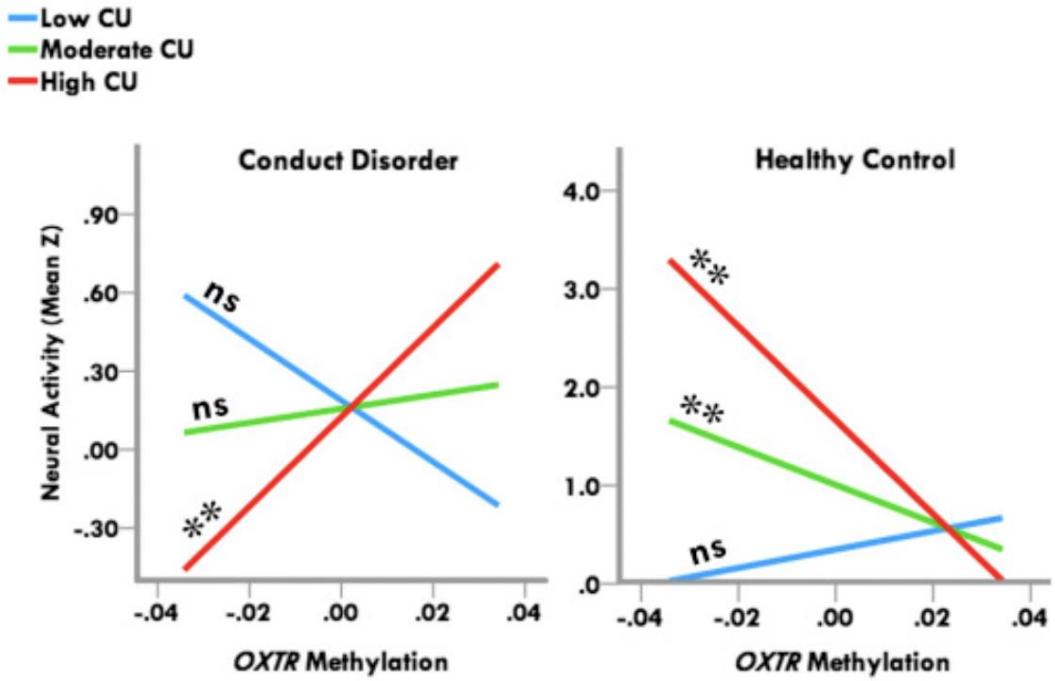


Figure 35. Differing directions of association between overall neural activation to emotional faces and DNA methylation of the *OXTR* gene according to CD diagnostic status and level of CU traits (low, moderate, high) and group (CD vs Healthy) in adolescent males (Aghajani et al., 2018).[2]

It has been suggested that genetic or epigenetic modifications can only be considered markers of psychiatric disorder when considered in the context of other variables, such as affective traits (Gescher et al., 2018; Moul, Dobson-Stone, Brennan, Hawes, & Dadds, 2015). In light of this observation, and given the results reported in this thesis, findings from previous

[2] This figure is taken from the original published work by Aghajani et al, the different y axis scales on the graphs reflect the different intra-group variance in neural activation for each of the groups. The original figure legend is included below.

Oxytocin receptor gene (methylation (OXTR Meth) 3 callous-unemotional (CU) traits 3 diagnosis interactions impact neuroprocessing of emotion recognition, within the midcingulate, insular, temporoparietal, precuneal, and supplementary motor regions. The interaction effect within these regions (F 1,51 = 14.39, p , .001, R 2 change = .172) was due to increasing OXTR Meth levels relating to neural hyperactivity in youths with conduct disorder at high CU levels (B = 17.10, p = .009), but to hypoactivity in healthy control youths at either high or moderate CU levels (high: B = 247.75, p = .002; moderate: B = 219.18, p = .006). Scatterplots visualize the direction of associations, in which neural activity strength (y axis) as indexed by z values averaged across all illuminated voxels is plotted against average OXTR Meth levels (x axis), at low, moderate, and high CU levels, in conduct disorder versus healthy control participants. **p , .05. ns, not significant (p. .05). (Aghajani et al., 2018)

studies that have reported particular epigenetic modifications associated with a psychiatric disease phenotype without also considering measures of affective traits should be interpreted with caution. For example, in previous work, levels of *MAOA* gene promoter methylation were positively associated with ASPD in incarcerated males (Checknita et al., 2015). However, as a measure of CU traits was not included in this study, additional effects, which may occur in differing directions for participants with high vs. low levels of CU traits, could have cancelled each other out, and thus led to an over-simplified picture of the association between *MAOA* methylation and ASPD. Also, the findings presented in chapter 3 highlight a potential limitation of using findings such as this to identify a diagnostic biomarker for antisocial behaviour until we have also studied this modification in TD populations (i.e., elevated *MAOA* methylation may be observed in individuals with ASPD and high CU traits, but also in healthy controls with low CU traits, as I observed with *SLC25A24* methylation in chapter 3). Rather, determining the way this pattern of methylation interacts with other aspects of the individual, such as level of CU traits, may provide a more complete understanding of why some individuals with this epigenetic modification present with antisocial behaviours, while others do not. The findings from females presented in chapter 3, alongside those from Aghajani et al (2018) in males (Aghajani et al., 2018), highlight the importance of considering CU traits in relation to antisocial behaviour in both sexes. Taken together, these findings provide further support for the inclusion of a CU-trait specifier in the diagnosis of CD, in addition to sex-specific criteria (this will be discussed in more detail in section 6.3.4).

6.3.3. Markers for Distinguishing between CD and TD

The 3-way relationship I observed between epigenetics, affective (CU) traits and the CD behavioural phenotype demonstrates the importance of considering interactions between

several different clinical and behavioural measures when trying to identify 'biomarkers' of psychopathology in general, and CD and CU traits in particular. Thus, it appears that distinguishing psychiatric 'disease' groups from healthy control groups based on biological markers alone is not a trivial endeavour. A more promising approach for future studies may be to integrate data from multiple modalities, which provides the opportunity to observe combinations of factors that are associated with a specific phenotype. For this, data integration methods that can extract biological insight from multimodal data will likely be required (Larranaga et al., 2006). For example, deep learning-based approaches that incorporate genetic, epigenetic, clinical, and neuropsychological data may allow us to discover integrative features associated with anti-social behavioural phenotypes that cannot be explained by a single data type. Work to develop machine learning classifiers that can model high dimensional multimodal data to differentiate between clinical and non-clinical groups is already underway in other areas of neuroscientific and psychiatric research. For example, recent work from the field of degenerative brain diseases reports the success of using such models for early diagnosis of Alzheimer's disease (Lee, Nho, et al., 2019; Lu et al., 2018; Venugopalan, Tong, Hassanzadeh, & Wang, 2021), and preliminary studies using these modelling techniques to predict disease progression have also provided promising results (e.g. (Liang et al., 2021)). Although this research is still in its infancy, software tools which incorporate gene expression, methylation, and SNP data are already freely available, such as the multimodal longitudinal data integration framework (*MildInt*, (Lee, Kang, Nho, Sohn, & Kim, 2019)). Future work building on the existing findings from genetic, epigenetic and neuropsychological studies on CD and CU traits to apply these tools to antisocial behavioural phenotype will be a valuable development in the field. Integrating data measures from areas of genetic or environmental risk may also ultimately enable us to develop reliable

prognostic signatures for CD progression, and tailor intervention and prevention strategies accordingly.

6.3.4 Sex Differences in Other Neurodevelopmental Disorders: The wider Perspective

Several authors have suggested that CD is neurodevelopmental in nature (Fairchild et al., 2019; Raine, 2018). Considering psychopathy as a sexually dimorphic neurodevelopmental disorder has also been proposed as a useful next step for better understanding the differences that emerge in early life between males and females with CD and high CU traits (Tully et al., 2021). Sex differences in symptom presentations and their underlying biological mechanisms have also been reported for a number of other psychiatric/neurodevelopmental disorders. One example of this is in ASD. As with CD, the sex distribution of youth with an ASD diagnosis is skewed towards males, with an estimated ratio of around 3:1 in males to females (Loomes, Hull, & Mandy, 2017). Sex-by-diagnosis interaction effects on brain structure have also been observed in ASD. For example, elevated volume in the temporal-parietal lobe is reported in females with ASD as compared to TD females, but this case-control distinction is absent in males (Lai et al., 2013). Other studies have reported structural neural differences associated with ASD in males, but not in females with ASD (e.g. Beacher et al., 2012; Schaer, Kochalka, Padmanabhan, Supekar, & Menon, 2015; Zeestraten et al., 2017). These findings may be particularly relevant for researchers and clinicians seeking to better understand and treat CD where sex-by-diagnosis interaction effects on brain correlates have also been reported. For example, theoretical models that have been put forward to explain the difference in prevalence in ASD may also inform theoretical models of CD. In particular, two of the most widely accepted theories to explain why ASD is more commonly diagnosed in males centre around the concept of female protective factors (Werling & Geschwind, 2013)

and the ‘Extreme Males Brain’ theory (Baron-Cohen, 2005). Possibly, aspects of these theories may also be applicable to help explain the sex-differences in the neural correlates of CD. For example, the ‘Female Protective Effect’ theory of ASD suggests that certain sex-specific factors ‘protect’ females from reaching the diagnostic threshold and so those who do are likely carrying more substantial genetic/environmental contributions than males with this diagnosis (Werling & Geschwind, 2013). Drawing on this model, future studies on CD (and CU traits) which incorporate sex-specific analyses may allow researchers to determine whether a similar effect also occurs for females with CD, and thus better elucidate the differences in the developmental pathways to CD for males and females (Tully et al., 2021). Thus, is seems integrating knowledge from research into other neurodevelopmental disorders will be an important next step in advancing CD research (Svensson et al., 2018). This may be particularly relevant given that youth with one type of neurodevelopmental issue commonly display additional neurodevelopmental issues, as described by the ‘ESSENCE’ theory (Gillberg, 2010). This means that, in practice, clinicians aiming to treat youth with CD will likely encounter considerable co-morbidity with neurodevelopmental disorders, and so treatments that address neurocognitive deficits common to multiple conditions may be an efficient mean of symptoms amelioration in youth with CD symptomatology.

ADHD is another psychiatric disorder with neurodevelopmental origins, which is more commonly diagnosed in males than females (Biederman et al., 2002; Gudjonsson et al., 2016). ADHD and CD are highly co-morbid (Storebo & Simonsen, 2016) and are understood to be clinically related (Rubia, 2011). Sex differences are reported in both the clinical presentation and symptom severity of ADHD (Gershon, 2002). As with CD, these differences in symptom presentation may contribute to the higher rates of ADHD diagnoses in males. It is suggested that the predominant ADHD phenotype in females is characterized by more

inattentive than hyperactive/impulse-control behaviours, which are less easily recognised as problematic (Gaub & Carlson, 1997; Quinn, 2008). Sex differences in the relationship between brain structure and ADHD symptomatology are also reported. For example, in females with ADHD only, reduced cortical surface area is observed in the PFC, as compared to TD females (Dirlikov et al., 2015). Whereas, in males, ADHD diagnosis is associated with reduced cortical surface area in the primary motor regions (and this finding is not observed in females (Dirlikov et al., 2015).

These findings, which demonstrate the existence of sex difference in several neurodevelopmental disorders, may also provide principles that can be used by clinicians working with patients with CD. Specifically, methods for tailoring treatment according to these sex differences may also be relevant for those working with patients with CD, as the sex-specific neurocognitive impairments and patterns of symptom presentations observed in males and females with CD may require distinct therapeutic approaches to. For example, compensatory strategies are reported to be employed by females with both ADHD (Mowlem, Agnew-Blais, Taylor, & Asherson, 2019; Quinn & Madhoo, 2014) and ASD (Livingston & Happe, 2017) to mask their behavioural symptoms, and this has been suggested to partially account for the sex differences in the reported prevalence of these disorders (Schuck, Flores, & Fung, 2019; Young et al., 2020). The use of 'camouflaging' strategies is therefore also an area worth investigating in females with CD, and if appropriate, therapeutic techniques used to overcome these masking behaviours in ADHD/ASD patients may be transferable to work with females with CD.

Females with life-course persistent CD symptoms suffer the worst mental (Odgers et al., 2008) and physical (Bardone et al., 1998) health outcomes and, in order to address this, it may be valuable for clinicians to consider the sex-specific recommendations for treating other

neurodevelopmental conditions with sex differences. Some of the guidelines for working with females with ADHD may be particularly useful for clinicians working with females with CD in order to improve rates of treatment response. Specifically, incorporating targeted psychotherapeutic work into treatment to address the social-relational and psychosexual problems in females with CD (as is already part of ADHD treatment methods, *(*Young et al., 2020)) may be valuable. Additionally, considering how hormonal changes (e.g. during the menstrual cycle or pregnancy) interact with (or exacerbate) symptoms and may alter the effectiveness of some medications (see (Quinn, 2005)) may be of benefit to clinicians working with females with CD.

In summary, these findings demonstrate the importance of further research into the sex differences in psychopathologies at a neurobiological level, in order to develop our understanding of the distinct biological underpinnings of these conditions in males and females. There are also implications of these differences for treatment and prevention strategies that must be considered by clinicians working with individuals with these diagnoses in order to maximise the effectiveness of targeted interventions in both sexes.

6.3.5. Implications for Future Research on CD

The findings from studies presented in this thesis, and the consistent findings of sex differences in the clinical presentation (Ackermann et al., 2019; Cao, Sun, Dong, Yao, & Huang, 2018; Konrad et al., 2021) and neurobiological markers (Gonzalez-Madruga et al., 2020; Smaragdi et al., 2017; Villemonteix et al., 2021) associated with CD in previous work underline the importance of considering biological sex when investigating this disorder. In particular, researchers should avoid amalgamating the sexes in CD neuroimaging studies, as

effects which are either present in only one sex or are of opposite directions in the two sexes may counterbalance each other and appear as null findings (Smaragdi et al., 2017). As previously mentioned, females are underrepresented in the CD literature (Freitag et al., 2018), thus it would be most valuable for studies with low participant numbers to focus solely on females. Larger mixed-sex studies would benefit from ensuring that they are suitably powered to test for sex-by-diagnoses interactions. Prospective longitudinal studies are also needed to explain the mechanisms behind the observed sex-specific trajectories of CD (Moffitt & Caspi, 2001). Another potential challenge for future research on CD is to be mindful of the previously reported sex differences, while also respecting gender diversity and ensuring the inclusivity of research practices going forward (Cameron & Stinson, 2019). Given that non-binary gender identity is becoming increasingly common in youth (Diamond, 2020), understanding how gender identity interacts with biological sex in relation to CD symptomatology is also likely to be an important area for further study.

Findings reported in this thesis also provide further evidence for considering variations in CU traits both youth with CD and TD youth. The heterogeneity within CD provides a challenge to researchers of disruptive behaviour disorders (Frick & Viding, 2009), indeed it has been suggested that the approach of considering CD as a single diagnosis (one conduct disorder as opposed to multiple conduct *disorders)* is inherently limited (Viding & McCrory, 2020). However, the practice of subgrouping youth with CD according to their level of CU traits is supported by a comprehensive research base. For example, twin studies demonstrate that conduct problems in the presence of high levels of CU traits are more heritable, whereas environmental factors contribute more to development of CD in coincidence with lower levels CU traits (Viding, Blair, Moffitt, & Plomin, 2005; Viding, Jones, Frick, Moffitt, & Plomin, 2008). Also, psychophysiological and neuroimaging research consistently reports

muted response to emotional stimuli in the group with CD and high levels of CU traits (e.g. Blair et al., 2014; de Wied, van Boxtel, Matthys, & Meeus, 2012) which contrasts with the repeated reports of elevated response to threatening stimuli in youth with CD and low CU traits (Blair et al., 2014; Sebastian et al., 2014; Viding et al., 2012)). I observed that methylation of the *SLC25A24* gene relates to level of CU traits in a fundamentally different way in female youth with CD as compared to TD females, which would not have been apparent had I not considered the interaction between group status and CU traits in this analysis. This highlights the importance of including a measure of CU traits and examining its correlates in both youth with CD and TD youth. The findings that these differences in methylation also relate to variation in brain structure and function during an emotional face processing task also emphasize the importance of previous recommendations (e.g. Caldwell et al., 2019; Raschle et al., 2018) to include CU traits as a variable in neuroimaging research.

6.3.6 Implications for Interventions, Treatment and Clinical Management of CD

Acknowledging both the differences between males and females, as well as between youth with high vs. low levels of CU traits has important implications for clinical practice.
The evidence presented for sex differences in the clinical presentation (Ackermann et al., 2019; Konrad et al., 2021) and neurobiological correlates (González-Madruga et al., 2020; Smaragdi et al., 2017) of CD demonstrates that sex-specific intervention and treatment strategies are vital (Smaragdi et al., 2020). The existing literature indicates that interventions aimed at addressing antisocial behaviours have the greatest efficacy when they are introduced mid-childhood (Piquero et al., 2016). For example, the Stop Now and Plan (SNAP) intervention has been shown to aid the reduction of aggression, conduct problems and internalizing disorders in children aged 6-11 (Burke & Loeber, 2015; Koegl, Farrington,

Augimeri, & Day, 2008). This programme has been successfully used to ameliorate CD symptomatology in both boys (Burke & Loeber, 2015) and girls (Pepler et al., 2010). Although recent research demonstrates that treatment outcomes for youth with CD using this programme also vary according to sex-specific subgroups (Smaragdi et al., 2020). Developing evidence-based treatment strategies for CD in non-binary youth will also likely be a necessary future development in clinical work.

The differences in type and severity of neuropsychological deficits between youth with high vs. low levels of CU traits also indicate that distinct approaches are needed to address the dissimilar behaviours included in CD symptomatology. Clearly, including both sex and level of CU traits will be important for optimising treatments and developing targeted strategies for identifying relevant neurocircuitry that should be targeted to treat the specific psychological or affective deficits seen in CD (Anderson & Kiehl, 2014). The evidence presented in this thesis demonstrate that differences in structure and function relating to CU traits are present across multiple brain regions in both cortical and subcortical regions. This suggests that the present focus on the amygdala and frontal cortical circuits in relation to CD and CU traits (Blair, 2013; Koenigs, 2012; Moul, Killcross, & Dadds, 2012) may need to be expanded so that clinicians adopt a more holistic approach to addressing neurocognitive deficits. In youth with CD and high levels of CU traits, behavioural training interventions based on improving emotion-recognition (Dadds, Cauchi, Wimalaweera, Hawes, & Brennan, 2012), enhancing coping skills for responding appropriately to emotional distress (Muratori et al., 2017) and rewarding emotional skill development (Datyner, Kimonis, Hunt, & Armstrong, 2016) have all shown promising results. In adults with psychopathy and criminal histories there has also been some success using real-time EEG and fMRI neurofeedback exercises to normalize activation of the insula and PFC (Konicar et al., 2021; Konicar et al., 2015; Sitaram et al.,

2014) and subsequently reduce antisocial proclivities in this group (Konicar et al., 2021; Konicar et al., 2015). Thus, delivering neurofeedback training to youth with CD and high CU traits that specifically targets circuits associated with empathy and prosocial behaviour has been highlighted as a promising next step for ameliorating psychopathological symptoms (Paul & Bennett, 2021). In contrast, in youth with low CU traits, neurofeedback training which enhances the activation of circuitry involved in co-ordinating emotional regulation may be of more value, as emotional dysregulation (and subsequent displays of reactive aggression) are more common in this group (Blair, 2014). It will also be important to consider how these interventions may need to be sex-specific.

6.4 Limitations

6.4.1 Challenges of MRI Research in Participants with CD

Neuroscientific research in psychiatric disorders has often struggled to recruit enough participants to perform adequately powered studies (Turner, Paul, Miller, & Barbey, 2018) and research on CD is no exception. However, recent initiatives to address this, such as the multi-site FemNAT-CD study (Freitag et al., 2018), Enhancing Neuroimaging Genetics through Meta-analysis (ENIGMA; (Thompson et al., 2014) and IMAGEN (Quinlan et al., 2017)) projects have been major developments in the field. These collaborations allow more precise estimates of effect sizes and the increased sample sizes reduce the likelihood of both Type 1 and Type 2 errors (Klapwijk, van den Bos, Tamnes, Raschle, & Mills, 2021). Longitudinal multicentre projects have also been identified as a promising approach for recruiting adequate numbers of females with CD and high CU traits to study the trajectory of this group into early adulthood and beyond (Tully et al., 2021). However, at present the reproducibility of neuroimaging is still an on-going issue, which requires attention (Poldrack

et al., 2018) and thus multi-site work to replicate the findings from previous studies in CD participants will be of value .

There are also some specific challenges for developmental neuroimaging studies (Klapwijk et al., 2021), particularly the increased in-scanner motion observed in younger participants may confound results (Ducharme et al., 2016; Satterthwaite et al., 2012). Similarly, in case-control studies where the disease phenotype is associated with impulse control, such as CD, lower scan quality may be problematic among clinical groups (Klapwijk et al., 2021). This effect was recently demonstrated by a multi-site ASD study, where implementing post factum stricter quality control measures resulted in the exclusion of 1818 of the original 3145 participants and was accompanied by an attenuation in between-group differences (Bedford et al., 2020). Similarly, a recent meta-analysis of ADHD DTI studies found that the majority of studies included significant case-control group differences in head motion and that, in general, for those where the groups did not differ in head motion there were also no significant DTI findings (Aoki et al, 2018). Another important factor for case-control neuroimaging studies is having the capability to differentiate between the effects of the main variables of interest (e.g. CD, CU traits) and other factors which affect the brain (Peterson, 2003). For example, comorbid disorders (e.g. Rubia et al., 2008; Schiffer et al., 2013; Seleem et al., 2020), medication (Pape et al., 2021) and illicit substance use (Kroll et al., 2020) may all influence brain structure and function. In the study presented in chapter 5, the comorbidity rates of other psychiatric diagnoses did differ between males and females, thus I cannot rule out the possibility that this may have contributed to the sex-specific effects I observed. In this study, I determined that it was not appropriate to control for ADHD comorbidity in youth with CD, due to the overlap of symptoms of this disorder with CD symptoms. However, this means that I cannot disentangle neuroimaging findings associated with CD and those

associated with ADHD symptomatology. A possible solution to this issue would be for future research in this area to include an 'ADHD-only' clinical control group. This method was used effectively in a recent FemNAT-CD study that investigated the neural correlates of reward processing in adolescents with CD-only, compared to those with CD and ADHD diagnoses, and those with ADHD-only (Baumann et al., 2021).

In the studies presented in this thesis, I excluded participants who used psychoactive medications. This was done to reduce the likelihood of this being a confounding factor which influenced my results. However, by doing this I also made my sample of youth with CD less representative of the general population of youth with CD, where a higher proportion may be taking these medications (Pringsheim, Hirsch, Gardner, & Gorman, 2015). Similarly, in the studies presented in chapters 3 and 4, participants who were smokers or taking hormonal contraceptives were excluded, and in all studies, participants who were currently intoxicated were excluded. However, one key limitation of the research presented in chapters 3 and 4 of this thesis is the fact that we cannot be sure of participants' levels of substance use, prior to, or at the time of, data collection. Although we removed participants who self-reported that they were smokers and requested that participants abstained from alcohol or drugs in the hours preceding testing sessions, we did not explicitly measure drug or alcohol levels therefore it is possible that there was intra-individual variation between participants in their levels of substance misuse. This may subsequently have influenced DNA methylation levels in ways not accounted for by our analysis, thus causing a potential confound. This may be particularly relevant as having a CD diagnosis increases adolescents' risk of engaging in both alcohol and illicit substance misuse behaviours (Hopfer et al., 2013) and also exposure to both alcohol (Harlaar and Hutchinson, 2013) and recreational drugs (Wong et al., 2011) have been previously shown to influence DNA methylation. These specific features of my

participant groups should be noted when seeking to apply my findings to the general population of youth with CD.

6.4.2. What About Age-of-Onset?

In the studies reported in this thesis, I did not distinguish between participants based on age-of-onset of CD symptoms, thus I cannot rule out the potential effects of this aspect of heterogeneity within CD on my findings. Specifically, as CD in females is more associated with adolescent onset (Berkout, Young, & Gross, 2011; Konrad et al., 2021), some of the sex differences I observed may instead reflect differences according to age of symptom onset (but because of the low numbers of females with childhood-onset CD it is nearly impossible to study age-of-onset in relation to CD in females). However, while some studies have reported differences between individuals with adolescent-onset, life-course persistent and childhood-limited symptoms in terms of their levels of cognitive impairments (Johnson, Kemp, Heard, Lennings, & Hickie, 2015), environmental risk factor susceptibility (Barker & Maughan, 2009), symptom severity and long-term prognoses (Moffitt & Caspi, 2001), other recent research has presented more mixed findings. For example, genetic liability appears to account for a similar amount of the variation in CD symptoms for both the childhood-onset (62%) and adolescent-onset (65%) subtypes (Silberg, Moore, & Rutter, 2015) and Odgers et al (2008) demonstrated no difference in adult outcomes between individuals with CD according to age-of-onset (Odgers et al., 2008; Odgers, Robins, & Russell, 2010). Also, a number of structural neuroimaging studies have reported similar structural neural correlates of CD, regardless of age of symptom onset. For example, considerable overlap in the GMV reductions observed in males with the two age-of-onset subtypes of CD has been reported (Fairchild et al., 2011) along with no significant differences in cortical thickness or surface area (Fairchild et al., 2015). Of particular relevance to the studies presented in this thesis, research also indicates

there is no significant difference in brain response between males with CD in different age-of-onset groups in response to angry as compared to neutral facial expressions (Passamonti et al., 2010).

6.4.3 Challenges of Epigenetic Research in Children and Adolescents with Psychiatric Disorders

Epigenetic research in psychology using carefully phenotyped individuals provides researchers the opportunity to identify novel biomarkers associated with the phenotype, which may subsequently prove to be useful therapeutic targets (Kular & Kular, 2018). Specifically, changes in the methylation status of target loci may be useful measures to indicate disease progression (Bagot, Labonte, Pena, & Nestler, 2014) and/or treatment efficacy (e.g. (Adriani et al., 2018; Lisoway, Zai, Tiwari, & Kennedy, 2018; Yehuda et al., 2013)). In such a way, this research has the potential to enable clinicians to develop improved strategies for early diagnosis, prevention and treatment of psychopathological symptoms (Bowley, 2021). However, in order for such studies to be of value, they must yield robust results that can be replicated.

Identifying epigenetic targets and markers of early environmental exposures may allow clinicians to develop effective evidence-based strategies for treating youth who present with genetic and/or environmental risk factors for psychopathologies (Bianco-Miotto, Craig, Gasser, van Dijk, & Ozanne, 2017; Murgatroyd & Spengler, 2011). However, studies in the area of developmental human behavioural epigenetics have a number of specific limitations (Provenzi, Brambilla, Borgatti, & Montirosso, 2018). First, the majority of research currently published is from cross-sectional or retrospective study designs. Thus, findings from these studies cannot provide researchers with the capacity to develop causal interpretations.

Prospective and longitudinal studies are needed to understand the nature of associations between genetic and epigenetic factors and behavioural or clinical phenotypes. However, unlike genome sequencing (which can be done using a single tissue sample from one time-point), epigenome sequencing in longitudinal studies will require multiple samples over time from the tissue(s) of interest (Carter et al., 2017). This means these studies will rely heavily on long-term engagement in epigenomic investigations from enrolled participants, which may be particularly challenging when working with adolescents with psychiatric disorders, who are known to be less compliant, particularly those with externalising disorders (Beech, Carter, Mann, & Rotshtein, 2018). Sufficient sample sizes in longitudinal studies are crucial for reducing the risk of false positive discoveries posed by inadequately powered studies. This was recently demonstrated by a meta-analysis investigating the association between neonatal (cord blood) DNA methylation and prosocial behaviours in childhood (e.g., Luo et al., 2021). The authors reported no significant association between these factors across the four included cohorts, in contrast to previous reports from smaller scale studies which have demonstrated that DNA methylation at birth is associated with similar constructs, namely social communication deficits (Rijlaarsdam et al., 2021), CU traits (Cecil et al., 2014) and conduct problems (Cecil et al., 2018).

Another on-going issue in the area of epigenetic research surrounds the efficacy of using peripheral tissue samples, such as blood or saliva, when studying disorders that predominantly manifest in the brain (Bakulski, Halladay, Hu, Mill, & Fallin, 2016). This may be of particular importance when studying children and adolescents, as adolescence is a period characterized by significant biological development, which is often associated with changes in DNA methylation (Han et al., 2019) These changes may occur at a tissue-specific

rate, and so concordance of DNA methylation between different tissues may also vary as a function of age (Xu & Taylor, 2014).

Finally, the principle challenge for epigenetic studies in psychology, and other fields, is to distinguish between alterations induced by the psychopathological 'disease' mechanisms, and those which pre-existed in the cell-of-origin (Mancarella & Plass, 2021), or are a consequence of other behaviours (e.g. smoking or vaping (Xie, Rahman, Goniewicz, & Li, 2021)). In adult participant groups, previously collected samples from a time of relative 'biological stability' may be a useful baseline to aid this process. However, the rapid changes in the epigenetic profiles of cells during childhood and adolescence make it harder to define this 'stable' period. Thus, differentiating between pre-existing and disease-associated epigenetic changes in youth will be challenging. Nevertheless, prospective longitudinal studies investigating the progression of epigenetic markers alongside environmental factors from infancy would be a valuable future research development for this area.

6.4.4 Problems of Using the DSM-5 Criteria Originally Based on Males for Mixed-sex Diagnosis – Need for Sex-specific Criteria?

The sex-specific symptom presentations, and sex differences in types of aggressive behaviour repeatedly observed in youth with CD, may indicate that some items in the current DSM criteria are unsuitable for diagnosing CD in females (Zoccolillo, Tremblay, & Vitaro, 1996). The need for sex-specific diagnoses thresholds has also been suggested (Moffitt et al., 2008; Zoccolillo et al., 1996). If the current diagnostic criteria is not wholly applicable to females with CD, or additional criteria are needed specifically for females, then it is possible that individuals included in the studies presented here have not been correctly assigned to the CD and TD groups, and this may subsequently have influenced the CD x CU interaction effects reported in chapter 3 and 4. The findings from the SEM model relating *OXTR* genotype to

CD symptom score may also have been inaccurate if there are CD symptoms specific to females which are not included in the current CD diagnostic criteria and so are not assessed in the KSADS-PL CD-supplement (Kaufman et al., 1997) which was used to assess CD symptom score, according to the DSM-5 diagnostic criteria, in this work.

As our understanding of the underlying biological mechanisms of female CD, and how these relate to observable behaviours, increases, researchers in this field will be better able to determine whether the current diagnostic criteria are appropriate for use in both sexes. Possibly, additional sex-specific CD classifiers that incorporate neurocognitive and affective impairments and biological markers could help create criteria for CD, which can be adequately applied to males and females, thereby increasing the validity of the diagnosis (Wakefield, Pottick, & Kirk, 2002).

6.5 Overall Summary and Conclusions

This thesis has presented the results of three studies including females with CD. In the first study, I observed a CD x CU interaction effect on levels of DNA methylation in a region on chromosome one, which corresponded to the location of the *SLC25A24* gene. I also found that an increased level of methylation at this locus was associated with a reduction in GMV in brain areas, including the SFG, dlPFC, supramarginal gyrus, secondary visual cortex and ventral PCC. I further demonstrated that, in a sub-sample of these participants in which the CDxCU interaction was again observable in the region of the *SLC25A24* gene, *SLC25A24* methylation was positively associated with brain activity during an emotional face processing task in both cortical and subcortical brain regions. Specifically, in response to faces in general, increased methylation of that gene was associated with elevated response in the right

hemisphere regions of the ventral caudate, para-hippocampal region, superior temporal cortex and mid-temporal region. However, in response to angry faces, greater activation in the left AI, left occipital lobe and left calcarine cortex in association higher methylation was observed. In response to fearful faces, greater methylation was associated with increased response in the right ventral posterior cingulate, SFG, and in the left dorsal ACC. Taken together, the findings presented in chapters 3 and 4 indicate that the relationship between the epigenetic modification of DNA methylation at the *SLC25A24* gene and level of CU traits is opposite in females with CD, compared to TD females, and is also associated with variations in brain structure and function. The neuroimaging findings, particularly in relation to the CD with high CU trait females, were inconsistent with previous findings in males, which also suggests that the neurobiological underpinnings of this phenotype are sex-specific. Finally, in chapter 5 an SEM approach was used to investigate the factors associated with CD symptom variation, using a model incorporating genetic, clinical, demographic, and neuroimaging data. Although overall *OXTR* genotype did not explain a significant amount of the variation in CD symptoms, in either males or females, significant associations were found between other variables in the model, which were in-line with previous findings (e.g. IQ negatively associated with CD symptoms, CU positively associated with CD symptoms). There was also evidence of some sex-specific associations - most significantly that *OXTR* genotype was associated with left amygdala response to angry faces in males-only.

To conclude, the findings presented in this thesis have provided novel evidence for the biological mechanisms behind CD symptomatology and CU traits in females and reveal distinct associations between epigenetic modifications and affective traits in the psychiatric 'disease' group compared to healthy controls. I also provided evidence of sex-specific associations between *OXTR* genotype data and brain function during emotional face

processing and demonstrate how an SEM approach can be used to probe the associations between clinical, demographic and genetic variables in relation to a psychiatric phenotype. This work can provide a valuable contribution to the field of CD by adding to our knowledgebase of how epigenetic, genetic, and structural and functional brain are related to CD in females.

APPENDIX A: SUPPLEMENTARY MATERIALS FOR CHAPTER 3

Appendix A1. R Script for Methylation Pre-processing and Analysis

```
############################################### #
#   Methylation Analysis - Full Script       #
#              L.Farrow                  #
############################################### #

# load libraries ####
library(minfi)
library(limma)
library(gplots)
library(ggpubr)
library(tidyverse)
library(sva)
library(maxprobes)
library(doParallel)

# load own function ####
source("~/Desktop/Frankfurt/R_files/LB_MET_N122/Scripts/ComplexHeatmapfunction.R")
source("~/Desktop/Frankfurt/R_files/LB_MET_N122/Scripts/qqplot.R")

#### Setup Global variables ####
options(stringsAsFactors = F)
home <- getwd()
rawdata <- "~/../Desktop/FarrowEpigen/2019-069-ILL_METUKF_N=122/"
outputfolder <-
paste0("~/Desktop/Frankfurt/R_files/LB_MET_N122/METUKF_Epic/Output_ICUsubscales
",Sys.Date())
baseDir <- "~/Desktop/Frankfurt/R_files/LB_MET_N122/METUKF_Epic"
dir.create(outputfolder)
setwd(outputfolder)
fdrcutoff=0.01
list.files(baseDir)

# read in data ####
targets <- read.metharray.sheet(baseDir)
targets$Basename=paste0(baseDir, "/",
                        targets$SentrixBarcode_A, "/",
                        targets$SentrixBarcode_A, "_",
                        targets$SentrixPosition_A)
methylA<-read.metharray.exp(targets=targets, verbose=T)

# QUALITY CONTROL ####
# identify failed 'noisy' probes ####
Bad_probes <- detectionP(methylA)
proberemoval <- Bad_probes>0.01
rowtotalprobes <- rowSums(proberemoval)
idxremoval <- rowtotalprobes>12
```

```
cleanedRGset <- methylA[!idxremoval,]

# cleanup
rm(Bad_probes)
rm(proberemoval)
rm(rowtotalprobes)
rm(idxremoval)
rm(methylA)
gc()

# NORMALIZATION ####
# Sample and probe exclusion needs to be done before SV analysis
# normalization ####
GRset.funnorm <- preprocessFunnorm(cleanedRGset)
save(GRset.funnorm, file=paste0(outputfolder,"/GRset.funnormRAW.RData"))
# cleanup
rm(cleanedRGset)
gc()
# exclude SNPs and Xreactive probes ####
GRset.funnorm <- addSnpInfo(GRset.funnorm)
GRset.funnorm <- dropLociWithSnps(GRset.funnorm, snps=c("SBE","CpG"), maf=0)
GRset.funnorm <- dropXreactiveLoci(GRset.funnorm)
# Sample exclusion ####
Samplestoexclude=c()

# Sex prediction ####
# plot sex prediction
pdf(paste0(outputfolder,"/Genderplot.pdf"))
plotSex(GRset.funnorm)
plotSex(GRset.funnorm)
gender=getSex(GRset.funnorm)
Samplestoexclude=c(Samplestoexclude,which(gender$predictedSex != "F"))
dev.off()

# Extracting M values ####
Mvalue_processed <-getM(GRset.funnorm)

Mvalue_processed[ is.infinite(Mvalue_processed)] = NA
idx<- complete.cases(Mvalue_processed)
Mvalue_processed<- Mvalue_processed[idx,]
Beta_processed <- getBeta(GRset.funnorm)

#Get top 1000 probes by variances ####
vrncs<- apply(Mvalue_processed, 1, var)
top_v <- order(vrncs, decreasing =TRUE)
index <- top_v[1:1000]
HV_Mvalue<- Mvalue_processed[index,]

# cluster analysis ####
dstncs <- dist(t(HV_Mvalue), method = "euclidian")
```

```
Hc <- hclust(dstncs, method = "ward.D2")
Samplestoexclude=c(Samplestoexclude,which(cutree(Hc, k=2)==2))
pdf(paste0(outputfolder,"/HCluster100var.pdf"))
plot(Hc)
abline(h=120, col="red")
dev.off()

# Annotation
annotation=getAnnotation(GRset.funnorm) #must be loaded from the GRSet.funnorm
EpiAnnot=annotation[rownames(HV_Mvalue),] # select same genomic regions as from
Object
targets=pData(GRset.funnorm) ## needs targets to be defined

pdf(paste0(outputfolder,"/All_samples_Heatmap.pdf"))
complexheatmap<-myplot(HV_Mvalue, "Mvalue_processed", "1000 top variable loci")
dev.off()


Samplestoexclude=c(Samplestoexclude, which(GRset.funnorm$Medcationbin=="yes"))
Samplestoexclude=c(Samplestoexclude, which(GRset.funnorm$SmokingBin=="yes"))

Samplestoexclude=unique(Samplestoexclude)
GRset.funnorm=GRset.funnorm[,-Samplestoexclude]

# save cleaned dataset####
save(GRset.funnorm, file=paste0(outputfolder,"/GRset.funnorm.RData"))
load("GRset.funnorm.RData")

# Regression Modelling ####
# Define Datatype and standardize data for model

GRset.funnorm$Age <- scale(GRset.funnorm$Age)
GRset.funnorm$ICUimp_total_sum_imp <- scale(GRset.funnorm$ICUimp_total_sum_imp)
GRset.funnorm$PDS <- scale(GRset.funnorm$PDS)
GRset.funnorm$TOTALIQ <- scale(GRset.funnorm$TOTALIQ)
GRset.funnorm$Group <- as.factor(GRset.funnorm$Group)
GRset.funnorm$Site <- as.factor(GRset.funnorm$Site)
GRset.funnorm$Ethnicity <- as.factor(GRset.funnorm$Ethnicity)
GRset.funnorm$Ccept <- as.factor(GRset.funnorm$Ccept)
wilcox.test(GRset.funnorm$PDS~GRset.funnorm$Group)
chisq.test(table(GRset.funnorm$Site, GRset.funnorm$Group))
chisq.test(table(GRset.funnorm$Ethnicity, GRset.funnorm$Group))
wilcox.test(GRset.funnorm$Age~GRset.funnorm$Group)
wilcox.test(GRset.funnorm$TOTALIQ~GRset.funnorm$Group)
summary(aov(GRset.funnorm$TOTALIQ~GRset.funnorm$Ethnicity))
summary(aov(GRset.funnorm$ICUimp_total_sum_imp~GRset.funnorm$Ethnicity))
summary(aov(GRset.funnorm$correctedicu~GRset.funnorm$Ethnicity))

# MODEL DEFINITION ####
## Full Model
```

```
mod3 <- model.matrix(~ Group*correctedicu + Age +  Site +
             Ethnicity + TOTALIQ, data=pData(GRset.funnorm))

## Null model
mod01 <- model.matrix( Age +  Site +
             Ethnicity + TOTALIQ, data=pData(GRset.funnorm))

# recall Mvalues after QC is done
Mvalue_processed <-getM(GRset.funnorm)
Mvalue_processed[ is.infinite(Mvalue_processed)] = NA
idx<- complete.cases(Mvalue_processed)
Mvalue_processed<- Mvalue_processed[idx,]

# surrogate variable analysis####
gc()
n.sv = num.sv(Mvalue_processed, mod3,
           method="leek") ##
print(n.sv)
gc()
sva.results <- sva(as.matrix(Mvalue_processed), mod1, mod01, n.sv = n.sv)
mod1=cbind(mod1, sva.results$sv) # like this you add all SVs to the models no matter how
many there were
mod01=cbind(mod01, sva.results$sv)
design=mod1
save(sva.results, mod3, mod03,Mvalue_processed,
file="Designmatrices_GroupICU_0.01nominal.RData")
# load("Designmatrices_ICU.RData")
## remove large objects
rm(sva.results)
gc() #clean and free the memory

# TEST MODELS ####
fit=lmFit(Mvalue_processed, design)
fit2=eBayes(fit)

rm(fit) # remove and clean memory
gc() #garbage collection
save(fit2, file="BayesFitmodel_Final.RData")
ncores= detectCores()
registerDoParallel(cores = ncores)

 dmrs_int <- bumphunter(GRset.funnorm, design = design, nullMethod="bootstrap", coef=6,
                cutoff = 0.95, B=250, type="M")
 save(dmrs_int, file = "dmrs_bhoutput_cueffect_95cutoff.RData")
dmrs$table[,]
idx<-(is.infinite(dmrs$table$area))
dmrs<- dmrs[-idx]
sum((dmrs$pvaluesMarginal<0.05)==TRUE)
idx2 <- (dmrs$pvaluesMarginal<0.05)
dmrs<- dmrs[idx2]
```

Table A2. Participant Numbers From Each Data Collection Site in the Chapter 3 Study

Data Collection Site	**Number of Participants**	
	CD	***TD***
1	4	*3*
2	4	*6*
4	12	6
5	12	17
7	19	27

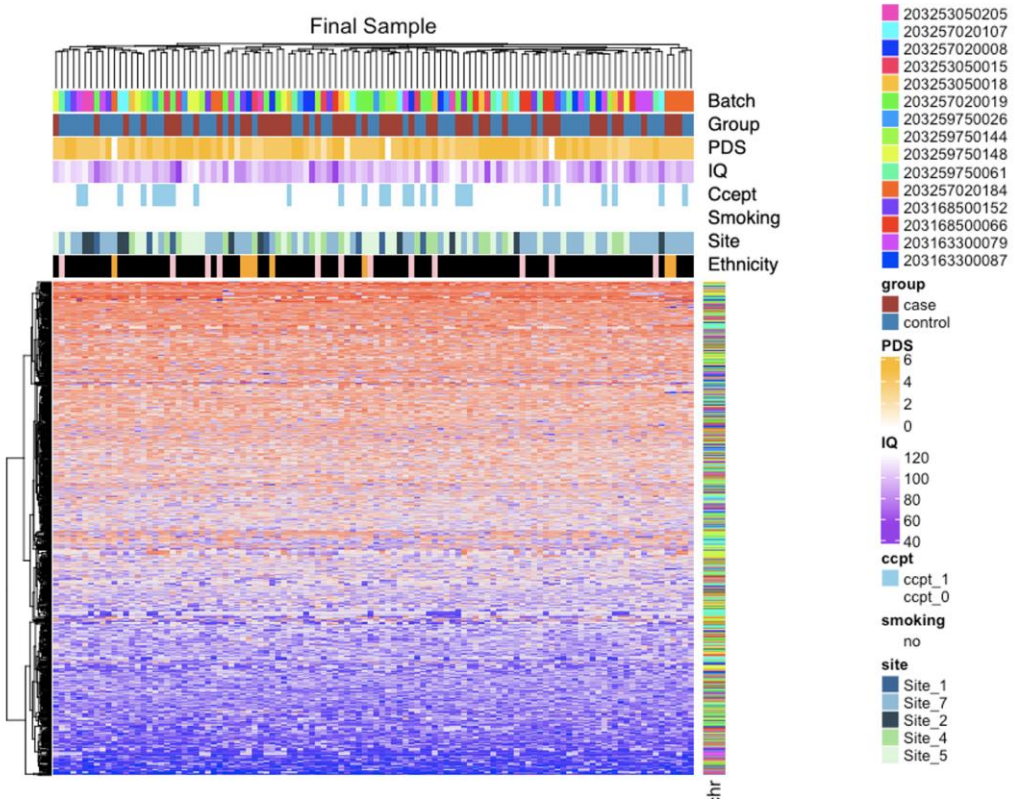
Notes: Site 1= Universitätsklinikum Aachen, Site 2= Johann Wolfgang Goethe Universität Frankfurt am Main, Site 4= University of Southampton, Site 5= Universität Basel, Site 7 = The University of Birmingham.

Chi-squared testing confirmed no significant association between group and data collection site X^2 (2, N = 110) = 4.24, p = .375

Quality Control Checks of Methylation Data after Pre-processing

Figure A3. Heat maps and hierarchical clustering plots

a) Epigenetic Data post-QC sample correlation check -Heatmap with dendrogram: Visual inspection confirmed neither clustering of methylation values for matched variables, nor batch effects were present in the final sample.



b) Hierarchical clustering plot (dendrogram) showing 4 distinct groups in the dataset

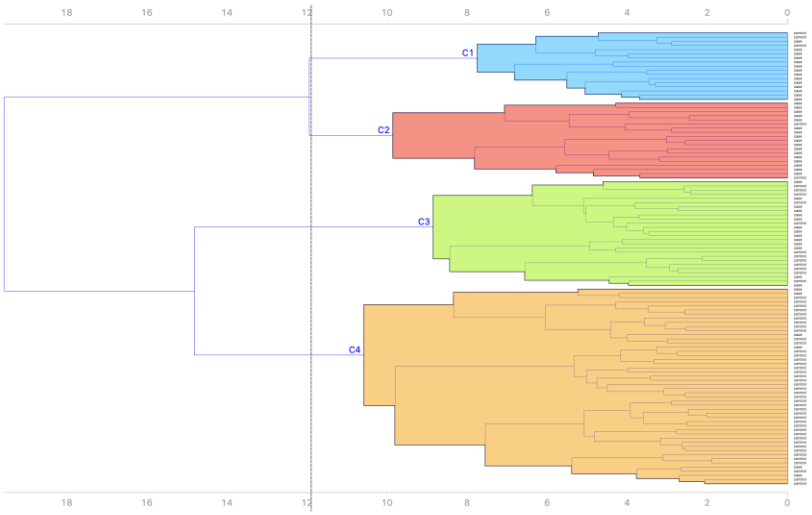


Figure A4. Histogram plot confirming normally distributed M values for DMR without outliers

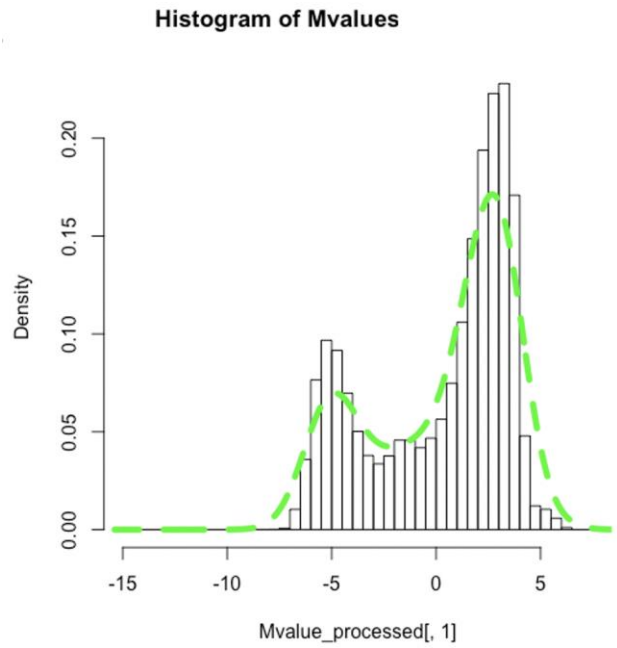


Figure A5. QQ plots for 3 EWAS models for (a) Main effect of Group, (b) Main effect of CU, (c) CDxCU interaction

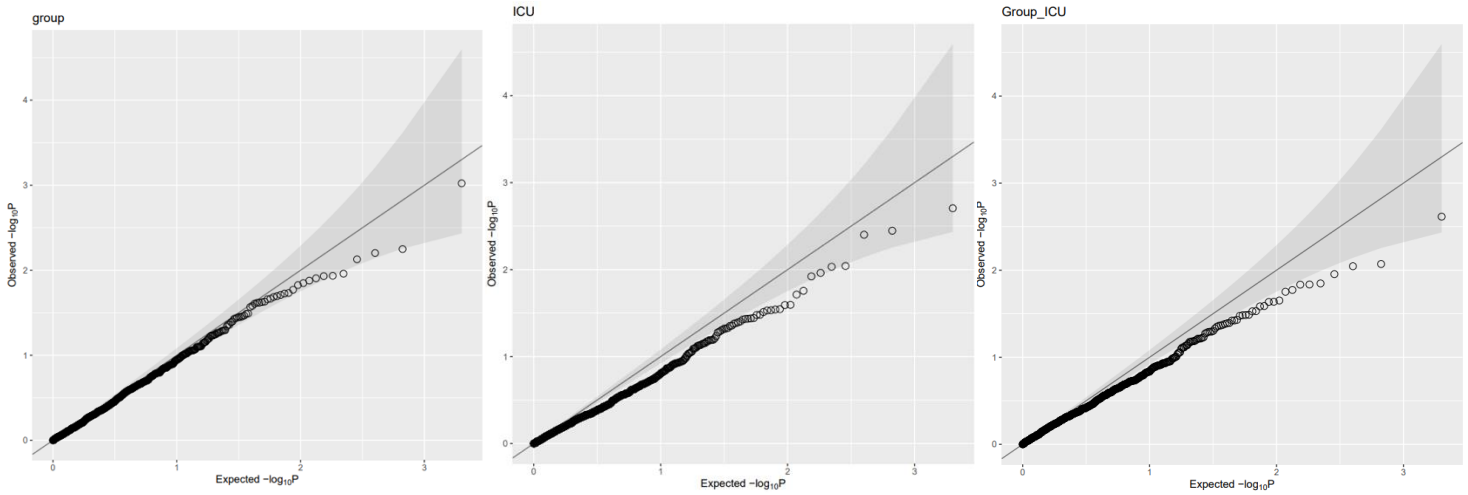
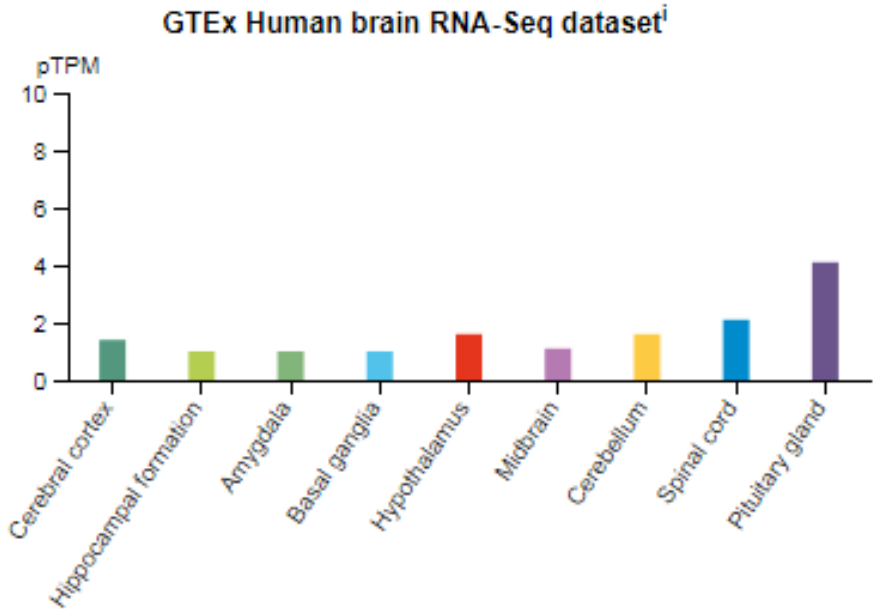


Figure A6 . GTEx bar plots of *SLC25A24* gene expression across brain tissues**.**



Selection criteria for ROIs for VBM analysis was; (i) region had been previously identified in the literature as of interest in relation to Anti-social behaviour/CD /CU traits and, (ii) *SLC25A24* gene is reported to be expressed[11] in post-mortem tissue taken from that brain region

Figure A7. 3D Rendering of the 4 ROIs tested: the amygdala (green), basal ganglia (yellow), cerebellum (red) and hippocampus (blue)

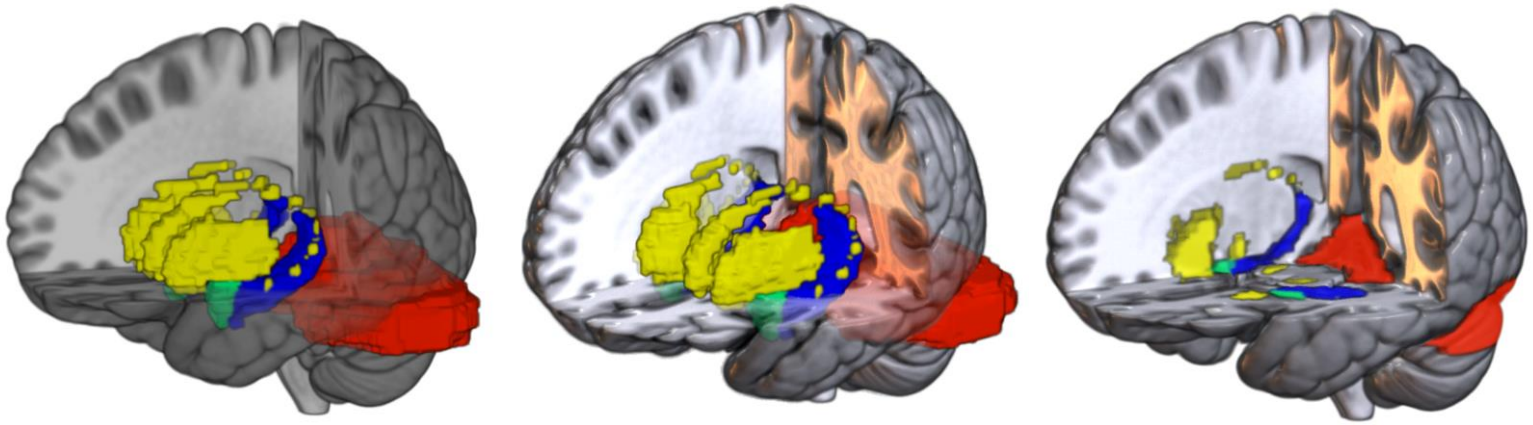


Table A8. GMV Clusters Associated with *SLC25A24* Methylation

Covariates	Brain region	BA	L/R	Peak Voxel	k	Z	Cluster-corrected P-value
PDS, SES	SFG	10	L	-17 48 20	528	4.57	<.001
	dlPFC	9	L	-37 42 38	108	3.99	<.001
	Supramarginal Gyrus	40	L	-48 -32 34	290	3.74	<.001
	Ventral PCC	23	R	7 -24 37	235	3.57	<.001
	Secondary Visual Cortex	18	L	-11 -92 -2	177	3.57	<.001
	Secondary Visual Cortex	18	R	17 -87 3	140	3.54	<.001
	dlPFC	9	L	-41 23 39	84	3.37	<.001
	Supramarginal Gyrus	40	L	-61 -35 21	97	3.36	<.001

	Secondary Visual Cortex	18	R	10 -96 20	79	3.33	<.001
PDS, SES, GAD	SFG	10	L	-17 48 20	490	4.51	<.001
	dlPFC	9	L	-37 42 38	97	3.93	<.001
	Angular Gyrus	39	L	-43 -46 25	73	3.90	<.001
	Secondary Visual Cortex	18	L	-11 -92 -1	230	3.66	<.001
	Supramarginal Gyrus	40	L	-48 -32 34	101	3.65	<.001
	Supramarginal Gyrus	40	L	-58 -27 38	174	3.60	<.001
	Primary Visual Cortex	17	R	17 -87 3	150	3.56	<.001
	Dorsal PFC	31	R	6 -24 38	266	3.54	<.001

	Supramarginal Gyrus	40	L	-57 -28 21	214	3.53	<.001
	Pre-Motor region	6	R	50 -1 53	73	3.47	<.001
	dlPFC	9	L	-41 23 39	160	3.45	<.001
PDS, SES, MDD	SFG	10	L	-17 48 20	500	4.49	<.001
	dlPFC	9	L	-37 42 38	111	3.97	<.001
	Primary Visual Cortex	17	R	17 -87 3	162	3.66	<.001
	Supramarginal Gyrus	40	L	-48 -32 34	242	3.66	<.001
	Secondary Visual Cortex		L	-12 -91 -2	197	3.60	<.001
	Ventral PCC	8	R	7 -24 37	235	3.53	<.001
	dlPFC	9	L	-41 23 39	131	3.41	<.001
	Supramarginal Gyrus	40	L	-60 -34 21	152	3.41	<.001

Notes: SFG = GAD = Generalized Anxiety Disorder, MDD = Major Depressive Disorder, Superior Frontal Gyrus, dlPFC =Dorso-lateral Prefrontal Cortex, PCC = Posterior Cingulate Cortex

APPENDIX B: SUPPLEMENTARY MATERIALS FOR CHAPTER 4

Table B1. Participant Numbers from Each Data Collection Site in the Chapter 4 Study

Data Collection Site	**Number of Participants**	
	CD	***TD***
1	3	*3*
2	*7*	*7*
4	*10*	*3*
7	11	18

Notes: Site 1= Universitätsklinikum Aachen, Site 2= Johann Wolfgang Goethe Universität Frankfurt am Main, Site 4= University of Southampton, Site 7 = The University of Birmingham.
Chi-squared testing confirmed no significant association between group and data collection site X^2 (2, N = 62) = 5.46, p = .141

APPENDIX C: SUPPLEMENTARY MATERIALS FOR CHAPTER 5

Appendix C1. *OXTR* SNPs and Weightings for Composite Risk Score Calculation

SNP no.	***OXTR* SNP**	**Risk Allele**	**Beta Weight**
1	rs1042778	T	0.018
2	rs11131147	A	0.049
3	rs112478465	A	0.031
4	rs143825102	T	-0.020
5	rs1488466	C	0.041
6	rs1488467	C	0.037
7	rs17297971	A	0.001
8	rs2139184	A	-0.024
9	rs2228485	G	-0.059
10	rs2254298	A	0.007
11	rs2324728	T	-0.004
12	rs237884	G	0.002
13	rs237885	T	0.014
14	rs237889	T	-0.008
15	rs237898	T	0.001
16	rs237902	A	0.0063
17	rs34605596	G	0.082
18	rs34955659	T	-0.07
19	rs35014760	C	-0.030
20	rs36047964	T	0.012
21	rs4493422	T	0.046
22	rs4564970	C	0.309
23	rs4686302	T	0.032
24	rs53576	A	-0.002
25	rs56898713	T	0.091
26	rs59746083	G	0.089
27	rs62242634	C	-0.027
28	rs62242635	A	0.031
29	rs6770632	A	-0.092
30	rs6801703	A	-0.013
31	rs6802389	C	0.006
32	rs7632287	A	0.001
33	rs9872310	G	0.0013
34	rs9872425	T	-0.026

Total Composite Score for Each Participant = $\sum \beta iNi$ *(i=1 to i=34)*
β= beta weighting
N = number of risk alleles for that SNP

Table C3. SEM Variables Correlation Table

Variables	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.
1. Sex	-0.07	-	0.01	-0.4	0.21*	0.12*	-0.08	-	-0.01	-	-0.05	-0.04	0	0.05	0.02	0.01	0.0	-
2. Site	1	.011	-.15*	0.16*	-0.04	0.04	-0.04	0.0	0.04	0.01	0.02	0.03	-0.01	0.04	0.06	<0.0	-	-
3. Age		1	-	0.77*	<0.0	0.16*	-0.06	0.0	0.02	0.05	0.03	0.01	0.03	0.06	0.01	0.05	0.0	-
4. Total IQ			1	-	-	-	0.38*	0.0	0.1	0.1	0.08	0.09	0.1	0.05	0.07	-	-	0.06
5. PDS				1	-0.06	0.08	-0.03	0.0	<0.0	0.06	0.02	0.01	0.01	0.01	-0.02	0.08	-	0.01
6. CU					1	0.60*	-	-	-0.05	-0.09	-0.08	-0.09	-0.04	0.04	<0.0	0.06	0.0	-
7. CD						1	-	-	-0.06	-0.05	-0.02	-0.09	-0.05	-0.05	-0.06	0.08	0.0	-
8. SES							1	0.0	0.01	0.05	0.05	0.02	<0.0	-0.02	-0.03	-	0.0	0.1
9. L BOLD response: all								1	0.69**	0.89**	0.63**	0.89	0.59	0.9	0.61	-0.06	-0.07	-0.06
10. R BOLD response: all faces>fixation									1	0.59**	0.86**	0.62**	0.89**	0.64**	0.87**	<0.01	0.04	<0.01
11. L BOLD response: neutral>fixation										1	0.70**	0.68**	0.42**	0.70**	0.44**	-0.05	-0.04	-0.01
12. R BOLD response: neutral>fixation											1	0.48**	0.66**	0.50**	0.62**	<0.01	0.05	0.04
13. L BOLD response: anger>fixation												1	0.68**	0.73**	0.49**	-0.07	-0.06	-0.04
14. R BOLD response: anger>fixation													1	0.51**	0.69**	0.03	0.03	-0.03

15. L BOLD response: Fear>fixation														1	0.71**	-0.03	-0.07	-0.09
16. R BOLD response: Fear>fixation															1	<0.01	0.04	-0.01
17. rs6770632																1	0.0	0.01
18. rs53576																	1	-
19. *OXTR* RS																		1

Table C4: Regions across all participants showing main effect in the Amygdala at *P*< .005 FWE-corrected for contrasts; All faces>Fixation, Anger>Fixation, Fear>Fixation, Neutral>

Contrast	L/R	Peak Voxel	k	Z	T	FWE corrected P-value
All Faces >Fixatio	L	-21 0 -12	19	Inf	11.5	<.001
	R	21 -3 -12	14	Inf	9.15	<.001
Anger>Fixation	L	-21 0 -12	20	Inf	11.5	<.001
	R	21 -3 -12	19	Inf	9.1	<.001
Fear>Fixation	L	-21 0 -12	17	Inf	12.11	<.001
	R	21 -6 -12	16	Inf	8.70	.001
Neutral>Fixation	L	-21 0 -12	13	Inf	9.80	.001
	R	21 -6 -12	12	7.20	67.5	.001

Table C5. Regions in female participants showing main effect in the Amygdala at *P*< .005 FWE-corrected for contrasts; All faces>Fixation, Anger>Fixation, Fear>Fixation, Neutral>Fixation

Contrast	L/R	Peak Voxel	k	Z	T	FWE corrected P-value
All faces>Fixation	L	-21 -3 -12	20	Inf	9.54	<.001
	R	21 -6 -12	14	7.39	7.98	<.001
Anger>Fixation	L	-21 -3 -12	21	Inf	9.29	<.001
	R	21 -3 -12	11	7.17	7.70	0.001
Fear>Fixation	L	-21 -3 -12	15	Inf	8.65	.001
	R	21 -6 -15	10	6.26	6.61	.002
Neutral>Fixation	L	-24 -3 -12	19	7.77	8.46	<.001
	R	21 -6 -15	8	6.25	6.60	.003

Table C6. Regions in male participants showing main effect in the Amygdala at $P < .005$ FWE-corrected for contrasts; All faces>Fixation, Anger>Fixation, Fear>Fixation, Neutral>Fixation

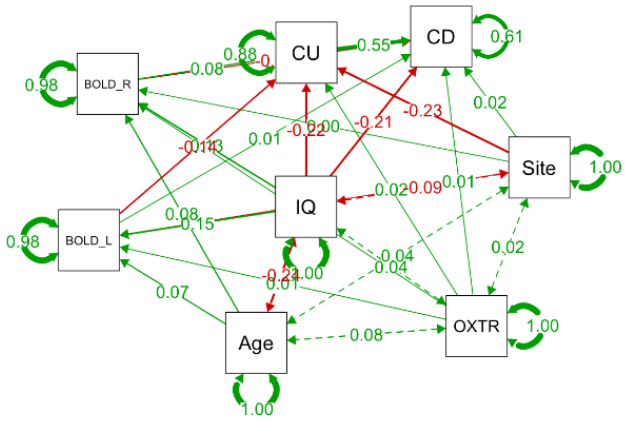
Contrast	L/R	Peak Voxel	k	Z	T	FWE corrected P-value
All faces>Fixation	L	-21 0 -12	9	7.27	7.93	.002
	R	24 0 -12	8	5.99	6.35	.003
Anger>Fixation	L	-21 0 -12	12	6.63	7.12	.001
	R	27 0 -15	12	5.74	6.06	.001
Fear>Fixation	L	-21 0 -12	12	Inf	8.99	.001
	R	24 -6 -12	8	6.16	6.56	.002
Neutral>Fixation	L	-21 0 -12	3	5.14	5.37	.011
	R	-	-	-	-	-

Appendix C7. SNP rs6770632 and SNP 53576 SEM Model Results for Single-sex Participant Groups

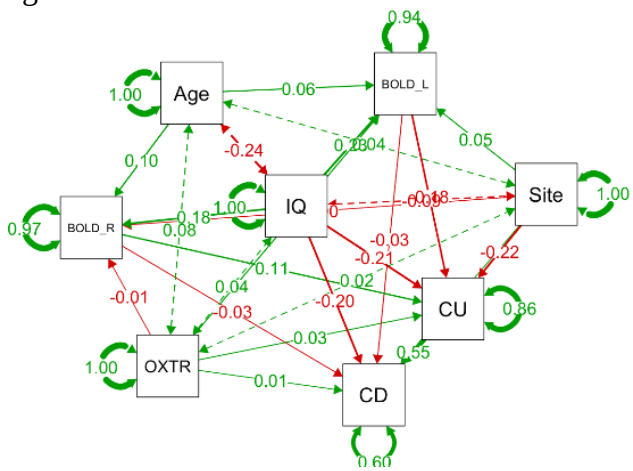
a) rs6770632 -Female

b)

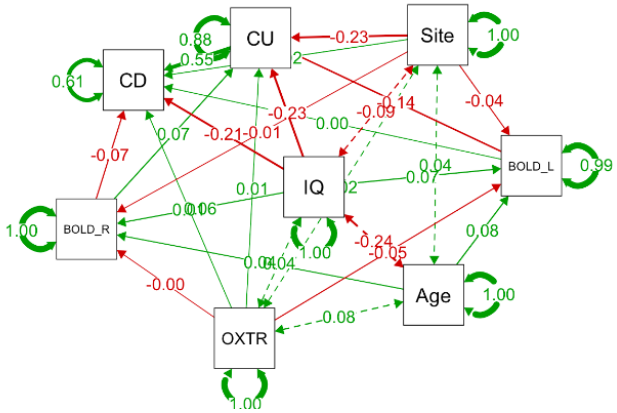
All Faces> Fixation



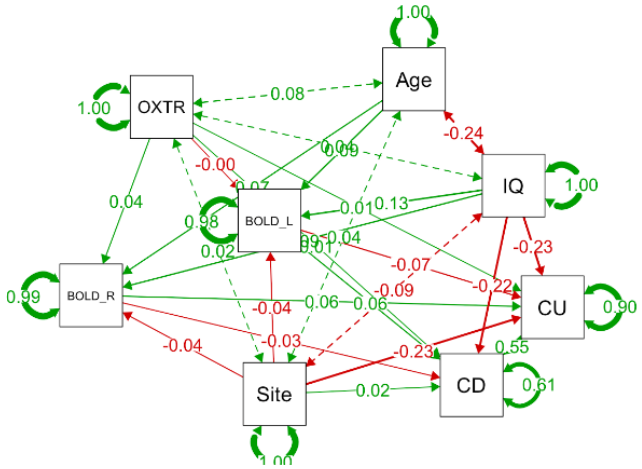
Anger>Fixation



Fear>Fixation

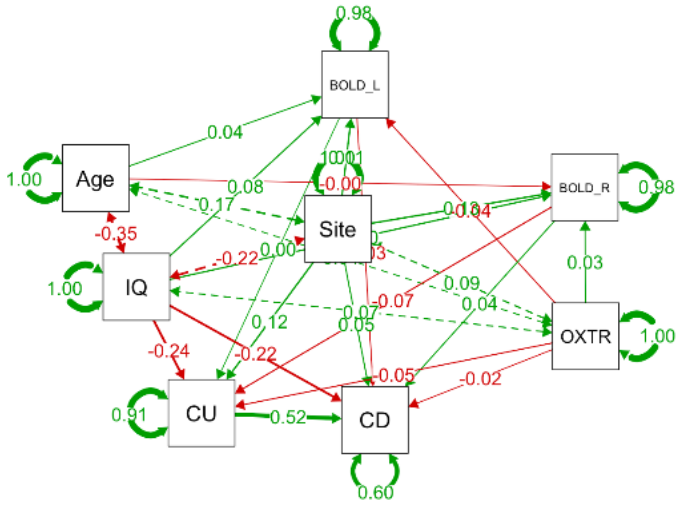


Neutral> Fixation

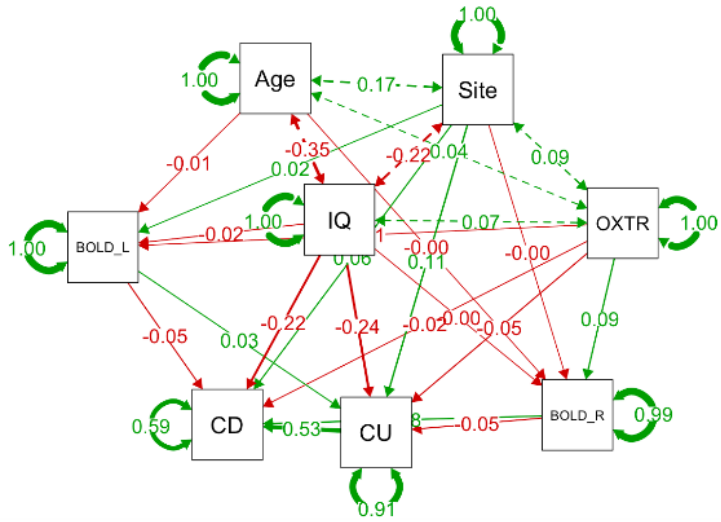


c) rs6770632 -Male

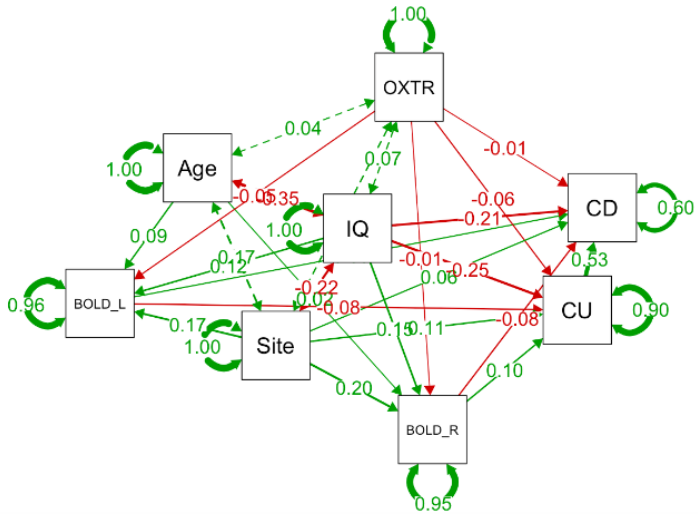
All Faces>Fixation



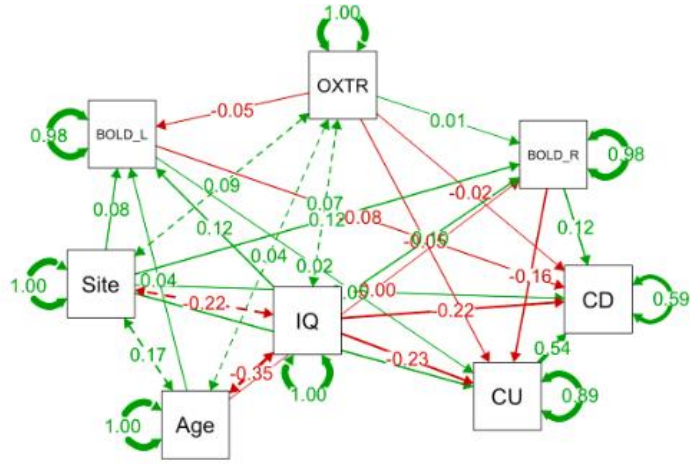
Anger >Fixation



Fear>Fixation

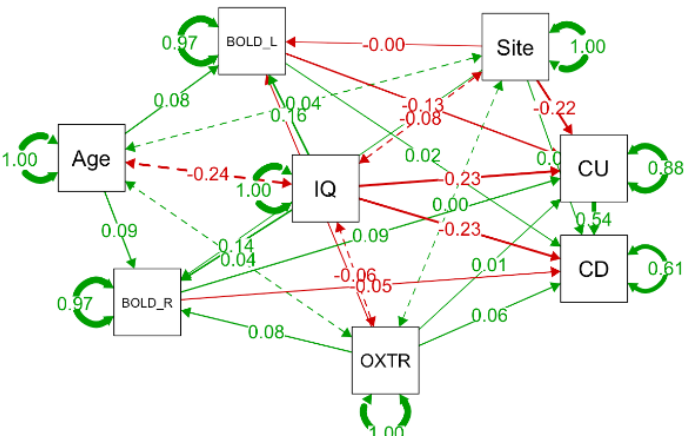


Neutral> Fixation

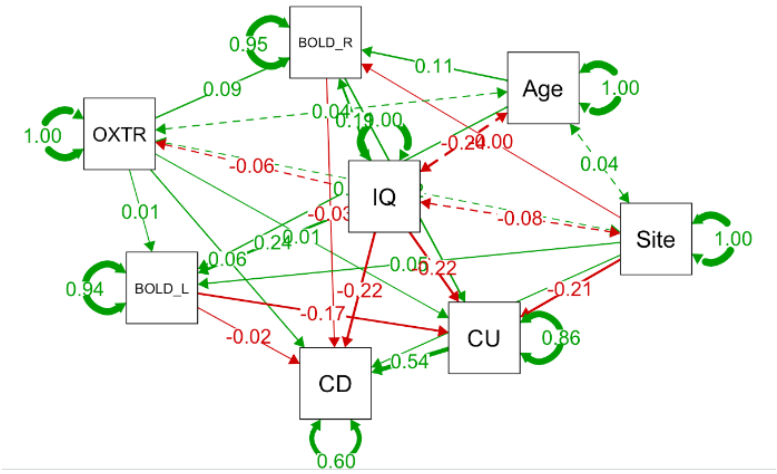


d) rs53576 -Female

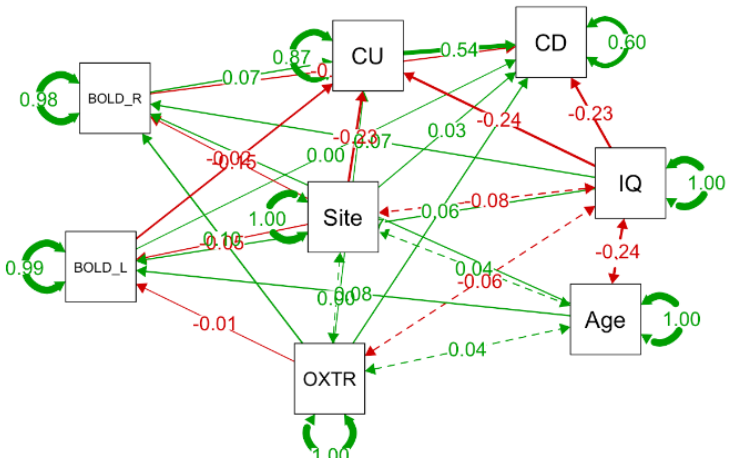
All Faces> Fixation



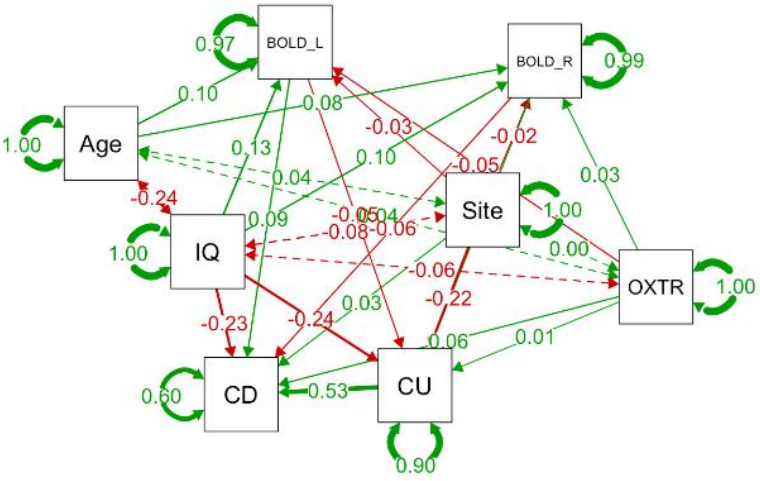
Anger>Fixation



Fear>Fixation

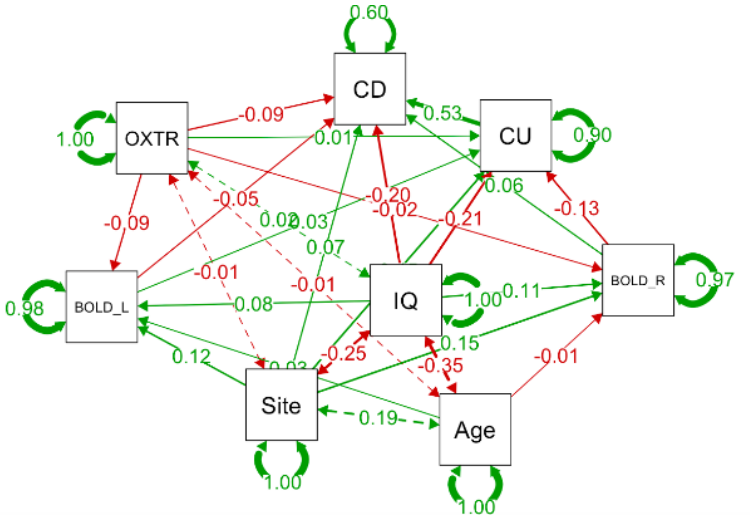


Neutral> Fixation

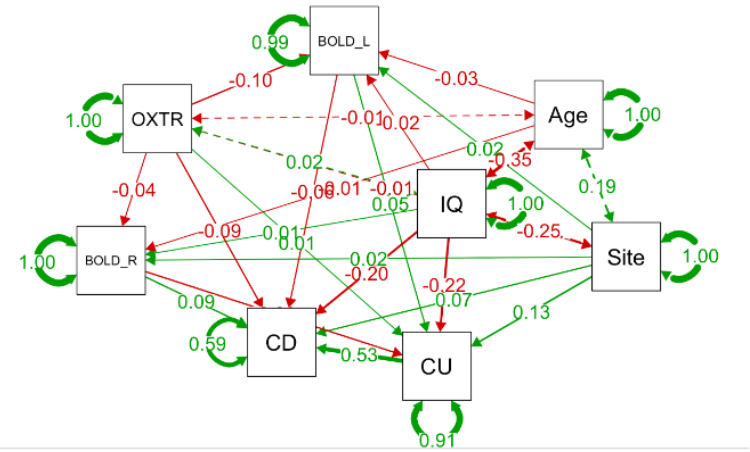


e) rs53576 -Male

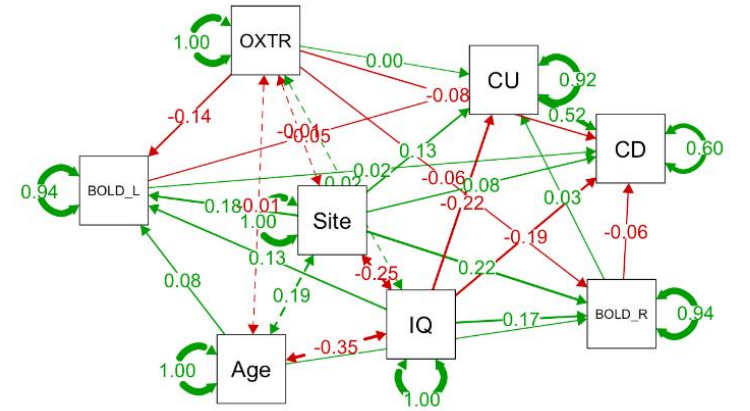
All Faces>Fixation



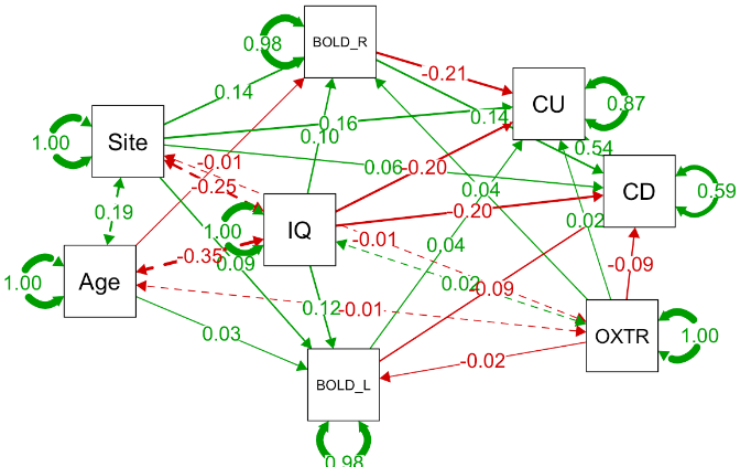
Anger >Fixation



Fear>Fixation



Neutral> Fixation



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